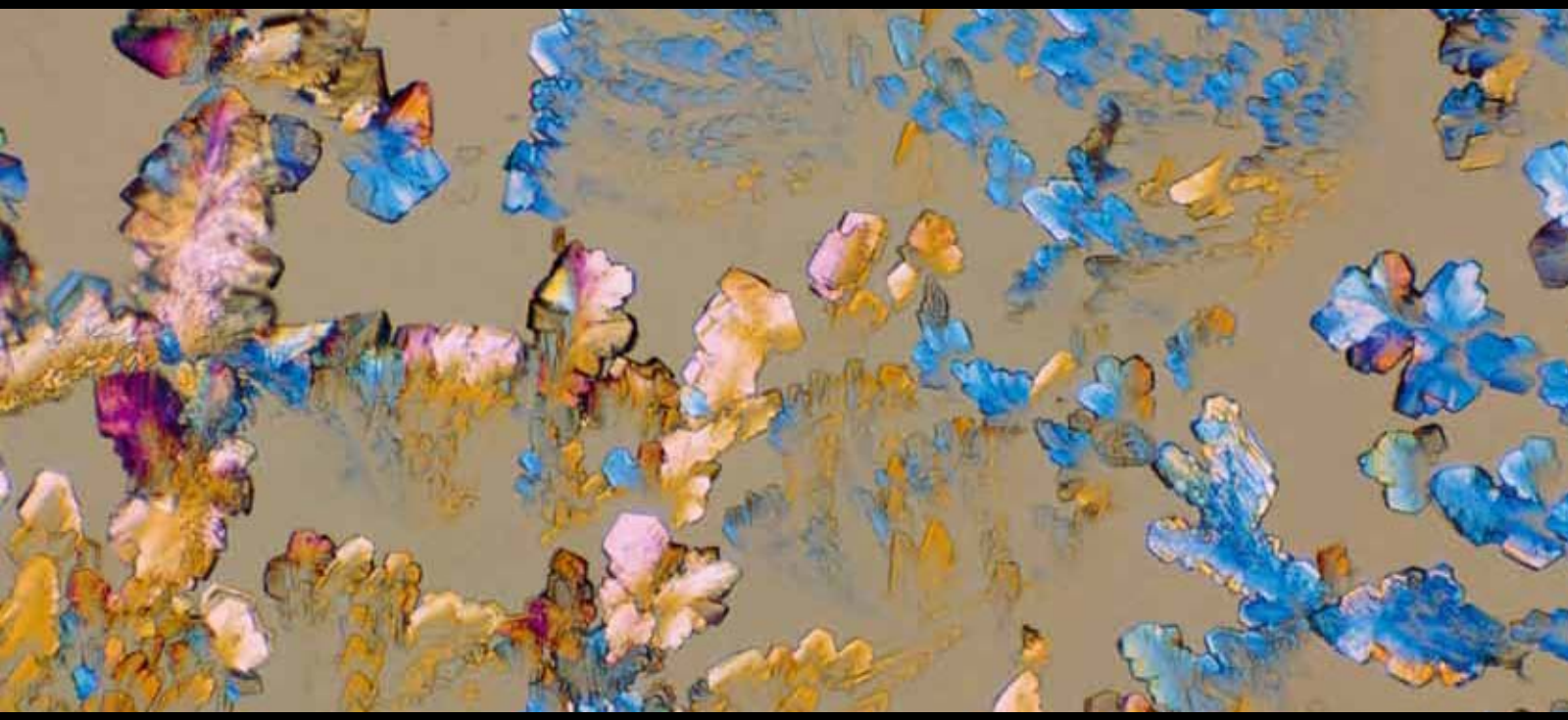
Journal of Amino Acids

Functional Residues in Proteins

Guest Editors: Shandar Ahmad, Jung-Ying Wang, Zulfiqar Ahmad, and Faizan Ahmad



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Editorial Board

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Editorial

Functional Residues in Proteins

Shandar Ahmad,[1] Jung-Ying Wang,[2] Zulfiqar Ahmad,[3] and Faizan Ahmad[4]

[1] *National Institute of Biomedical Innovation, Osaka, Japan*
[2] *Lunghwa University of Science and Technology, Taiwan*
[3] *Department of Biology, Alabama A&M University, Normal, AL 35762, USA*
[4] *Centre for Interdisciplinary Research in Basic Sciences, India*

Correspondence should be addressed to Shandar Ahmad, shandar@nibio.go.jp

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We are delighted to present a special issue on the subject of functional residues of proteins. Finding focused articles within the time frame of a special issue is always challenging, which is further compounded by authors' reluctance in making their contributions to new journals. We have been fortunate enough that some leading researchers in the field agreed to support this special issue and contributed their work at our request. We present here nine articles on various aspects of the subject. We received some more submissions, which could not be accommodated because of a strict quality control, we tried to maintain. Finally compiled special issue starts with a very interesting commentary on post-translated modifications leading to normal and disease-associated biological functions, contributed by T. M. Karve and A. K. Cheema. This is followed by the analysis of a specific biological system of ATP synthetase, contributed by Z. Ahmad et al., exploring its catalytic sites and focusing on charged residues. R. Prasad et al. then describe a related system of ABC transporters and review the state of the knowledge on its functional residues. Functional residues in a protein do not always follow a universal pattern, and this is demonstrated by R. Kumar et al. in describing the dynamic structure of estrogen receptors. This paper is then followed by a special group of functional residues, that is, cysteins in sulfurtransferases by N. Nagahara. Viola et al. present an excellent study of catalytic machinery in the biosynthesis of amino acids. Subsequntly, K. M. Fukasawa et al. then present their analysis of functionally relevant zinc-binding sites in metalloproteases. Subsequently, M. S. Khan et al. provide an elaborate discussion on the mechanism of serpin inhibition, specially focusing on the role of polymerization. Finally, M. A. Kabir et al. present an investigation of a eukaryotic chaperon, involved in protein-folding.

Thus, the special issue covers a wide range of systems and describes an overview of some of the most significant studies on the subject of finding functional residues in proteins. We would like to express our most sincere gratitude to the authors who agreed to contribute and referees who provided useful feedback, allowing the papers to meet the high standards of publication.

We hope that the readers of Journal of Amino Acids will find these articles of great interest and look forward to any comments to improve our future efforts.

Shandar Ahmad
Jung-Ying Wang
Zulfiqar Ahmad
Faizan Ahmad

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Review Article

Small Changes Huge Impact: The Role of Protein Posttranslational Modifications in Cellular Homeostasis and Disease

Tejaswita M. Karve[1] and Amrita K. Cheema[2]

[1] *Department of Biochemistry, Cellular & Molecular Biology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, 3900 Reservoir Road, NW, Washington DC 20057, USA*
[2] *Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine, Washington, DC 20057, USA*

Correspondence should be addressed to Amrita K. Cheema, akc27@georgetown.edu

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Posttranslational modifications (PTMs) modulate protein function in most eukaryotes and have a ubiquitous role in diverse range of cellular functions. Identification, characterization, and mapping of these modifications to specific amino acid residues on proteins are critical towards understanding their functional significance in a biological context. The interpretation of proteome data obtained from the high-throughput methods cannot be deciphered unambiguously without a priori knowledge of protein modifications. An in-depth understanding of protein PTMs is important not only for gaining a perception of a wide array of cellular functions but also towards developing drug therapies for many life-threatening diseases like cancer and neurodegenerative disorders. Many of the protein modifications like ubiquitination play a decisive role in various drug response(s) and eventually in disease prognosis. Thus, many commonly observed PTMs are routinely tracked as disease markers while many others are used as molecular targets for developing target-specific therapies. In this paper, we summarize some of the major, well-studied protein alterations and highlight their importance in various chronic diseases and normal development. In addition, other promising minor modifications such as SUMOylation, observed to impact cellular dynamics as well as disease pathology, are mentioned briefly.

1. Introduction

With current advances in the fields of systems biology and proteomics, the interest in deciphering protein modifications and their impact on the cellular microenvironment and disease pathophysiology is greatly enhanced. Proteins are large macromolecules comprised of a specific sequence of amino acids. Although protein folding and refolding play a critical role in protein function, the modification of amino acids and their side chains contributes significantly to the structural and functional diversity of the proteins. These modifications impart complexity to the eukaryotic proteomes that is several orders of magnitude greater than the coding capacity of the genome. The common modifications include phosphorylation, acetylation, glycosylation, ubiquitination, acetylation, and hydroxylation. Posttranslational modifications (PTMs) of proteins influence the enzyme activity, protein turnover and localization, protein-protein interactions, modulation for various signaling cascades, DNA repair, and cell division.

Given the pivotal role of PTMs in the regulation of cellular environment, there is a constant effort to develop novel, highly sensitive, and sophisticated PTM identification techniques. Some of these techniques are targeted towards identifying specific PTMs like the modification of the histone tails recognized by specially designed probes while other techniques are more robust like surface-enhanced Raman spectroscopy and mass spectrometry [1–3]. A novel technology called "multidimensional protein identification technology or MudPIT," which is a combinatorial method chromatography in conjunction with mass spectrometry,

has been efficient in discovering global PTM [4]. Traditional biochemical methods like Western blotting and SDS-PAGE are widely used to confirm the high-throughput results obtained from the spectroscopic methods as well as for understanding the biological significance of PTMs *in vivo*. In addition, there have been successful attempts in developing *in silico* algorithms that can reliably predict various PTMs in a given protein sample. Other artificial PTMs like biotinylation, which attach a prosthetic group to a protein, are frequently used to understand protein-protein interaction(s) that results from the changes in three-dimensional structure of protein. Although more than 150 protein modifications have been reported, a detailed assessment of each modification is beyond the scope of this paper. We have focused on major modifications, which have received significant attention by the research community in the past few decades.

2. Acetylation

One of the most common protein modifications is the acetylation of lysine residue. The acetylation of proteins is mainly a cotranslational and posttranslational process. The histone acetylation as well as deacetylation is of particular interest due to its role in gene regulation [5, 6]. This occurs on the lysine residue of histone proteins at the N-terminal tail of lysine and is facilitated by the enzymes, histone acetylases (HATs), or histone deacetylases (HDACs). A single lysine alteration on histones significantly impacts the cellular homeostasis since the acetylation status of histones regulates various transcription factors, molecular chaperones, and cellular metabolism [7, 8]. In addition, regulation of histone acetylation by the HATs and HDACs has a well-established link to aging and various neurological and cardiovascular diseases [9–12]. Finally, at least one HDAC family, MYST proteins, is shown to participate in a diverse array of functions in health and disease to modulate the fate of stem cells and chromatin state [13].

In prokaryotes, the acetylation of glutamic acid and aspartic acid has also been observed. The conversion of glutamic acid to N-acetylglutamic acid is an important intermediate step for ornithine synthesis in bacteria [14].

Recent studies not only have linked the process of protein acetylation with a number of diseases but also have shown that amino acid acetylation significantly contributes to the overall pathophysiology of the diseases [9–12]. One such study has noted that the increased acetylation of the cytoskeletal proteins, especially microtubule proteins, in response to the reactive oxygen species (ROS) and thus suppression of SIRT2 aggravates the mitochondrial dysfunction in the CPEO (chronic progressive external ophthalmoplegia) syndrome patients [15]. Conversely, the study showed that the lysine hyperacetylation of the OGG1 enzyme, an important DNA repair enzyme, in response to the ROS, is a required step for the activation of the DNA repair system [16]. Similarly, another member of the deacetylases family, SIRT1, has been shown to be downregulated in oxidative stress-induced endothelial cells. However, pretreatment with a pharmacological agent like resveratrol was shown to attenuate the SIRT1 levels as well as eNOS acetylation. Thus, identification of the eNOS as a substrate for SIRT1 in the endothelial cells has been a pivotal step in understanding the pathology and mechanism of the cardiopulmonary diseases and vasculature [17].

Acetylation of proteins and carbohydrates has also been evaluated as a target for cancer therapy [9, 18]. Chammas et al. have reported the use of N-acetylation as well as O-acetylation of the surface tumor antigens as a tool for therapeutic development against different types of melanomas and leukemias [9].

A number of acetoproteins have been implicated in the cognitive disorders like dementia and Alzheimer's disease [10]. In the case of dementia, lysine acetylation of tau proteins results in "tau tangles" while in Alzheimer's disease lysine hyperacetylation of β-amyloid peptide results in impaired cognition [10]. Additionally, mouse models have demonstrated that alterations in histone acetylation pattern play a role in age-dependent memory impairment [11]. Thus, these studies indicate that targeting lysine acetylases and deacetylases might be promising avenue for developing novel therapies for neurodegenerative diseases. The widespread and dynamic nature of lysine acetylation and the nexus that exists between epigenesis-directed transcriptional regulation and metabolism has been comprehensively reviewed [19].

3. Carbonylation

Protein carbonylation can result from excessive oxidative stress in a biological system. It is an irreversible PTM which may lead to the formation of nonfunctional proteins, in turn leading to many diseases. Many disorders including autoimmune diseases and cancer are mediated by increased production of reactive oxygen as well as nitrogen species (ROS and RNS) in the cell. However, the role of ROS and RNS in protein oxidation is less understood. Protein carbonylation has received significant attention as an indicator of oxidative or genotoxic stress [20, 21]. Investigation of the Murphy Roth's Large (MRL) mouse model, used widely in tissue regeneration studies, suggested that common environmental contaminants like trichloroethene (TCE) increased ROS and RNS leading to the production of high amounts of nitrotyrosine and protein carbonyl(s), a classic hallmark response to high oxidative stress [22]. These byproducts are seen to induce a number of autoimmune diseases like systemic sclerosis and fasciitis. Elevated levels of protein carbonyls and nitrotyrosine have also been observed in other instances with high oxidative stress, mitochondrial disorders, indicative of hypocitrullinemia and loss of glutathione (iGSH) [23]. Thus, carbonylation of amino acids like proline, arginine, lysine, threonine, glutamate, and aspartate (see Table 1) is irreversible and results in a nonfunctional protein, which then participates as a mediator in a number of chronic diseases, especially the ones that are influenced by the status of oxidative stress in a cell [24]. A recent proteomics study has detailed the identification of carbonylation pattern as a fingerprint for oxidative stress [25]. The carbonylation pattern can thus be an informative

Table 1: Comparative analysis shown for 20 amino acids with possible functional modification(s) observed for each amino acid residue. For more information, refer to the text and citations therein.

Amino acids	Acetylation	Carbonylation	Glycosylation and glycation	Hydroxylation	Methylation	Nitration	Palmitoylation	Phosphorylation	Sulfation	Ubiquitination
Alanine	√									
Isoleucine	√									
Leucine	√									
Valine	√									
Phenylalanine				√						
Tryptophan			√	√		√				
Tyrosine			√	√		√		√	√	
Asparagine			√	√						
Cysteine		√					√			
Glutamine					√					
Methionine					√					
Serine	√		√		√			√		
Threonine	√	√	√					√		
Aspartic acid	√	√		√						
Glutamic acid	√	√								
Arginine	√	√	√		√					
Histidine	√	√			√					
Lysine	√	√		√	√					√
Glycine	√									
Proline	√	√		√						

tool for the identification of stress as well as a marker for therapy for many mitochondrial, neurological, and cardiovascular diseases. Another proteomics study conducted in mouse model for an early stage of alcoholic liver disease (ALD) identified biomarkers for early stage ALD wherein a carbonylated protein expression pattern was highlighted [26].

At least one study that focused on sepsis in mouse models concluded that N-acetylcysteine, an antioxidant, induces cellular antioxidant defense and prevents nitration of tyrosine residues and protein carbonylation. These results indicate the therapeutic potential of N-acetylcysteine for treating sepsis patients [27]. Protein carbonylation, particularly of UCH-L1 forming carbonyl-modified UCH-L1 and its interaction with other proteins like tubulin, was thought to be one of the causes of familial as well as sporadic Parkinson's disease (PD) [28]. The carbonyl-modified UCH-L1 is proposed to be an investigative tool to explore the underlying molecular mechanism of PD development [28]. This carbonyl modification may be of therapeutic value targeting the familial and/or sporadic PD [28]. Choi et al. demonstrated the irreversible carbonylation of protein to methionine sulfone as an indicator of oxidative stress damage in cases of the sporadic PD and Alzheimer's as well as other neurodegenerative diseases [29]. In recent years, proteomic tools and methods for the identification of sites of protein carbonylation have been widely developed [30].

4. Glycosylation and Glycation

Another well-studied cotranslational and posttranslational mechanism is the addition of a sugar moiety to proteins, lipids, or other organic molecules inside or outside of the cell. Glycosylation being an enzyme-directed reaction is site as well as substrate specific, tightly regulated and reversible. On the other hand, glycation is a random event that most often leads to the formation of defective or non-functional biomolecules.

Glycans resulting from glycosylation are classified under one of five known classes: N-linked glycans, O-linked glycans, C-linked glycans, phosphoglycans, and glypiation (GPI-anchored). O-linked glycans have been shown to participate in the diverse cellular processes and development [31, 32]. Glycosylation, a covalent modification, plays a central role in protein localization, protein-protein interactions, structural stability of the cell, immune responses, and modulating of cell signaling [32, 33]. Thus, any dysfunctional glycans formed in the cell could lead to diseases including cancer, liver cirrhosis, diabetes, and exacerbated HIV infection [34, 35]. A novel glycosylation prediction tool developed by Szabá et al. utilizes currently available databases of the T-cell antigens and autoantigens glycosylation [36].

O-glycosylation, as well as phosphorylation, has been shown to have a beneficial effect in Alzheimer's disease by reducing the formation of neurofibrillary tangles in neurons [37]. Glycosylation of prion (PrP), a cell surface protein and a transmissible agent, is a determinant of the final disease outcome in the host [38]. Recent characterization of glycosylation sites on apolipoprotein E (apoE) revealed a novel glycosylation site in addition to the already known sites as well as at least 8 new complex glycans in secreted and cellular apoE [39]. The involvement of apoE in Alzheimer's disease, atherosclerosis, and immune responses is well documented, and this novel information can help gain insight towards understanding the mechanistic role of glycosylated apoE residues in these diseases [39]. Improper or incomplete glycosylation in the Fc receptor for immunoglobulin A has been shown to impact the IgA-mediated immune response which in turn affects many diseases including HIV, alcoholic liver cirrhosis, and other neuropathies [34, 40].

Other glycation products called (AGEs) have been implicated in cardiovascular diseases, cataract, and diabetes mellitus apart [41, 42]. The end products are commonly used as markers to evaluate the disease prognosis since inhibiting a subclass of the AGEs has been shown to benefit physiological conditions like diabetes and arthrosclerosis [43–46]. Finally, glycosylation is shown to be a contributing factor in cancer cell transformation via Src(s) as well as in regulating various signaling pathways like Wnt-β catenin pathway, thereby affecting the disease physiology and final outcome [47].

5. Hydroxylation

Hydroxylation is an important detoxification reaction in the cell and is mostly facilitated by the group of enzymes called hydroxylases. It is also one of the few reversible, post-translational modifications and hence has a prominent relevance to the cellular physiology.

Proline hydroxylation-mediated modification of collagen has been studied extensively since it has significant implications on the structural physiology of the cell [48]. Some cancers or metabolic disorders like scurvy are linked to the lack of proline hydroxylation due to ascorbate deficiencies, an important component of the reaction [49]. The enzyme prolyl 4-hydroxylase that catalyzes the conversion of 4-hydroxyproline to collagen, is one of the most well-studied enzymes in this group [50, 51].

Proline hydroxylation is an important step in activating antioxidant defense against hypoxia via hypoxia inducible factor (HIF) [52]. Under normoxia, proline hydroxylation acts as a regulatory step for HIF1α and 2α to bind to the von Hippel-Lindau tumor suppressor (pVHL) E3 ligase complex, which targets both the factors for rapid degradation by ubiquitin-proteasome complex [52]. Under hypoxic conditions, however, the abrogation of proline hydroxylation as well as asparagine hydroxylation is necessary for the continual action of the HIF transcription factor. Thus, hydroxylation of asparagine and proline acts as a "hypoxic switch" for induction of HIF under the low oxygen conditions [53]. Further, proline hydroxylation of HIF similar to that under normoxic conditions was shown to be protumorigenic [54, 55]. The inhibition of normoxic HIF1α was suggested as a therapeutic alternative in such cases [55, 56]. A number of *in silico* prediction tools augment the understanding of the complexity of the process as well as designing novel therapies for diseases like cancer and cholestatic liver disease [57].

Other amino acids that undergo hydroxylation include phenylalanine, tyrosine, and tryptophan, all of which have aromatic side chains [58]. Several genetic diseases are linked to the lack of hydroxylation of aromatic amino acids like that of phenylketonuria (PKU) and hyperphenylalaninemia, due to a defect in phenylalanine hydroxylase, an enzyme that converts phenylalanine to tyrosine [59]. Tyrosine hydroxylase is used as a molecular target to treat hypertension [60–62]. This enzyme is also known to act as an autoantigen in autoimmune polyendocrine syndrome (APS) type I. On the other hand, tryptophan hydroxylation, catalyzed by tryptophan hydroxylase, is a critical regulatory step in the production of an important neurotransmitter, serotonin [63].

6. Methylation

Protein methylation has a tremendous impact in health and disease, spanning from embryonic to postnatal developmental stages in numerous physiological conditions such as cancer, lipofuscinosis, and occlusive disease [64–68]. The most commonly methylated amino acid residues are lysine and arginine with lysine methylation receiving special consideration due to its role in epigenetics and chromatin remodeling [69].

Histone methyltransferases (HMTs), also sometimes referred to as the histone lysine methyltransferases (HKMTs), specifically target lysine residues in histones, which regulate gene expression [69]. Histone methylation together with the histone acetylation also has been shown to control cellular RNA synthesis including activation and inhibition of specific RNAs types, metabolism, and degradation *in vivo* [69, 70].

In addition, there is a growing number of lysine methyltransferases that methylate nonhistone proteins on lysine residue that are being constantly identified [69]. These can methylate proteins like p53 (Figure 1), ERα, NF-κB, and pCAF and other transcription factors that have been implicated in tumorigenesis and other metabolic disorders like inflammatory and immune responses. In addition to regulating gene expression, they regulate the protein stability by dominating the downstream effector responses that are responsible for the cell fate [69].

Postnatal developments like erythropoiesis, development of immune system, and other cell signaling cascades are regulated not only by chromatin methylation/demethylation but also by targeting specific amino acid residues for post-translational modification(s) in a given context [64, 65, 71].

The role of methyltransferases in various diseases has been widely noted. In the case of lysosomal storage diseases, methylation of a specific lysine of the mitochondrial ATP synthase plays central role in the accumulation and storage of this protein in the form of aggregates in the lysosomal bodies [68]. Levels of homocysteine, a methylation product of methionine, plays a major role in cardiovascular diseases as well as neurological disorders like Parkinson's disease [66, 72]. These diseases are exacerbated by the excessive accumulation of homocysteine due to the lack of removal or inefficient metabolism of this compound [72]. However, optimal amount of homocysteine is necessary for the normal functioning of the body, and its metabolism is extremely sensitive to internal vitamin B levels. [72]. Therapeutic approaches targeting plasma homocysteine are being proposed to counter the harmful effects of elevated homocysteine in patients [72]. Inaccurate methylation of oncoproteins in various cancers is commonly observed in conjunction with the upregulation of many lysine demethylases [67].

7. Nitration

Protein nitration and carbonylation are the by-products of the protein oxidation reactions. Nitration is a reversible and a stable post-translational modification that is initiated when amino acids are exposed to nitrating agents or oxidative stress [59]. Formation of protein carbonyls and nitroderivatives is classic hallmark of exposure to genotoxic stress [73]. Nitroproteins are thought to be involved in a plethora of diseases. Nitrotyrosine, a chief nitration product, is associated with many neurodegenerative diseases, lung diseases, inflammation, cardiac diseases, and cancer [74]. In addition, nitrotyrosine has been implicated in the regulation of various cell signaling pathways thus activating or inhibiting certain cellular transduction signals depending on the context of cellular physiology at any given time [75, 76].

A recent study has highlighted that the conversion of a tyrosine 253 of HDAC2 to nitrotyrosine not only abrogates its activity in the cell but also targets it for rapid proteasomal degradation and ultimately affects the gene regulation in the cell. This study is particularly important for novel cancer therapies that explore the option of HDAC inhibitors [77]. Furthermore, this study proposes a mechanism explaining the underlying effect of nitrosative stress in the context of a neoplastic transformation. It should be noted that certain flavonoids have been shown to inhibit protein nitration as well as induce cellular antioxidant defense response [78, 79]. Such phytochemicals seem to have a potential therapeutic value, especially in cases of cancer and ischemic retinopathy. At least one mechanistic study has provided strong evidence in recommending dietary supplements like epicatechin and N-acetylcysteine (NAC), both of which inhibit tyrosine nitration, to alleviate diabetic retinopathy and ischemic retinopathy that result from excessive nitrosative stress [79].

An *in vitro* mass spectrometric analysis of Lewy bodies in Parkinson's disease was able to capture the classical hallmark of the disease, an increase in 3-nitrotyrosine (3-NT) modification for various proteins [80]. Such elegant studies not only further our understanding of protein nitrosylation but also shed light on the intrinsic complexity of this modification that can be useful in designing targeted therapy for such a debilitating neurological malady [81].

Additionally, 3-NT and nitrated A2E proteins have recently been proposed as biomarkers for age-related macular degeneration (AMD) since their accumulation increases with age and specifically with increased nitrative stress [82]. 3-NT is routinely used as a biomarker for protein damage that is induced by oxidative inflammation [83]. Continual overproduction of nitric oxide or NO is a classical marker of the cellular inflammation, and this overproduced NO not

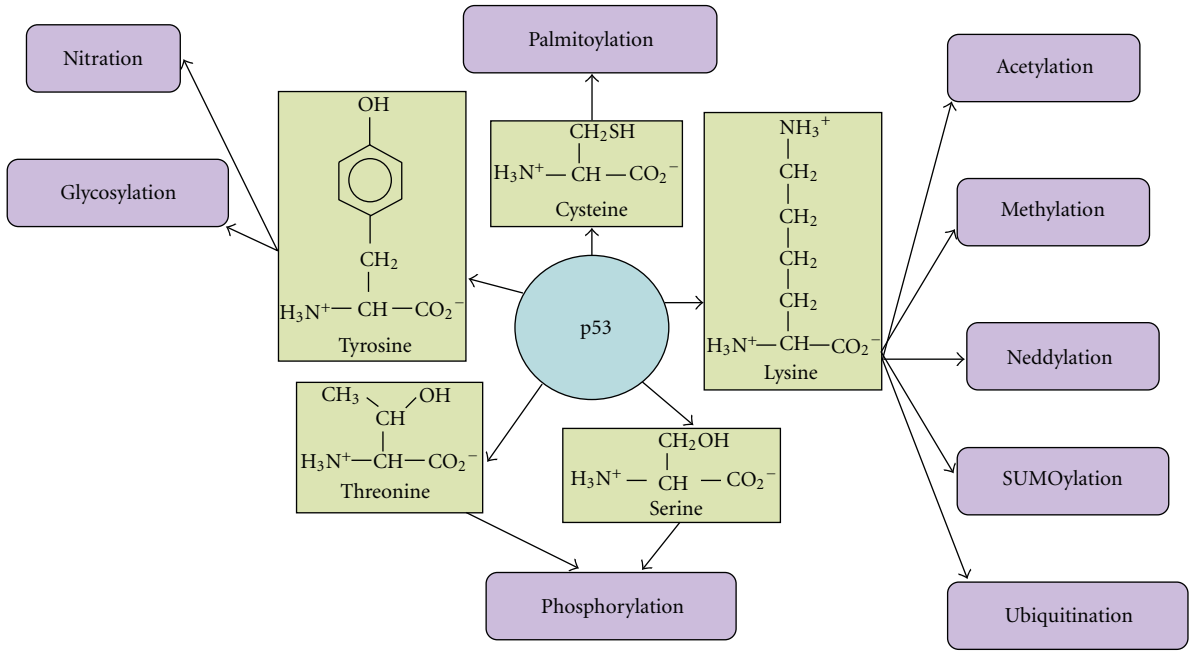

Figure 1: Post-translational modifications for p53, a tumor-suppressor protein, responsible for maintaining the genomic stability in a cell. The figure illustrates various posttranslational modifications that are frequently observed in p53 with varied functional implications in normal and/or diseased condition. The amino acid residues that most often undergo the respective modifications in a given context have been highlighted. For details of posttranslational alterations in p53 refer to [113–127].

only can damage the organelles but is also a contributing factor for cell death mainly by mediating apoptosis [84]. Nitrative controlled apoptosis in hepatic stellate cells (HSCs) is crucial because protein nitration plays a significant role in liver fibrosis prognosis [84].

Peroxynitrite, another harmful nitration product formed from NO and superoxide ion, along with nitrotyrosine was shown to contribute to amyloid β-peptide-induced toxicity and tau protein neurofibrillary tangles in Alzheimer's patients [85]. Peroxynitrite and cellular nitroproteins are also linked with inflammation of human colonic epithelium, a symptom of irritable bowel syndrome (IBS) [86].

8. Palmitoylation

Palmitoylation is a unique reversible cysteine thioaylaction modification that consists of covalent attachment of the fatty acid, mainly palmitic acid, to the protein molecule [87, 88]. This modification is unique, partly because of its reversible nature as compared to the other lipid-protein modifications like prenylation/isoprenylation and myristoylation, both of which are irreversible and co-translational reactions. However, these reactions tend to make proteins more hydrophobic as they add lipid molecule(s) to the protein structure. These modifications are also involved in cell trafficking, membrane stimuli, and protein-protein interactions [87, 88]. Palmitoylation was shown to play a regulator role in the G-protein linked cell signaling pathways via modification of the regulators of G protein signaling (RGS) proteins [89]. Palmitoylation of a specific cysteine residue in the RGS protein not only regulates the activity of the protein but also is an important mediator for localization and targeting of certain G-proteins and for modulating G-protein signaling [89]. However, in the case of thyrotropin-releasing hormone (TRH) receptor type 1 (TRH-R1), palmitoylation is not required specifically for G-protein signaling but for maintaining the inactive form of the receptor, since the unpalmitolyated form is constitutively active and leads to oversecretion of thyrotropin and prolactin [90]. Apart from the G-protein trafficking, palmitoylation is closely involved in the neural inflammatory response including demyelinating diseases as well as T-cell autoimmune responses [91]. Hence, a novel approach using the stability of palmitoylated proteins to present as antigens to the MHC-class II immune response is being proposed in order to benefit the patients with autoimmune diseases including multiple sclerosis [91].

Reduced or dysfunctional palmitoylation has been linked to many diseases and disorders [92–96]. In diabetic vascular disease, efficient palmitoylation of the endothelial nitric oxide synthase (eNOS) is a required step for efficient bioavailability of eNOS so it can be targeted to the plasma membrane [93]. Lack of eNOS palmitoylation, as observed in insulin-deficient or insulin-resistant patients, leads to the chronic inflammatory response in these patients [93]. Inefficient palmitoylation of the γ-secretase, a component of the β-amyloid aggregate, in Alzheimer's disease adversely affects the proper trafficking and functional potential of the neurons [92]. Again, lack of the distinct cysteine palmitoylation of the Huntington protein (HTT), a player in Huntington's disease,

increases neural toxicity and enhances rapid formation of inclusion bodies, further deteriorating the patient prognosis [94].

In some of the disorders, protein palmitoylation can worsen the disease outcome. Cysteine 172 residue of Hepatitis virus C core protein (that forms the viral nucleocapsid) undergoes an essential step of palmitoylation, in order to efficiently multiply the virion particles and thus sustain an active infection in the host cells [95]. Palmitoylated oncogenic NRAS is a proposed target for developing therapies against NRAS-associated malignancies like acute myeloid leukemia (AML) as well as other types of NRAS-amplified leukemias [96]. With such a contradictory role for the palmitoylation, researchers have developed novel probes that can be utilized for the fluorescence microscopy and mass spectrometry analysis of protein palmitoylation [97].

9. Phosphorylation

Phosphorylation, addition of a phosphate group to an amino acid, is one of the central reversible, post-translational modifications that regulate cellular metabolism, protein-protein interaction, enzyme reactions, and protein degradation for a myriad of proteins, which results in intracellular signaling cascades [98, 99]. This reaction is mediated by a number of protein kinases (PKs) in the cell. Conversely, dephosphorylation or removal of a phosphate group is an enzymatic reaction catalyzed by various phosphatases (PPs) [98]. The ERK1/ERK2-MAPK signaling (mitogen-activated protein kinase), a central cell proliferation pathway which intercepts with the receptor tyrosine kinases (RTKs) pathway, and cell cycle progression proteins like cyclin-dependent kinases (CDKs) are some of the networks that are affected by the phosphorylation/dephosphorylation status of proteins [98]. Thus, a proper balance of action between the PKs and PPs is a key to maintain cellular homeostasis. Autophagy, a cell death mechanism, is also phosphorylation-dependent [100]. At least, one report has shown that the autophosphorylation event of the Atg1 protein is a "regulatory switch" that determines the initiation of the process [100].

Serine/threonine (Ser/Thr) and tyrosine are the most commonly observed phosphorylated amino acid residues and have been frequently implicated in progression of cancers [101–103]. For example, okadaic acid present in shell fish poisoning rapidly stimulates Ser/Thr phosphorylation in an intact cell while simultaneously inhibiting many phosphatases thus inducing phosphorylation-mediated signaling cascades which promote uncontrolled cell proliferation [102, 104]. Dysregulated phosphorylation has been implicated in neurological diseases like Parkinson's and dementia that harbor the accumulation of the Lewy bodies [104]. Ser-129 phosphorylation of α-synuclein protein is responsible for the build-up of proteolytic Lewy aggregates [104]. In case of lung cancer, at least one distinct threonine (T163) phosphorylation event on the protein Mcl-1, induced by nicotine (an active ingredient of tobacco), is responsible for chemoresistance in these tumors [105]. Thus, a single phosphorylation event in this case is responsible for cell survival due to blocking of the antiapoptotic function of the protein Mcl-1 and promoting tumorigenesis. The role of protein phosphorylation/dephosphorylation in cancers and their huge impact in disease pathophysiology has been extensively reviewed multiple times over the years, with a steady stream of new discoveries of protein phosphorylation and their effects in disease pathology [106–109]. The phosphorylation of eNOS, on the other hand, is a key to its own regulation that is central in many inflammatory and autoimmune diseases [110]. The regulation of the NF-κB cascade, which controls chemokine and cytokine responses and inflammation, is activated in response to the stimuli via phosphorylation. This abnormal phosphorylation of the NF-κB cascade is a classical hallmark of cancers and chronic immune disorders [111]. Thus, the molecular targeting of specific kinases and phosphatases is seen as a promising strategy in treating cancer as well as other inflammatory diseases [111, 112].

The effects of the process of protein phosphorylation on cell physiology are enormous. As such, it is beyond the scope of this paper to cover every aspect of this ubiquitous modification. However, we have made an effort to highlight few important features of phosphorylation itself as well as combined effects with other PTMs.

10. Sulfation

N-sulfation or O-sulfation, facilitated by the addition of a sulfate group by oxygen or nitrogen, respectively, is another post-translational protein modification, commonly observed for membrane as well as secreted proteins [128–130]. Sulfated proteins have been observed to play a role in protein-protein interactions, G-protein receptor signaling, chemokine signaling, and immune responses [131, 132]. However, their precise role in cellular regulation still remains somewhat enigmatic [129]. Gao et al. showed that tyrosine sulfation was involved in cellular calcium transportation and mediating association between the chemokine receptor (CXCR3) and IFN γ-inducible protein-10 (IP-10) [133].

As mentioned above, tyrosine sulfation is a key player in many diseases including autoimmune response, HIV infection, lung diseases, multiple sclerosis, and cellular enzyme regulation [131, 134–137]. Heavy sulfation of high-molecular weight glycoconjugates (HMGs) produced by cystic fibrosis (CF) respiratory epithelia was shown to adversely affect the association between HMG and airway secretions and possibly create a breeding ground for harmful bacteria like *P. aeruginosa* and *S. aureus* in the CF airways and thus contributing to the pathogenesis of the disease [136]. Other lung diseases like chronic obstructive pulmonary disease (COPD) and asthma are equally aggravated due to induction of chemokine signaling by the tyrosine sulfation and thus affecting the downstream molecular players along with the leukocyte trafficking and airway inflammation [135, 138].

Tyrosine sulfation still remains one of the major post-translational modifications that is involved in multiple disorders [131, 135, 136, 138]. Thus it is also being proposed as a molecular target for developing a prophylaxis against HIV1 infection as it greatly diversifies the antigen availability and presentation beyond the standard 20 amino acids

[134]. Given its role and potential as a target for the drug development, there are few sulfation site(s) prediction tools, like that of random forest algorithm, being tested [139]. Techniques like mass spectrometry that are highly sensitive and specific for "sulfoproteome" analysis due to the presence of the sulfoester bond in the sulfated amino acids are frequently used [129, 140].

11. Ubiquitination

Ubiquitination is a highly dynamic, coordinated, and enzymatically catalyzed post-translational modification that targets proteins for degradation and recycling [141]. Proteins that are targeted for degradation are tagged by a covalent attachment of a small regulatory protein, ubiquitin (Ub) [141, 142]. This process is called ubiquitination which is a multistep enzymatic process [142]. It consists of three main enzyme classes that act in a specific order: Ub activating enzymes (E1), Ub conjugating enzymes (E2), and Ub ligases (E3) [143]. Proteins targeted for degradation can be mono-Ub or poly-Ub which is dependent on the type and localization of the substrate. Some of the notable multi-subunit E3(s) are anaphase-promoting complex (APC) and the SCF complex (Skp1-Cullin-F-box protein complex) that generally destine the target protein for the proteasomal degradation.

Another group of proteins called ubiquitin-like proteins (ULPs), which also follow the traditional path of sequential E1-E2-E3 processing for undergoing ubiquitination modification, need a mention [144]. They modify cellular targets in a pathway that is parallel to Ub but they maintains its distinctiveness [144]. Three of these ULPs that have received a lot of attention are NEDD8, Sentrin/SUMO, and Apg12 [144]. Ubiquitination is critical in almost every cellular process as well as a major player in almost any disease or disorder. Ubiquitination has a role in modulating diverse cellular functions like cell proliferation and differentiation, autophagy, apoptosis, immune response, DNA repair, neural degeneration, myogenesis, and stress response [145–147]. It also affects the outcome of many life-threatening diseases like cancer, neurodegenerative disorders, HIV infection, Herpes, and liver diseases [148–151]. A bi-functional ubiquitin editing protein called A20 was shown to regulate NF-κB signaling, affecting gene transcription, cell proliferation, and inflammatory responses [147]. It is also linked with the inhibiting Beclin-1 ubiquitination, an autophagy inducer protein, thus limiting the autophagic response [145]. A transcription factor that regulates the cellular antioxidant defense, NFE2L2, is stabilized and thus protected from the Ub-proteasomal degradation via six conserved cysteine residues on the N-terminal of the NFE2L2 [128]. NFE2L2-mediated gene regulation has been proposed as a therapeutic alternative not only in cancer but also for neurodegenerative diseases that show high oxidative stress like Batten's and Parkinson's diseases [152, 153]. Even though, these studies propose usage of ubiquitination inhibitors that are effective in cancer and neurological diseases as they stabilize tumor suppressor proteins and antioxidant defense mediators, the process of proteasomal degradation can be equally helpful in alleviating these diseases [154, 155]. One of the examples where ubiquitination might be beneficial is the proteasomal degradation of nuclear as well as oncogenic IκB protein, a player in the NF-κB signaling [147]. However, there are at least a few drugs, for example, Bortezomib, an FDA approved cancer treatment drug, that are inhibitors of ubiquitination and commonly used as a part of the treatment regimen for the cancer and other neural diseases [154].

12. Conclusion

Despite considerable efforts to understand the relevance of posttranslational modifications in the cellular context, we are still in the process of unraveling the complexity of these modifications and their tremendous impact. Sophisticated technological advances like high resolution mass spectrometry and reliable *in silico* tools are now increasingly available for identification and characterization of these site-specific protein alterations. One such novel application is the role of glycosylation resulting in the formation of disorderly proteins or intrinsically unstructured proteins (IUPs). Disorderly proteins are newly discovered proteins that are heavily modified by the post translational mechanisms resulting in non-functional or dysfunctional protein molecules. These proteins have been shown to play an essential role in gene transcription, protein expression, enzyme activities, cell signaling cascades, and so forth. Due to their role in disease pathology and cellular homeostasis, these protein molecules are actively sought after molecular targets for developing drugs for cancer treatment as well as other chronic diseases. Other well-known protein modifications like phosphorylation are key players in expanding the avenue of translational medicine for heterogeneous diseases like cancer.

With the constant addition of new post-translational modifications, verification of newly identified proteins changes by traditional methods and correlating the biological significance is a challenging task. We are just beginning to grasp the enormity of the field and its effect on the normal development and disease pathophysiology.

Continued search and evaluation of various functional modifications of proteins and understanding their interaction in various biological pathways have important implications in the successful development of novel prognostic markers as well as therapeutic targets for cancer, severe neurodegenerative diseases, and other debilitating genetic disorders.

Conflict of Interests

The authors have no conflict of interests.

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Review Article

Role of Charged Residues in the Catalytic Sites of *Escherichia coli* ATP Synthase

Zulfiqar Ahmad,[1] Florence Okafor,[1] and Thomas F. Laughlin[2]

[1] *Department of Biology, Alabama A&M University, P.O. Box 610, Normal, AL 35762, USA*
[2] *Department of Biological Sciences, East Tennessee State University, Johnson City, TN 37614, USA*

Correspondence should be addressed to Zulfiqar Ahmad, zulfiqar.ahmad@aamu.edu

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Here we describe the role of charged amino acids at the catalytic sites of *Escherichia coli* ATP synthase. There are four positively charged and four negatively charged residues in the vicinity of of *E. coli* ATP synthase catalytic sites. Positive charges are contributed by three arginine and one lysine, while negative charges are contributed by two aspartic acid and two glutamic acid residues. Replacement of arginine with a neutral amino acid has been shown to abrogate phosphate binding, while restoration of phosphate binding has been accomplished by insertion of arginine at the same or a nearby location. The number and position of positive charges plays a critical role in the proper and efficient binding of phosphate. However, a cluster of many positive charges inhibits phosphate binding. Moreover, the presence of negatively charged residues seems a requisite for the proper orientation and functioning of positively charged residues in the catalytic sites. This implies that electrostatic interactions between amino acids are an important constituent of initial phosphate binding in the catalytic sites. Significant loss of function in growth and ATPase activity assays in mutants generated through charge modulations has demonstrated that precise location and stereochemical interactions are of paramount importance.

1. Introduction

A typical 70 kg human generates approximately 2.0 million kg of ATP, the cell's energy currency, in a 75-year lifespan by converting food into useable energy by oxidation. ATP is generated by ATP synthase from ADP and inorganic phosphate (Pi) [1, 2]. ATP synthase is not only the essential means of cellular energy production in animals but also in plants and almost all microorganisms. ATP synthase is the final enzyme in the oxidative phosphorylation pathway and is responsible for ATP synthesis by oxidative or photophosphorylation in the membranes of bacteria, mitochondria, and chloroplasts. It is the smallest known biological nanomotor. In order to synthesize ATP, a mechanical rotation mechanism is used where subunits rotate at approximately 100 times per second. Basic [3] functional aspects of ATP synthase remain the same in both prokaryotes and eukaryotes [4].

Membrane bound F_1F_o ATP synthase enzyme is structurally identical and highly conserved among different species. ATP hydrolysis and synthesis occur in the F_1 sector, whereas proton transport occurs through the membrane embedded F_o [2, 5]. ATP synthesis is the result of proton gradient-driven clockwise rotation of γ (as viewed from the outer membrane), while ATP hydrolysis occurs from anticlockwise rotation of γ-sub unit. Detailed reviews of ATP synthase structure and function may be found in [6–16].

A number of diseases such as Leigh syndrome, ataxia, Batten's diseases, Alzheimer's, angiogenesis, hypertension, cancer, heart disease, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, and tuberculosis that affect both human and animals have been associated with ATP synthase ([1, 17] and references therein). The presence of ATP synthase on the surfaces of multiple cell types, and its involvement in a number of cellular processes, makes this enzyme an attractive molecular target in the development of treatments for numerous diseases. [18–21]. One particular way in which ATP synthase can be used as a therapeutic target is to inhibit it and thereby deprive

abnormal cells of required energy leading to cell death [1, 17, 21, 22].

2. Inhibition of ATP Synthase

A wide range of natural and synthetic products are known to bind and inhibit ATP synthase [1, 17, 23, 24]. Biochemical and structural studies of ATP synthase have so far revealed about ten different inhibitor binding sites. A detailed list of known inhibitors and their actions on ATP synthase are discussed in reference [1, 17]. The inhibitory effects and the extent of inhibition on a molar scale are variable among different inhibitors. Some inhibitors prevent synthesis of ATP but not hydrolysis, or vice versa, while some are known to inhibit both synthesis and hydrolysis equally. Well-known inhibitors of ATP synthase are sodium azide (NaN3), aluminum fluoride (AlFx), scandium fluoride (ScFx), beryllium fluoride (BeFx), dicyclohexylcarbodiimide (DCCD), and 7-chloro- 4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) [11, 24–32]. Less well-known inhibitors of ATP synthase are peptides such as melittin, melittin-related peptide (MRP), ascaphin, aurein, caerin, dermaseptin, magainin II, and polyphenols such as resveratrol, piceatannol, quercetin, morin, and epicatechin [1, 18, 19, 21, 33–35].

The polyphenol piceatannol is one of the most portent inhibitors of ATP synthase [19, 22]. The binding site for polyphenols is at the interface of α-, β-, and γ-subunits of the F_1 sector. X-ray structure shows that the following polyphenol binding pocket residues γGln274, γThr-277, βAla-264, βVal-265, γAla-270, γThr-273, γGlu-278, γGly-282, and αGlu-284, are highly conserved among different species and are within 4 Å of the bound polyphenol compounds. Consequently, piceatannol and other inhibitory polyphenols can form both hydrophobic and nonpolar interactions with the above residues [22, 36, 37]. We hypothesize that molecular modulation of both polyphenol-binding pocket residues and polyphenol structures may synergistically affect ATP synthase activity and provide additional clues to catalytic site function.

The βDELSEED-loop of *E. coli* ATP synthase is known to be the binding site for several basic amphiphilic α-helical peptide inhibitors of ATP synthase. Examples are melittin, melittin-related peptide (MRP), bacterial/chloroplast ATP synthase ε-subunit, and SynA2 (the synthetic derivative of cytochrome oxidase). The α-helical basic peptide, melittin, is composed of 26 residues and is the primary component of honey bee venom (*Apis mellifera*). MRP is a 23-residue long peptide derived from frog skin (*Rana tagoi*). Both melittin and MRP are potent inhibitors of ATPase activity of *E. coli* ATP synthase [1, 21, 38, 39].

Most ATP synthase inhibitory peptides studied so far are from anuran (frogs) sources. These antimicrobial peptides (AMPs) are cationic, between 10 and 50 residues in length, and frequently include a C-terminal amide group [1, 40–42]. Previous mode of action studies indicate that AMPs appear to interact with negatively charged phospholipids and then insert into the bacterial cell membrane or that they may also move across the cell membrane by passive transport and there disrupt a number of cellular processes [43].

Lately, it was observed that some of the antimicrobial effects of amphibian AMPs may be through their inhibitory effects on ATP synthase [1, 21]. Melittin and other peptide inhibitors inhibit ATPase activity in a reversible and non-competitive fashion [38, 39, 44–47]. It is hypothesized that relatively short antibacterial or anticancer cationic peptides of approximately 10–30 amino acid residues with α-helical secondary structure may inhibit ATP synthase through their binding to the βDELSEED loop. For example, lysine-induced three positive charges of dermaseptin or four positive charges of magainin II interact with the five negative charges of βAsp-380, βGlu-381, βGlu-384, βGlu-385, or βAsp-386 and result in the inhibition of ATPase activity.

Of nearly 60 anuran-derived potential antimicrobial/anticancer peptides, only 13 have been tested for their inhibitory effects on ATP synthase [1, 21]. It was shown that MRP (melittin-related peptide) and MRP-amide strongly inhibited the ATPase activity of ATP synthase and that the presence of an amide group at the c-terminus of MRP caused a ~16% increase in inhibition of ATP synthase ATPase activity. Whether or not negative charges in the βDELSEED motif play any role in the structural stability of the catalytic sites through electrostatic interactions with site residues remains to be seen.

3. Structural and Functional Aspects of Charged Residues in the Catalytic Sites of ATP Synthase

Recent studies have illuminated the role of charged residues in Pi binding at the catalytic sites. Binding of inorganic phosphate (Pi) is an important step in the ATP synthase mechanism which has been extensively studied by biochemical approaches and may be directly coupled to subunit rotation [2, 11, 25, 48–53]. ATP synthase is the terminal enzyme of oxidative phosphorylation and photophosphorylation that synthesizes ATP from ADP and Pi. The energy for ATP synthesis comes from the transmembrane movement of protons down an electrochemical gradient that is generated by substrate oxidation or by light capture. As protons move through the interface between the a and c subunits in the membrane-bound F_o-sector of the enzyme, the free energy is transduced into mechanical rotation of a group of subunits ($\gamma\varepsilon c_{10-14}$) which comprise the "rotor". The helical coiled coil domain of the γ-subunit projects into the central region of the $\alpha_3\beta_3$ hexagon in the membrane extrinsic F_1-sector. The $\alpha_3\beta_3$ hexagon contains three catalytic sites at α/β interfaces. The "Stator" subunits b_2 and δ function to prevent co-rotation of $\alpha_3\beta_3$ with the rotor [6, 54–58]. In this paper we present a detailed description of the catalytic site charged amino acids, their role in Pi binding, their effects on the spatial orientation, and effect of their modulation on one another.

Figure 1 represents the simplest form of ~530 kDa *Escherichia coli* ATP synthase containing eight different subunits, namely, $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10-15}$, divided into two sectors

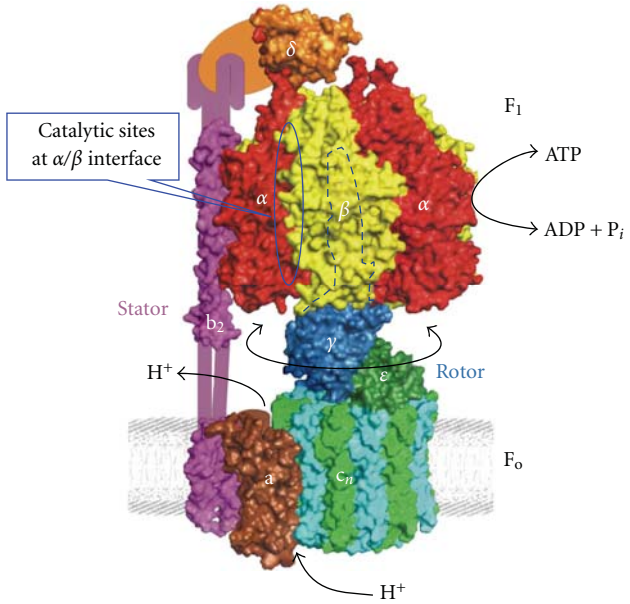

Figure 1: Escherichia coli ATP synthase structure: *E. coli* ATP synthase enzyme is composed of two sectors, water soluble F_1 and membrane bound F_o. Catalytic activity occurs at the interface of $\alpha\beta$/subunits of F_1 sector which consists of five subunits ($\alpha 3\beta 3\gamma\delta\varepsilon$) and proton conduction occurs at the Fo sector consisting of three subunits (ab_2c). One of the catalytic binding sites is identified with circle at the interface of α/β subunits. This model of *E. coli* ATP synthase is reproduced from Weber [6] with permission; copyright Elsevier.

F_1 and F_o. F_1 corresponds to $\alpha_3\beta_3\gamma\delta\varepsilon$ and F_o to ab_2c. Overall, F_1F_o-ATP synthase is structurally and functionally similar among sources with only a few exceptions such as in chloroplasts, where there are two isoforms, and in mitochondria, where there are 7–9 additional subunits. ATP hydrolysis and synthesis occur on three catalytic sites in the F_1 sector. The γ-subunit is comprised of three α-helices. Two of these helices form a coiled coil that extend into the central space of the $\alpha_3\beta_3$ hexagon. In recent nomenclature, the rotor consists of $\gamma\varepsilon c_n$, and the stator consists of $\alpha_3\beta_3\delta ab_2$. Current understanding of the F_1F_o ATP synthase structure, function, catalytic mechanism, and its role in human health and disease has been thoroughly reviewed by Senior's group and others [1, 2, 7, 8, 17, 20, 59].

Based on the binding of ATP, ADP, and Pi, the three catalytic sites located on the F_1 sector of ATP synthase are designated βTP, βDP, and βE by X-ray crystallographers [60, 61]. βE is the empty site into which Pi (inorganic phosphate) must initially bind for initiation of ATP synthesis. It has been proposed that the synthesis reaction in the three catalytic sites do not occur independently but in a consecutive manner [51]. In this "binding change mechanism", the three catalytic sites have different affinities for nucleotides at any given moment. Each catalytic site undergoes conformational transitions that lead to the following sequence: substrate binding (ADP and Pi) → ADP phosphorylation → ATP release. Experimental observations of rotation verified the predication made by Boyer [4, 51, 62] that catalysis requires the sequential participation of the three catalytic sites, with changing affinity for substrates and products, as it proceeds through the recurring mechanism, hence the term "binding change mechanism." Proton motive force is converted by F_o to a mechanical rotation of the rotor shaft, which drives conformational changes of the catalytic domains of F_1 causing synthesis of ATP by phosphorylation of ADP. Conformational changes in the catalytic sites are connected to γ-subunit rotation. γ-Subunit rotation in isolated $\alpha_3\beta_3\gamma$ subcomplex has been observed directly by Yoshida and Kinosita with colleagues in Japan and subsequently by several other labs [12, 63–68]. The focus of this paper, the role of charged residues at the catalytic sites of *E. coli* ATP synthase, is a fundamental issue, because catalytic site charged residues are also relevant to many other ATPases, GTPases, and their potential application to nanotechnology and nanomedicine [1, 11, 18, 69].

4. Catalytic and Motor Function of ATP Synthase

Determination of catalytic site Pi-binding residues has allowed a better understanding of the reaction mechanism of ATP synthesis and hydrolysis, and their relationship to the mechanical rotation of the γ-subunit. Characterization of catalytic site-charged residues can provide essential understanding in the following possible applications (1) development of effective modulator (inhibitory/stimulatory) molecules of ATP synthase catalytic function and (2) development of catalytic site mutants for biotechnological applications.

The analysis of charged catalytic sites residues involved in Pi binding has also helped answer the primary question of how the enzyme binds ADP and Pi rather than ATP at the catalytic sites? This is an often overlooked but crucial question in the mechanism of ATP synthesis. In active cells, the cytoplasmic concentrations of ATP and Pi are approximately in the 2–5 mM range, whereas that of ADP is at least 10–50-fold lower. Equilibrium-binding assays have established that both ADP and ATP bind to catalytic sites of purified F_1 and detergent solubilized F_1F_o with relatively similar binding affinities [71–74]. Obviously, a specific mechanism must have evolved for selectively binding ADP into catalytic sites while simultaneously preventing ATP binding during proton driven rotation and ATP synthesis. One hypothesis is that during ATP synthesis, the proton gradient-driven rotation of subunits impels an empty catalytic site to bind Pi tightly, thus stereochemically precluding ATP binding and, therefore, selectively favoring ADP binding [7]. A second fundamental question is how does subunit rotation affect Pi binding [49–51]? It was shown that Pi binding appears to be "energy linked", which entails a linkage to subunit rotation [56, 75, 76]. Therefore, for formulating a mechanism of ATP synthesis, it is of paramount importance to understand the features that determine Pi binding. Moreover, in the near future, it may be possible to use molecular features of Pi binding, derived from mutational and biochemical

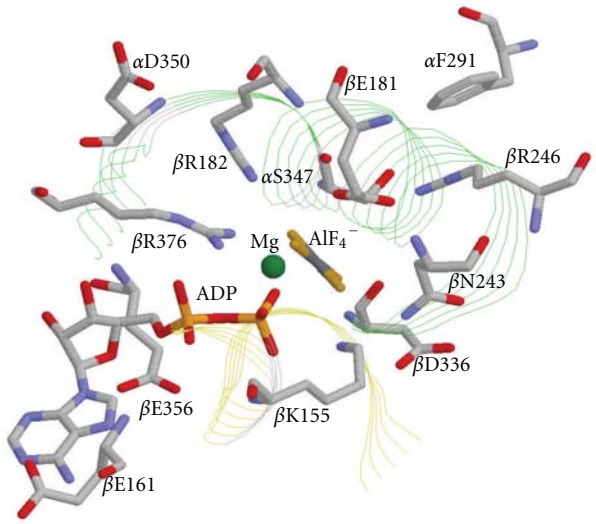

Figure 2: *Catalytic sites X-ray structure of ATP synthase showing spatial relationship of charged α and β-subunit residues.* The βDP site in the AlF_4^--inhibited enzyme structure is taken from [61]. *E. coli* residue numbering is used. Four positively and four negatively charged residue in close proximity to the bound phosphate analog AlF_4^- are identified. Rasmol software [70] was used to generate this figure using PDB file 1H8E [61].

studies, in the development of potent and novel molecular modulators of ATP synthase.

5. Characterization of Charged Residues at the Catalytic Sites

X-ray structural studies and mutagenic analyses of F_1 sector, the catalytic segment of ATP synthase, have so far identified four basic residues critical for catalysis, namely, αβLys-155, βArg-182, Arg-376, and βArg-246. Figure 2 shows the spatial orientation of these amino acids in close proximity to bound phosphate analog AlF_4^-. *E. coli* residue numbers are used throughout. Figure 2 also shows the spatial relationship between positive and negative charged residues.

Initial studies employed the MgATP- and MgADP-binding parameters in mutant enzymes βK155Q, βR182K, βR182Q, βR246A, βR246Q, βR246K, αR376K, and αR376Q. These studies used fluorimetric analysis with introduced βTrp-331 as a specific catalytic site probe, while analysis of the transition state formation was done using MgADP-fluoroaluminate and MgADP-fluoroscandium as transition state analogs [77–81]. Absent from these analyses was a direct measurement of Pi binding in the mutant enzymes. The above residues were clearly demonstrated to be involved in Pi binding with the subsequent application of Pi protection against NBD-Cl inhibition assays [11, 48, 82–85].

The βLys-155 is part of the Walker A sequence in the catalytic sites of ATP synthase. X-ray structures of native F_1 with bound MgAMPPNP and MgADP [5], of MgADP-BeFx inhibited F_1 [87], of MgADP-AlF_4- inhibited F_1 representing the transition state [61], and of MgADP-AlF_3 inhibited F_1 representing the late transition state/early ground state [88] all show the ε-amino group of βLys-155 very close (≤3 Å) to the γ-phosphate position. The βK155Q mutant lacks ATP synthesis and has very low F_1-ATPase activity (Table 1). Previous work had shown that βLys-155 plays a major role in binding MgATP, particularly at catalytic sites of high and medium nucleotide affinity, but not in binding MgADP [77]. βLys-155 is also critical for transition state formation [79, 80]. The earlier hypothesis that βLys-155 was important for Pi binding in ATP synthesis [2] was experimentally confirmed by Pi protection against NBD-Cl inhibition where Pi binding in the βE catalytic site is abolished in βK155Q. Therefore, residue βLys-155 is involved at all stages of ATP synthesis from Pi binding, to the transition state, to MgATP formation [84].

βArg-182 is another important positively charged phosphate-binding residue in the catalytic sites of ATP synthase [84]. Mutants βR182Q and βR182K lack ATP synthesis activity and have low F_1-ATPase activity (Table 1). Residue βArg-182 had been shown to be involved in MgATP binding at the site of highest affinity but not in MgADP binding. Transition state formation is abolished by βR182Q but retained in βR182K [78]. In this regard, it should be noted that βR182K F_1 does have somewhat higher ATPase activity (Table 1). βArg-182 was also hypothesized to be required for Pi binding in ATP synthesis [2], and

Table 1: ATPase activity of *E. coli* membrane bound or purified F_1 enzymes.

Mutation[a]	ATPase activity μmol/min/mg
Wild-type	28.0 (42.0)
Null	0.0013
βK155Q	(0.023)
βR182K	(0.250)
βR182Q	(0.020)
αR376K	(0.120)
αR376Q	(0.025)
αF291D	0.07
αF291E	0.09
βN243A	0.95
βN243D	0.033
βR246A	0.050 (0.25)
βR246K	(0.27)
βR246Q	(0.27)
βN243R	0.023
βN243R/βR246A	0.016
αF291R	0.035
αF291R/βR246A	0.52
αF291R/βN243R	0.028

[a]Wild-type, pBWU13.4/DK8; Null, pUC118/DK8. All mutants were expressed with the βY331W mutation also present, which does not significantly affect growth. Data are means of four to six experiments each. [b]Measured at 37°C and expressed as μmol ATP hydrolyzed/min/mg membrane protein. Each individual experimental point is itself the mean of duplicate assay tubes. Data in parentheses is from purified F_1 ATP synthase. Data taken from [48, 82–84, 86].

this confirmed that both βR182Q and βR182K mutations abolished Pi binding in the βE site. Therefore, residue βArg-182 is also involved in all stages of ATP synthesis from Pi binding through ATP formation [84].

The αArg-376 residue of *E. coli* ATP synthase has been described as the "arginine finger" based on G-protein literature and was thought to be a required ligand for the catalytic transition state. Nonetheless, this residue was not shown to be involved in MgATP or MgADP binding despite its apparent proximity to the γ-phosphate of MgAMPPNP in X-ray structures [65, 81]. Movement of this residue in and out of the catalytic site was inferred and was postulated to produce the rate acceleration ("positive catalytic cooperativity") linked to subunit rotation and full ("tri-site") catalytic site occupancy that is a hallmark of the mechanism [2]. Significant spatial displacements of residue αArg-376 have been noted in X-ray structures representing different reaction intermediates [5, 33, 61, 75, 87, 88]. Consequently, it was hypothesized that conformational freedom of this residue likely contributes to its importance in catalysis [87]. The previously hypothesized importance of this residue in catalysis [2] was confirmed by Pi protection against NBD-Cl inhibition in which Pi failed to protect αR376Q F_1 from NBD-Cl inhibition. However, just as the αR376K mutant was able to form the transition state [81], it was also able to support Pi binding. It is nevertheless strongly impaired in both ATP synthesis and hydrolysis, which suggests that this residue has other required function(s) such as in conformational movements or in H-bonding to other side chains that are specific to Arg and not supported by Lys [11, 84].

βArg-246 is the fourth positively charged residue within the Pi-binding subdomain of catalytic sites identified in the X-ray crystallographic structure (Figure 2) that is involved in Pi binding. βArg-246 is equivalent to βArg-260 in mitochondrial F_1 and is conserved among all species. Early random mutagenesis experiments revealed that mutations βR246H and βR246C impaired oxidative phosphorylation drastically and reduced ATPase activity in purified F_1 to ~1% of wild type [89, 90]. Further work showed that these mutations caused the unisite catalysis parameter K_dPi to change by 4 orders of magnitude, whereas the K_dADP was largely unaltered by βR246C, and the ATP hydrolysis reaction equilibrium constant changed to favor ATP over ADP plus Pi [91]. Computer simulations have drawn attention to βArg-246, where movement of the residue during rotation, conformational change of the sites [92], and a role in binding Pi in the transition state were predicted [93, 94]. Site-directed mutagenesis of βR246 to Gln, Lys, and Ala was used to examine the effects of each mutation on function. A variety of inhibitors and ligands known to bind or react in the catalytic sites close to the Pi-binding subdomain were utilized in combination with the mutant enzymes to establish the role of the βArg-246 side chain. Substitutions of the Arg side chain to Gln (removes charge and preserves bulk), to Lys (preserves positive charge), and to Ala (removes side chain and charge) were all examined. All three substitutions severely impeded growth by oxidative phosphorylation and reduced ATPase activity of purified F_1 to ~1% of wild type. Finally, as shown in Figure 3 Pi protection against NBD-Cl inhibition clearly demonstrated that βArg-246 residue side chain is an important constituent in binding Pi and in forming the transition state [48].

Pi binding assays using Pi protection against NBD-Cl were devoid of any nucleotide and enzymes were prepared so as to have all three catalytic sites essentially empty. Therefore, the sites were in βE conformation. In this conformation αArg-376 and βArg-246 lie 2.6 and 4.0 Å from βArg-182, whereas βLys-155 lies 9.5, 7.3, and 6.3 Å from αArg-376, βArg-182, and βArg-246, respectively [88]. In essence, the X-ray structure [87] showed that bound MgADP-BeFx mimicked bound MgATP. In assays of F_1-ATPase, it was shown that wild type and αR376Q were fully inhibited by MgADP-BeFx, whereas βK155Q and βR182Q were fully-resistant (Z. Ahmad, and A. E. Senior, unpublished work). These results supported the hypothesis that βLys-155 and βArg-182 are MgATP ligands, but that αArg-376 is not, and that the involvement of stringent stereochemical orientation factors plays a role in determining the functional interactions of αArg-376 [11, 84].

The four positively charged residues form a tetrahedral structure with βLys-155 at the apex and αArg-376, βArg-182, and βArg-246 on the base [11, 25]. A potential Pi-binding pocket can readily be envisaged at the center of this tetrahedron (see Figure 2). In ATP synthesis, the βE site will change to the βADP + Pi ("half-closed") site in association with γ-rotation [2, 61]. The X-ray structure of this conformation [61] shows that the residues αArg-376, βLys-155, and βArg-182 are each located ≤3.0 Å from the nearest oxygen atom of bound SO_4^{2-}) anion (modeling Pi), whereas βArg-246 is 4.5 Å from the sulfate. Thus, as the reaction proceeds, the three residues αArg-376, βLys-155 and βArg-182 close around the Pi and move it away from βArg-246 toward the site of transition state formation [11, 12, 48, 95].

The above results supported the following proposed molecular mechanism for ATP synthesis [2]. Initially, substrate Pi binds in the βE catalytic site using four basic residues as ligands, namely, αArg-376, βArg-182, βLys-155, and βArg-246 [11, 25, 48, 82–84, 86]. After binding of MgADP (in which these four residues are not involved), the catalytic transition state forms using αArg-376, βArg-182, and βLys-155 as direct ligands. Upon formation of MgATP, αArg-376 withdraws and no longer interacts, whereas βLys-155 and βArg-182 are still bound to the γ-phosphate. MgATP is released to the medium with the breaking of these bonds.

Historically, many attempts to measure Pi binding in purified *E. coli* F_1 using [^{32}P] Pi [50] or by competition with ATP or AMP-PNP in fluorescence assays of nucleotide binding [72, 96] failed to detect appreciable Pi binding at physiological Pi concentration. An assay devised by Perez et al. [97] in which the protection afforded by Pi against the inhibition of ATPase activity was induced by covalent reaction with 7-chloro-4-nitrobenzo-2-oxa-1, 3,-diazole (NBD-Cl) provided a measure of Pi binding. Orriss et al. [98] showed by X-ray crystallography that the covalent adduct formed by NBD-Cl is specifically in the βE catalytic site. Hence, protection afforded by Pi indicates that binding of Pi

	336 (*E. coli* residue numbers) 414
E. coli	DVSAFVPTNVISITDGQIFLETNLFNAGIRPAVNPGISVSRVGGAAQTKIMKKLSGGIRTALAQYRELAAFSQFASDLD
Salmonella	DVSAFVPTNVISITDGQIFLESNLFNAGIRPAVNPGISVSRVGGAAQTKIMKKLSGGIRTALAQYRELAAFSQFASDLD
Saccharomyces	DVSAYIPTNVISITDGQIFLEAELFYKGIRPAINVGLSVSRVGSAAQVKALKQVAGSLKLFLAQYREVAAFAQFGSDLD
Drosophila	DVSAYVPTNVISITDGQIFLESELFYKGFRPAVNIGLSVSRVGSAAQLKSVKKVAGSIKLSLAQYRELEDFAKFGSDLD
Salmo	DVSAYVPTNVISITDGQIFLESELFYKGFRPAVNIGLSVSRVGSAAQLKSVKKVAGSIKLSLAQYRELEDFAKFGSDLD
Xenopus	DVSAYIPTNVISITDGQIFLETELFYKGIRPAINVGLSVSRVGSAAQTRAMKQVAGTMKLELAQYREVAAFAQFGSDLD
Rattus	DVSAYIPTNVISITDGQIFLETELFYKGIRPAINVGLSVSRVGSAAQTRAMKQVAGTMKLELAQYREVAAFAQFGSDLD
Bos	DVSAYIPTNVISITDGQIFLETELFYKGIRPAINVGLSVSRVGSAAQTRAMKQVAGTMKLELAQYREVAAFAQFGSDLD
Homo	DVSAYIPTNVISITDGQIFLETELFYKGIRPAINVGLSVSRVGSAAQTRAMKQVAGTMKLELAQYREVAAFAQFGSDLD
Gallus	DVSAYIPTNVISITDGQIFLETELFYKGIRPAINVGLSVSRVGSAAQTRAMKQVAGTMKLELAQYREVAAFAQFGSDLD

(a)

	156 (*E. coli* residue numbers) 254
E. coli	KTVNMMELIRNIAIEHSGYSVFAGVGERTREGNDFYHEMTDSNVID-------KVSLVYGQMNEPPGNRLRVALTGLTMAEKFRD-EGRDVLLFVDNIYRYTLAGTE
Salmonella	KTVNMMELIRNIAIEHSGYSVFAGVGERTREGNDFYHEMTDSNVID-------KVSLVYGQMNEPPGNRLRVALTGLTMAEKFRD-EGRDVLLFVDNIYRYTLAGTE
Saccharomyces	KTVFIQELINNIAKAHGGFSVFTGVGERTREGNDLYREMKETGVINLEG--ESKVALVFGQMNEPPGARARVALTGLTIAEYFRDEEGQDVLLFIDNIFRFTQAGSE
Drosophila	KTVLIMELINNVAKAHGGYSVFAGVGERTREGNDLYNEMIEGGVISLKD-KTSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSE
Salmo	KTVLIMELINNVAKAHGGYSVFAGVGERTREGNDLYHEMIESGVINLKD-DTSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSE
Xenopus	KTVNMMELINNIAKAHSGLSVFAGVGERTREGNDFYHEMADSGVVNLENLGESKVSMVYGQMNEPPGNRLRVALTGLTIAESFRD-EGRDVLFFVDNIYRYTLAGTE
Rattus	KTVLIMELINNVAKAHGGYSVFAGVGERTREGNDLYHEMIESGVINLKD-ATSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSE
Bos	KTVLIMELINNVAKAHGGYSVFAGVGERTREGNDLYHEMIESGVINLKD-ATSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSE
Homo	KTVLIMELINNVAKAHGGYSVFAGVGERTREGNDLYHEMIESGVINLKD-ATSKVALVYGQMNQPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSE
Gallus	KTVLIMELINNVAKAHGGYSVFAGVGERTREGNDLYHEMIESGVINLKD-ATSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQDVLLFIDNIFRFTQAGSE

(b)

Figure 3: *Sequence alignment of α and β-subunit residues.* α and β-subunit amino acids from different species are aligned. Conserved positively charged residues are shown in blue color, and negatively charged residues are identified by red color. *E. coli* starting residue for α-subunit is αD336 and for β-subunit is βK155.

occurs at the βE catalytic site. By modifying the above assay for use with *E. coli* purified F_1 or membrane bound F_1F_o, further studies have to date investigated the relationship between Pi binding and catalysis for eight residues, namely αPhe-291, αSer-347, αGly-351, αArg-376, βLys-155, βArg-182, βAsn-243, and βArg-246. It was shown that the five residues αSer-347, αArg-376, βLys-155, βArg-182, and βArg-246 were grouped in a tetrahedral relationship,and are involved in Pi binding. The other three residues αPhe-291, αGly-351, and βAsn-243 are not involved in Pi binding [11, 25, 48, 82–84, 86]. In consequence, the presence of positively charged residues in the catalytic site explains the preferential binding of ADP over ATP.

It may be noted that [^{32}P]Pi binding was detected with a Kd(Pi) in the range of 0.1 mM using an alternative, pressure ultrafiltration method, and this result is consistent with data obtained from the NBD-Cl protection assay [99]. It is apparent that Pi dissociates more rapidly from *E. coli* F_1 than it does from mitochondrial F_1, undesirably, rendering the convenient centrifuge assay incompatible with the *E. coli* enzyme.

The story goes on with the presence of many charged/uncharged residues in close proximity to the Pi binding subdomain in the catalytic sites. These residues have been shown to exert either a direct or an indirect role in Pi binding. One of these residues is the charged αAsp-350 of the VISIT-DG sequence. αAsp-350 is part of the α-subunit VISIT-DG sequence, which is a highly conserved motif in this enzyme [25]. VISIT-DG sequence residues are of special interest in general and negatively charged αAsp-350 in particular, because of the close proximity to the known positively charged phosphate-binding residues. αAsp-350 is ~3 Å from αArg-376. It would be imperative to understand three specific aspects of αAsp-350 residue. First, is residue Asp-350 directly or indirectly involved in phosphate binding through αArg-376? Second, is αAsp-350 important for function through its role in maintaining the structural integrity of the Pi binding subdomain but not involved in Pi binding *per se*? Third, is the carboxyl side chain of αAsp-350 involved in the transition state at the catalytic site? Our hypothesis is that αAsp-350 interacts electrostatically with αR376. Such an interaction may provide proper orientation of αArg-376 side chain towards Pi.

6. Modulation of Charge in the Catalytic Sites

Understanding the role of charged residues and the effects of modifying them is important in understanding the molecular mechanism of ATP synthesis. NBD-Cl inactivation assay described earlier have shown that positively charged residues are functionally essential for Pi binding in the βE catalytic site of *E. coli* ATP synthase [11, 48, 83, 84]. The introduction

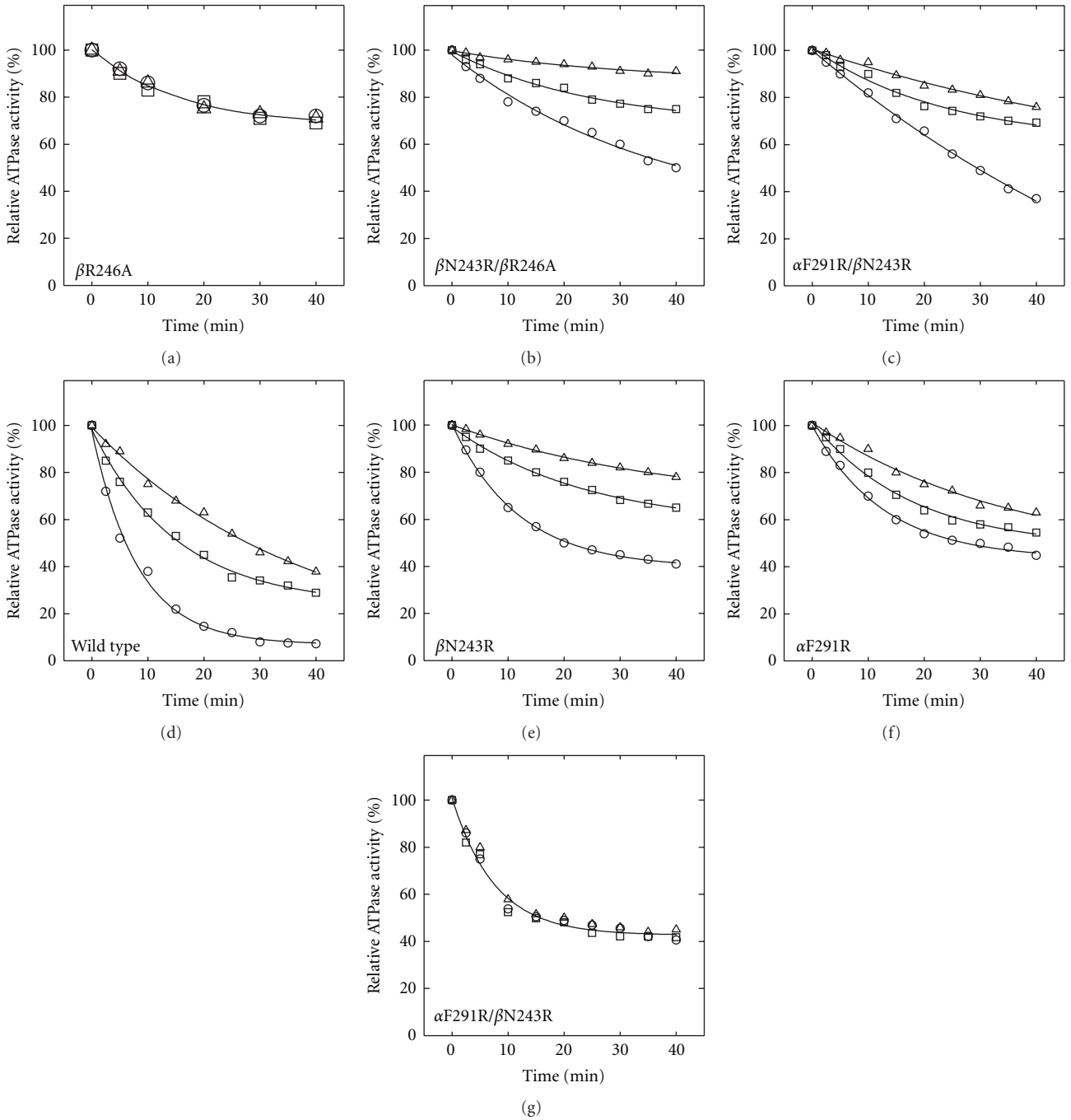

Figure 4: *Compensatory effect inserted arginine residue.* Membranes were preincubated with Pi at zero, 2.5 or 10 mM concentration as shown, for 60 min at 23°C. Then, NBD-Cl (125 μM) was added and aliquots withdrawn for assay at time intervals as shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. ◯, no Pi added; □, 2.5 mM Pi; Δ, 10 mM Pi. Data taken from [83].

of charged residues in place of uncharged residues in the vicinity of catalytically important residues has been shown to affect Pi binding by resulting in a loss or gain of ATPase activity [82, 83, 86]. Earlier work [82, 83] indicated that the introduction of negative charge in the Pi-binding pocket in the form of βN243D close to βArg-246 prevented Pi binding (Figure 5). Also, introduction of positive charge in the form of βN243R restored Pi binding in βR246A mutants. Similarly, the introduction of negative or positive charge in the form of αF291D/E/R with βR246 or βR246A resulted in a loss or gain of Pi binding (Figures 4 and 5) [83, 86]. These results suggested that modulation of charge in the Pi binding site could be used to understand the molecular mechanism of Pi binding. It is established that Arg residues occur particularly commonly in the Pi binding sites of proteins [100]. Therefore, varying the number of Arg residues in

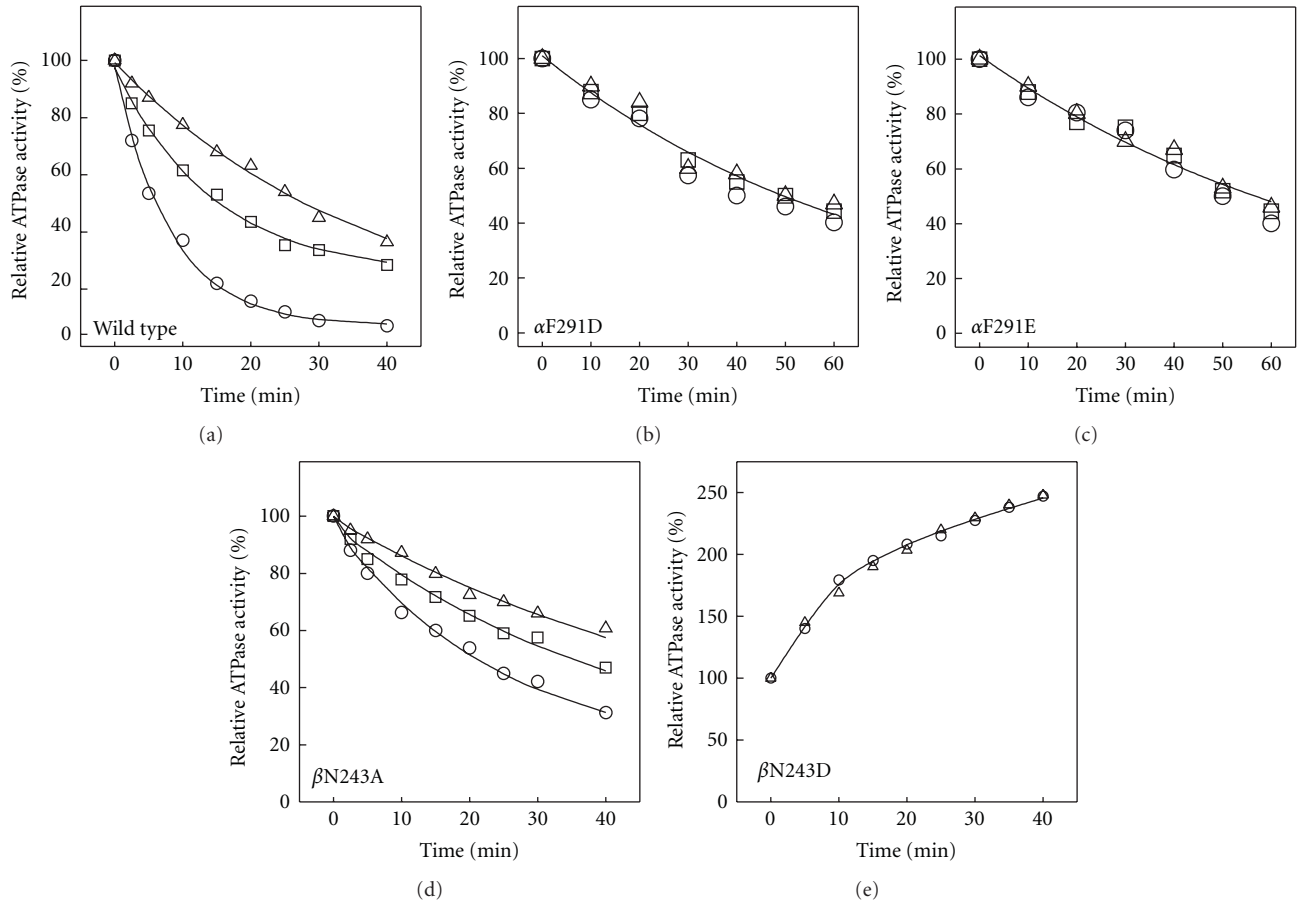

FIGURE 5: *Loss of Pi protection from inactivation by NBD-Cl inhibition with inserted negative charge.* Membranes were preincubated with Pi at zero, 2.5 or 10 mM concentration as shown, for 60 min at 23°C. Then, NBD-Cl (125 μM) was added and aliquots withdrawn for assay at time intervals as shown. ATPase activity remaining is plotted against time of incubation with NBD-Cl. ◯, no Pi added; □, 2.5 mM Pi; Δ, 10 mM Pi. Data taken from [82, 86].

the Pi binding site of ATP synthase can be an instructive approach.

Residue βAsn-243 lies 3.2 Å from βArg-246 in both AlF_3 and SO_4^{2-})-containing catalytic sites (nearest atom distances quoted) [83]. One experimental approach used was to introduce the mutation βN243R in a wild-type background (with βArg-246) and in presence of the βR246A mutation. Residue αPhe-291, located at the end of the Pi-binding pocket across the catalytic α/β interface, with its side-chain pointing toward the bound Pi analogs, is also a suitable location for introducing a new Arg. This residue lies at a distance of 3.2 Å from βArg-246 in the AlF_3-containing catalytic site and 7.5 Å in the SO_4^{2-}-containing catalytic site [61, 83, 88]. Arginine was introduced in the form of an αF291R mutation in the wild-type background and in the presence of the βR246A mutation. The actual distances of residues βArg-246, βAsn-243 and αPhe-291 were obtained from bound AlF_3 and SO_4^{2-} as determined by X-ray crystallography [61, 88], while speculative distances (in brackets) were calculated for mutant residues βAla-246, βArg-243 and αArg-291 using the "Deep View Swiss-Pdb Viewer" [101]. Apparently, mutations placed extra positive charge relatively close to Pi, and the βAla-246 mutation left a relatively large space into which a new Arg fits nicely.

7. Synergistic Stereochemical Interactions at the Catalytic Sites

The introduction of one or two extra positively charged Arg residues in the wild-type background at either β-243 or α-291, or both, has proven to inhibit Pi binding (Figure 4). Introduction of a new Arg at β-243 or α-291 in the βR246A background provided a significant compensatory effect on ATPase. ATP-driven proton pumping was also reinstated in the case of the αF291R/βR246A mutant. But these new arginines did not restore function to full normal [83].

The βR246A mutant did not show Pi binding, but both βN243R and αF291R mutations "rescued" Pi binding in combination with βAla-246 (Figure 4). Since neither βArg-243 nor αPhe-291 could be expected to assume the exact same stereochemical interactions that βArg-246 achieves, electrostatic interaction appears to be a significant factor. Presence of at least one positive charge at this general location is a requisite determinant of initial Pi binding in the catalytic site βE. βN243R or αF291R in the wild-type background

(representing one extra positive charge) did not prevent Pi binding, but the combination of αF291R/βN243R (two extra charges) abrogated Pi binding (Figure 4). Presumably the local concentration of charge in the latter becomes too disruptive and distorts the Pi-binding site [83].

A similar pattern of effects has been reported when transition state stabilization was assessed by assaying inhibition of ATPase activity by the transition state analogs MgADP-fluoroaluminate and MgADP-fluoroscandium. Previously, it was shown that [48] that both inhibitors are potent against wild-type ATP synthase but that each inhibit βR246A mutant only to small extent, which indicates that βArg-246 is intimately involved in transition state stabilization. It was found that either mutant residue βArg-243 or αArg-291 partly "rescued" transition state stabilization when present with βAla-246 [83]. Raising the number of positively charged residues to two (βN243R and αF291R mutants in wild-type background) had an adverse effect as reflected by a lesser inhibition of ATPase activity. Raising the number of local positive charges to three reduced transition state stabilization right back to where it was in βR246A. Interestingly, even in the best cases among the mutants (βN243R/βR246A or αF291R/βR246A) transition state stabilization was incomplete as compared to wild-type, which suggests the importance of stereochemical interactions [83].

In summary, all the results showed that Pi binding is notably affected by the local positive charges in catalytic site βE of ATP synthase. Positive charge in the vicinity of the natural βArg-246 is important because its removal in βR246A mutant can be compensated partially by introduction of one Arg at either β-243 or α-291. Thus, electrostatic interaction is an important determinant of Pi binding. The presence of two arginines by introduction of either βArg-243 or αArg-291 in the presence of βArg-246 does not prevent Pi binding, but the presence of all three arginines eliminates Pi binding. Effects on transition state stabilization followed a parallel pattern, but the restoration of Pi binding in βE catalytic sites by charge compensation was not sufficient by itself to restore full function [83].

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Review Article

Functionally Relevant Residues of Cdr1p: A Multidrug ABC Transporter of Human Pathogenic *Candida albicans*

Rajendra Prasad, Monika Sharma, and Manpreet Kaur Rawal

Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

Correspondence should be addressed to Rajendra Prasad, rp47jnu@gmail.com

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Reduced intracellular accumulation of drugs (due to rapid efflux) mediated by the efflux pump proteins belonging to ABC (ATP Binding Cassette) and MFS (Major Facilitators) superfamily is one of the most common strategies adopted by multidrug resistance (MDR) pathogenic yeasts. To combat MDR, it is essential to understand the structure and function of these transporters so that inhibitors/modulators to these can be developed. The sequence alignments of the ABC transporters reveal selective divergence within much conserved domains of Nucleotide-Binding Domains (NBDs) which is unique to all fungal transporters. Recently, the role of conserved but divergent residues of *Candida* Drug Resistance 1 (*CDR1*), an ABC drug transporter of human pathogenic *Candida albicans*, has been examined with regard to ATP binding and hydrolysis. In this paper, we focus on some of the recent advances on the relevance of divergent and conserved amino acids of CaCdr1p and also discuss as to how drug interacts with Trans Membrane Domains (TMDs) residues for its extrusion from MDR cells.

1. Introduction

The pathogenic *Candida albicans* accounts for approximately 50–60% causes of candidiasis particularly in immunocompromised human patients. But the infections caused by non-*albicans* species, such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* are also common particularly in neutropenic patients and neonates [1–4]. Of note, recently, the incidences of *albicans* and non-*albicans* species of *Candida* acquiring resistance to antifungals (particularly to azoles) have increased considerably which poses problems towards its successful chemotherapy [5–7]. On one hand, to combat antifungal resistance, search for better drugs with newer targets is underway; on the other hand, *Candida* cells have evolved a variety of strategies to develop resistance to common antifungals.

The main mechanisms of antifungal resistance to azoles include alterations in ergosterol biosynthetic pathway by an overexpression of *ERG11* gene which encodes the drug target enzyme 14∝-demethylase or by an alteration in target enzymes (point mutations) [3, 8, 9]. Reduced intracellular accumulation of drugs (due to rapid efflux) is another prominent mechanism of resistance in *Candida* cells [10]. Most commonly, genes encoding drug efflux pumps belonging to ABC (ATP binding cassette) and MFS (Major Facilitator) superfamilies of proteins are overexpressed in azole resistant *Candida* isolates which abrogates intracellular accumulation leading to enhanced tolerance to drugs (Figures 1(a) and 1(b)).

2. Efflux Pumps

Since ABC and MFS transporters are among the major players that contribute to azole resistance in clinical isolates of *Candida*, there is a spurt in research on all aspects of these genes and their encoded proteins [6, 7]. In this context, considerable attention is being paid to the structural and functional aspects of these proteins, which in turn could lead to better strategies for designing modulators/inhibitors of these pumps. The genome of *C. albicans* possesses 28 ABC and 95 MFS proteins; however, only ABC transporters CaCdr1p and CaCdr2p and MFS transporter CaMdr1p are known to be multidrug transporters which play major role in drug extrusion from resistant strains. In this review, we

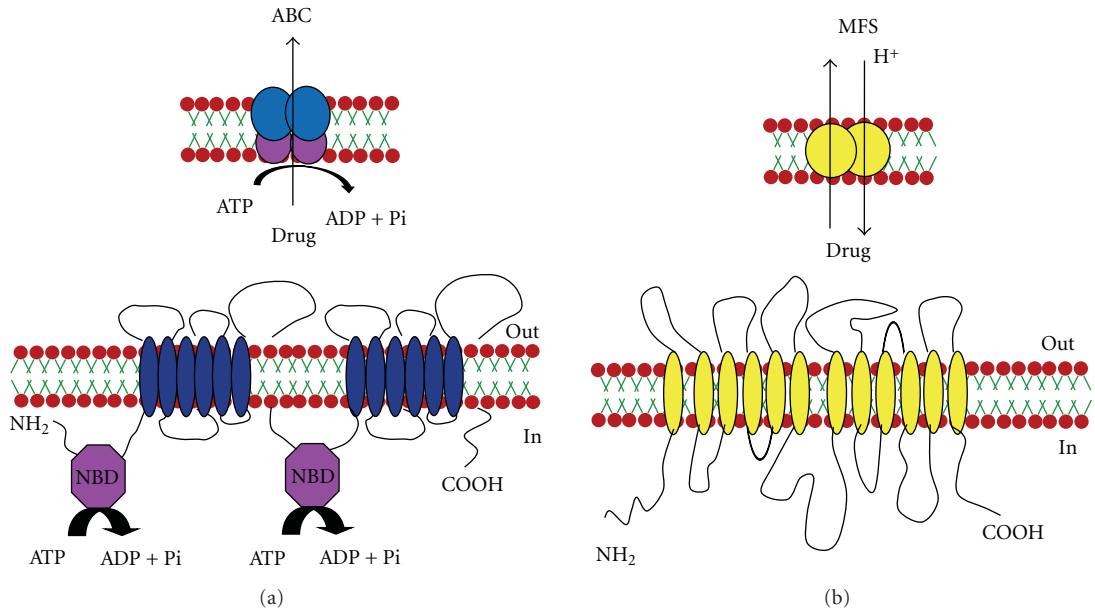


Figure 1: A cartoon representation of (a) ABC and (b) MFS transporters of Candida. The topology of ABC and the MFS transporters depicted here have the $(NBD\text{-}TMS_6)_2$ and the $(TMS)_{12}$ (Transmembrane Segments) arrangements, respectively. The NBDs (Nucleotide-Binding Domains) of the ABC transporters are responsible for the hydrolysis of ATP, which facilitates drug extrusion while the MFS transporters utilize proton gradient to expel drugs.

begin with a discussion on the structure and function of ABC proteins and then focus on the role of some of the critical amino acid residues of CaCdr1p in drug transport. For brevity, we have excluded MFS drug transporters from our discussion.

3. Structure and Function of ABC Efflux Proteins

ABC proteins are generally made up of two transmembrane domains (TMDs), each consisting of six transmembrane segments (TMS) and two cytoplasmically located nucleotide-binding domains (NBDs) which precedes each TMD (Figures 1(a) and 1(b)), [11, 12]. While it appears that several TMSs associate together to form the substrate binding site(s), this alone is probably not sufficient for substrate transport across the membrane bilayer. Vectorial transport of these substrates requires energy from the hydrolysis of ATP carried out at the NBDs. Given their varied roles and the greatly differing characteristics of substrates that members of this superfamily of proteins seem to efflux, it is hardly surprising that despite the overall conservation of the domain architecture of TMDs, their primary sequences are significantly different (Figure 2). On the other hand, NBDs of ABC transporters which power drug transport are highly conserved both in terms of primary structure and architecture (Figure 3).

4. Candida Drug Resistance 1 (CDR1)

CDR1 of *C. albicans*, the first ABC efflux pump characterized in any known pathogenic yeast, was isolated as a gene implicated in conferring resistance to cycloheximide in a *PDR5* disruptant hypersensitive strain of *S. cerevisiae* [13]. *CaCDR1* codes for a protein of 1501 amino acid residues (169.9 kDa), with a topology similar to that of ABC proteins Pdr5p and Snq2p of *S. cerevisiae*. On the other hand, its topology mirrors that of *STE6*, a -mating pheromone transporter of *S. cerevisiae*, as well as of the human MDR1 and CFTR. Despite a high structural and functional similarity between CaCdr1p and ScPdr5p, some distinct functional features tend to distinguish them. For example, both genes share overlapping specificities for cycloheximide and chloramphenicol but *CaCDR1* affects sensitivity to oligomycin while neither amplification nor disruption of Sc*PDR5* alters susceptibilities to this mitochondrial inhibitor [13]. It is worth mentioning that some of the close homologues of *CaCDR1* in *C. albicans* are also functionally distinct. For example, CaCdr2p that exhibits 84% identity with CaCdr1p has a distinct drug resistance profile [14]. The overexpression or deletion of *CaCDR3* and *CaCDR4*, the homologues of *CaCDR1* and *CaCDR2* interestingly do not affect drug susceptibilities of yeast cells [15]. The hydropathy plots of CaCdr1p and CaCdr3p show that both the proteins have similar topological arrangements where the hydrophilic domain containing the NBDs precedes the hydrophobic TMS [16]. The only apparent difference between the two proteins appears to be in the C-terminal where CaCdr3p has an extended loop connecting TM11 and TM12. In addition, there is stretch of 21 amino acids in the C-terminal of CaCdr3p which are absent in CaCdr1p [16]. Keeping in view, the importance of these regions in drug binding and transport, the subtle differences in the primary structures of these proteins could be responsible

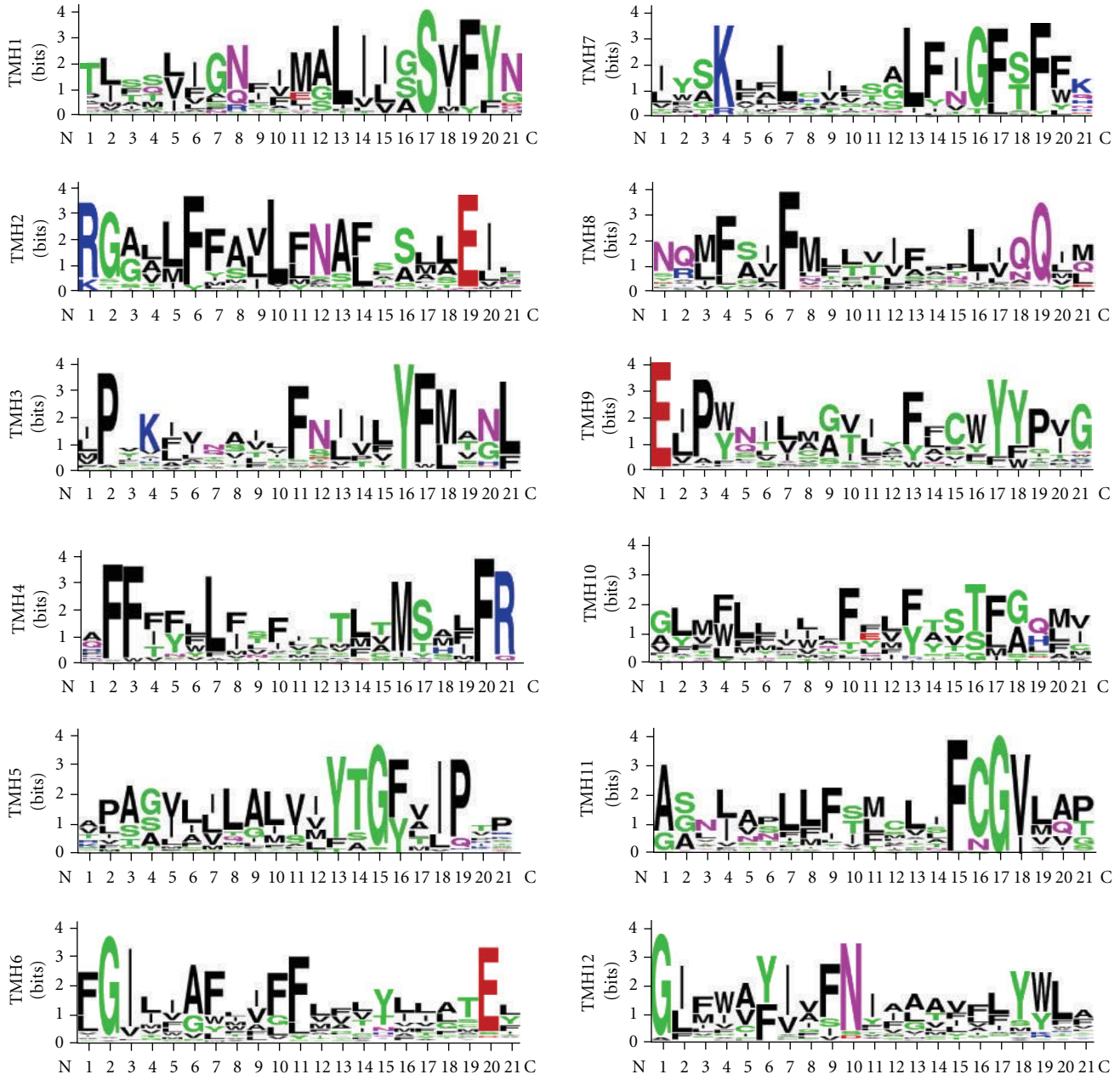


Figure 2: Sequence logos of CaCdr1p transmembrane segment (TMSs) residues with other fungal PDR transporters. Each logo consists of stacks of symbols, one stack for each position in the sequence. The scale indicates the certainty of finding a particular amino acid at a given position and is determined by multiplying the frequency of that amino acid by the total information at that position. The residues at each position are arranged in order of predominance from top to bottom, with the highest frequency residue at the top. The height of symbols within the stack indicates the relative frequency of each amino acid at that position. Colors such as green defines polar, blue correspond to basic, red to acidic, black to hydrophobic, and violet represent the amino acids that have polar amide group.

in governing their substrate specificity, hence only enabling some of them (CaCdr1p and CaCdr2p) to bind and transport drugs [17, 18].

To study the MDR proteins, a heterologous hyperexpression system is used where GFP tagged CaCdr1p/CaMdr1p has been stably overexpressed from a genomic *PDR5* locus in a *S. cerevisiae* mutant AD1-8u$^-$ [19]. The host AD1-8u$^-$ developed by Goffeau's group [20] was derived from a *Pdr1-3* mutant strain with a gain of function mutation in the transcription factor Pdr1p, resulting in constitutive hyperinduction of the *PDR5* promoter [19]. In previous studies, we have confirmed that GFP tagging of CaCdr1p (*CaCDR1-GFP*) and CaMdr1p (*CaMDR1-GFP*) did not impair its expression and the functional activity of the proteins [21, 22]. Figure 4 summarizes the strategy used for the expression of CaCDR1-GFP under *ScPDR5* promoter.

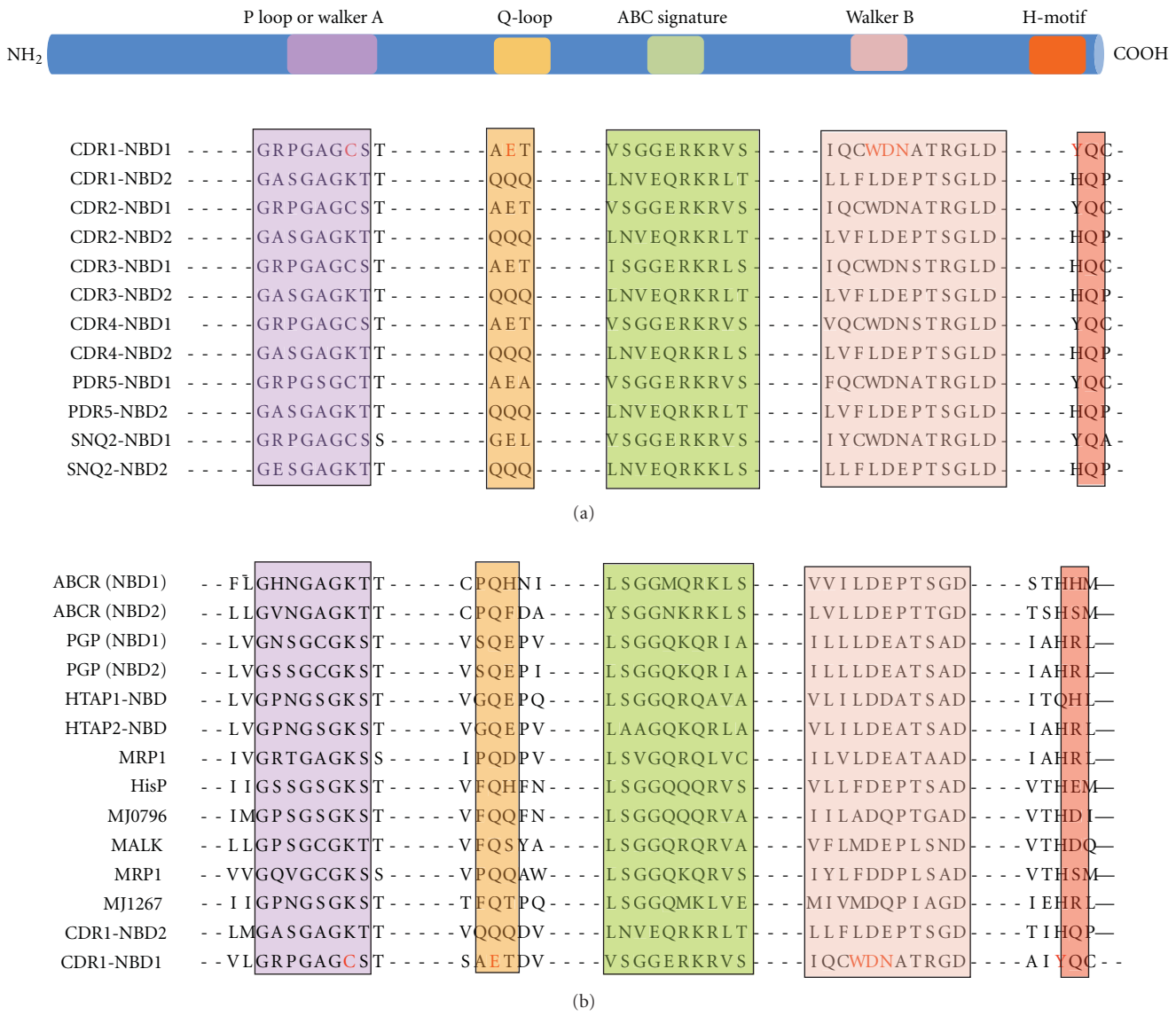


FIGURE 3: Sequence alignment of the conserved motifs from fungal ABC transporters. Comparison of the sequence alignment of the walker A, Q-loop, signature C, Walker B, and H-loop motifs of N- and C-terminal NBDs (NBD1 and NBD2) of CaCdr1p with known (a) fungal and (b) nonfungal ABC transporters. Conserved but unique residues are highlighted.

5. How Does CaCDR1 Power Drug Efflux?

The characteristic feature of CaCdr1p or of any other ABC drug transporter is that they utilize the energy of ATP hydrolysis to transport variety of substrates across the plasma membrane. The conserved NBDs located at the cytoplasmic periphery are the hub of such an activity. The NBDs of all ABC transporters, irrespective of their origin and nature of transport substrate, share extensive amino acid sequence identity within typical motifs [12]. For example, NBDs of ABC transporters have a β-sheet subdomain containing the typical Walker A and Walker B motifs, as an essential feature of all ATP requiring enzymes [23], along with an α-helical sub-domain that possesses the conserved ABC signature sequence. NBD domain sequences possess certain conserved amino acid stretches, which are considered to be critical for its functionality [24]. These include: the Walker A, with a consensus sequence GxxGxG*K*S/T, where "*x*" represents any amino acid, the Walker B motif, that is, hhhhD, where "*h*" represents any aliphatic residue, and an ABC signature, LSGGQQ/R/KQR. Structural and biochemical analyses of NBDs show that the well-conserved lysine residue of Walker A motif binds to the β- and γ-phosphates of ribonucleotides and plays a critical role in ATP hydrolysis [24]. Mutations of this lysine residue have been shown to reduce or abolish the hydrolysis activity and in some cases impair nucleotide binding [24]. Interestingly, though N-terminal NBD of CaCdr1p contains the conserved Walker A (GRPGAG*C*ST) and B (IQCWD) motifs, and an ABC signature sequence (VSGGERKRVSIA) [25], the commonly occurring lysine

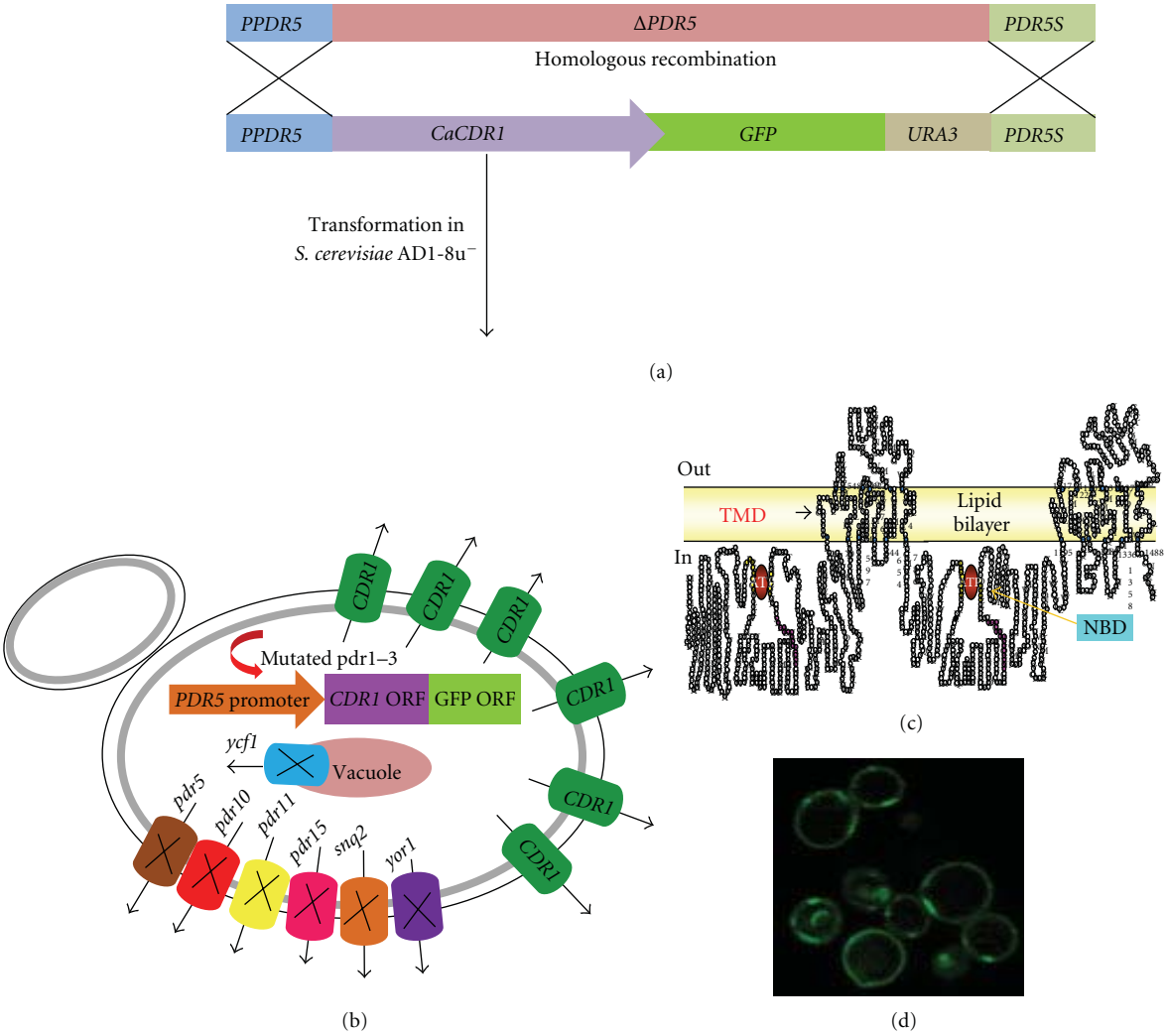


FIGURE 4: Overexpression of CaCdr1p in a heterologous system. (a) Strategy showing the cloning and transformation of *CaCDR1-GFP* in *S. cerevisiae*. (b) Pictorial representation of the host AD1-8u⁻ showing the Deleted ABC pump proteins (*pdr5*, *pdr10*, *pdr11*, *pdr15*, *snq2*, *yor1*, *ycf1*) and the hyper expressed *CaCDR1-GFP*. (c) Topology of CaCdr1p. (d) Localization of CaCdr1-GFP in the host strain AD1-8u⁻. The rimmed green fluorescent depicts overexpressing GFP tagged CaCdr1p.

residue within the Walker A motif is replaced with a cysteine. This replacement appears to be a unique feature of N-terminal NBDs (N-NBD) of most of the known fungal ABC-type transporters. In addition, degeneracy in Q loop and Walker B also exist in N-NBDs of all fungal transporters including in CaCdr1p. Notably, the C-terminal NBDs display degeneracy only in signature motifs (discussed later).

To ascertain the role of the uncommon cysteine of Walker A, active N-NBD of CaCdr1p was cloned and overexpressed and the soluble domain protein was purified and characterized. It was observed that an evolutionarily divergent Cys193 of Walker A of N-NBD was critical for ATP hydrolysis. The relative contribution of both the N- and C-terminal NBDs in ATP binding, hydrolysis, and transporter activity of native CaCdr1p (full protein) was examined wherein the atypical Cys193 of Walker A of N-NBD (C193K) and conserved Lys901 (K901C) in the Walker A of C-terminal NBD (C-NBD) were replaced [26]. The drug resistance profile of CaCdr1p mutant variant cells harboring C193K or K901C gave interesting insights into the functioning of the two NBDs. The cells expressing K901C showed enhanced hypersensitivity to drugs as compared to C193K variant which displayed partial sensitivity to select drugs. These observations clearly established that the two NBDs respond asymmetrically to the substitution of conserved residues of their respective Walker A motifs. The functional asymmetry of NBDs in CaCdr1p was also illustrated in another study where swapping of NBDs resulted in non-functional CaCdr1p chimeras and thus suggested that the two NBDs are not identical and nonexchangeable [27]. Interestingly, in the case of human P-gp, a close homologue of CaCdr1p, the issue of functional symmetry of two NBDs remains contentious. Approaches addressing this issue in P-gp provide data both in favor [28, 29] and against [30–32] functional asymmetry. This is in contrast with many other ABC transporters, for which there is evidence that the

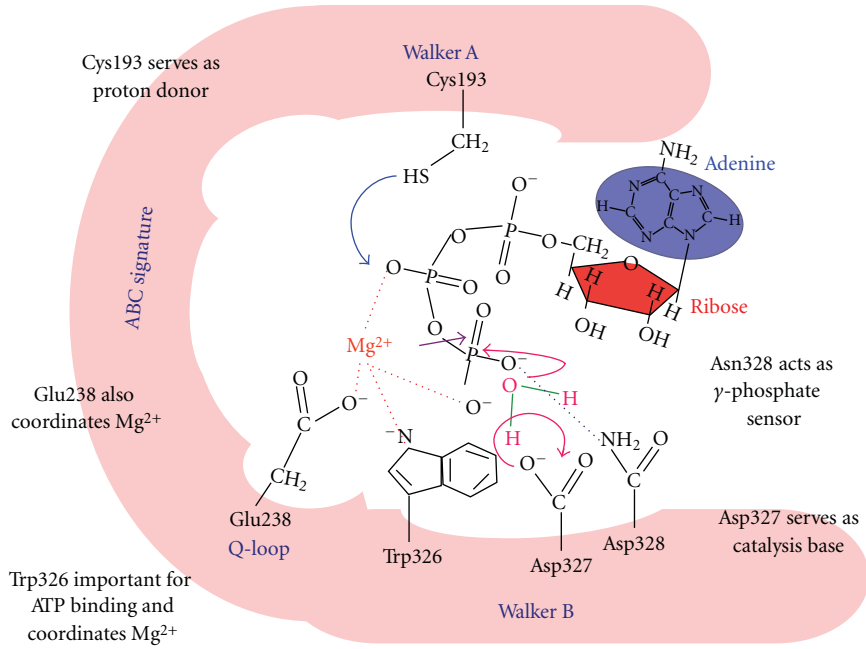
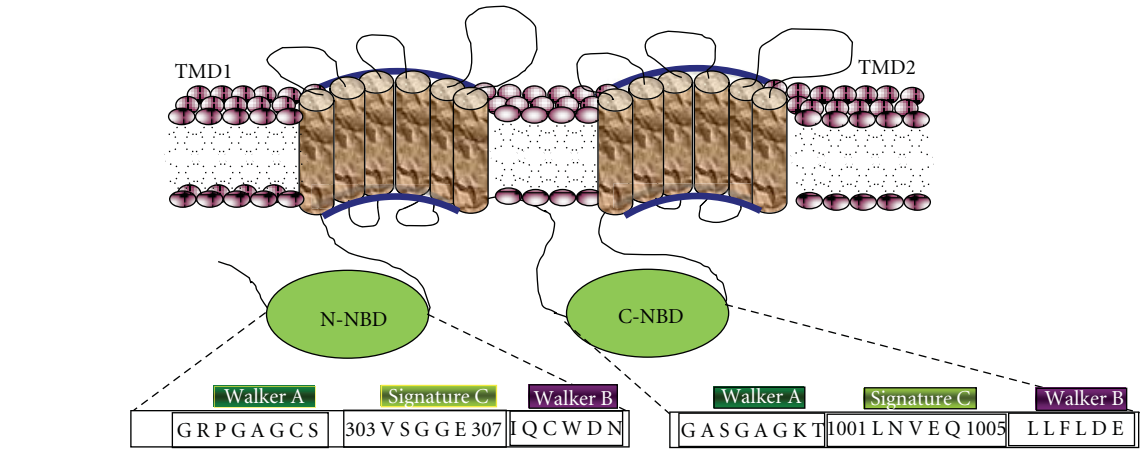


Figure 5: A hypothetical model depicting the N-terminal active site of CaCdr1p. The role of various residues involved in the catalytic mechanism for ATP hydrolysis by the N-NBD of CaCdr1p the details are discussed in the text.

two NBDs, although highly similar in sequence, may adopt different functional roles in the transport cycle [33].

Any functional asymmetry observed in the intact transporter is probably not entirely due to inherent properties of the NBD, and presumably also reflects either differences in the rate of hydrolysis or the effects of interdomain interactions. Two other residues of the N-NBD from CaCdr1p are also found to be important for domain functioning. As depicted in Figure 5, the unusual Trp326 in the Walker B motif of N-NBD, which is unique and conserved in all fungal transporters, is important for ATP binding and for the accompanying conformational change [34]. Thus, although the mutant with W326A appears capable of ATP hydrolysis, it does so with a much higher K_M value, indicating that the docking of the substrate in the binding pocket has been altered by the mutation. However, the protein appears capable of near-normal function in cells expressing the full-length protein carrying W326A mutation, implying that the conformational change that normally occurs upon ATP docking cannot by itself be responsible for the cross-talk by the domain with the TMDs. While the highly conserved Asp327 of N-NBD is shown to be the catalytic carboxylate in the context of other ABC transporters, in N-NBD of CaCdr1p, it does not appear to mediate catalysis *via* interaction with Mg^{2+} as is normally expected for similar transporters [34]. It has been shown that due to spatial proximity, fluorescence resonance energy transfer (FRET) takes place between Trp326 of Walker B and MIANS [2-(4-maleimidoanilino) naphthalene-6-sulfonic acid] on Cys193 of Walker A motif. These critical amino acids are positioned within the nucleotide-binding pocket of N-NBD to bind and hydrolyze ATP. The results show that both Mg^{2+} coordination and nucleotide binding contribute to the formation of the active site. The entry of Mg^{2+} into the active site causes the first large conformational change that brings Trp326 and Cys193 in close proximity to each other. It was also demonstrated that besides Trp326, typical Glu238 in the Q-loop also participates in coordination of Mg^{2+} by N-NBD. A second conformational change is induced when ATP, but not ADP, docks into the pocket. The unique Asn328 does sensing of the γ-phosphate of the substrate in the extended Walker B motif, which is essential for the second conformational change that must necessarily precede ATP hydrolysis.

It has been possible to deduce a picture of the catalytic mechanism for ATP hydrolysis by the N-NBD of CaCdr1p (Figure 5). The metal ion approaches the nucleotide binding pocket and forms a π-stacking interaction with the delocalized electron cloud of Trp326 in Walker B. This induces a large conformational change in the protein, bringing Cys193, Glu238, Trp326, Asp327, and Asn328 closer into the nucleotide binding pocket. At this point, the metal ion is sufficiently far from the MIANS on Cys193 to have no effect on its fluorescence intensity. However, Trp326 and MIANS on Cys193 are within 16 Å of each other at this point. ATP approaches with its phosphates directed towards the pocket. As in other ATPases, it may be assumed that the β and γ-phosphates also coordinately bind the metal ion and their negative charges are considerably masked. This is important since in the absence of the metal ion, the nucleotide does not dock into the active site. While other residues may also be involved in stabilizing the nucleotide within the pocket, Asn328 certainly acts as a sensor for the γ-phosphate. This induces the second conformational change



C. albicans	Cdr1p (N)	V S G G E R K R V S I A E	Cdr1p (C)	L N V E Q R K R L T I G V
C. albicans	Cdr2p (N)	V S G G E R K R V S I A E	Cdr2p (C)	L N V E Q R K R L T I G V
S. cerevisiae	Pdr5p (N)	V S G G E R K R V S I A E	Pdr5p (C)	L N V E Q R K R L T I G V
S. cerevisiae	Snq2p (N)	V S G G E R K R V S I A E	Snq2p (C)	L N V E Q R K K L S I G V
C. galbrata	Phd1p (N)	V S G G E R K R V S I A E	Phd1p (C)	L N V E Q R K R L T I G V
C. neoformans	CnAfr1p (N)	V S G G E R K R V S I A E	CnAfr1p (C)	L S V E A R K V T I G V E
A. fumigatus	AtrFp (N)	V S G G E R K R V S I A E	AtrFp(C)	L N V E Q R K R V T I G V
S. cerevisiae	Ste6p(N)	L S G G Q Q Q R V A I A R	Ste6p (C)	L S G G Q A Q R L C I A R
H. sapiens	Pgp (N)	L S G G Q K Q R I A I A R	Pgp (C)	L S G G Q K Q R I A I A R
H. sapiens	CFTR (N)	L S G G Q R A R I S L A R	CFTR (C)	L S H G H K Q L M C L A R
H. sapiens	MRP1 (N)	L S G G Q K Q R V S L A R	MRP1 (C)	L S V G Q R Q L V C L A R
H. sapiens	TAP1	L S G G Q R Q A V A L A R	TAP2	L A A G Q K Q R L A I A R

Figure 6: Topology of CaCdr1p and sequence alignment of signature motifs from various ABC transporters. The sequence alignment of signature motif residues in NBDs with those from other nucleotide-binding domains of some known ABC transporters is shown.

within the protein. Asp327 which acts as a catalytic base abstracts a proton from a water molecule, that is, part of the Mg-ATP complex present in the active site. The hydroxyl ion, thus, formed in turn attacks at the β-phosphate allowing it to, in turn, abstract a proton from the –SH of Cys193. The consequence of this is to simultaneously weaken the phosphodiester bond between β- and γ-phosphates, allowing the latter to leave. Once ATP is hydrolyzed, Asn328 no longer senses the γ-phosphate, and the conformation relaxes back to a more open one allowing ADP to leave (Figure 5).

The data thus far unequivocally show that the N-NBD of CaCdr1p and by extension those of other fungal transporters have evolved so as to use their unique substitutions to perform the task of ATP binding and hydrolysis. While it is not yet clear what evolutionary advantage these typical sequence variations might provide to the organisms, it is becoming more and more evident that it has mechanistic implications for the protein. We are yet to understand how the N-NBD works in conjunction with the C-NBD to give rise to a functional drug transporter. Does working in tandem require the ABC Signature sequence of one NBD to participate in ATP binding by the other, as is seen in other ABC transporters? Like in the N-NBDs, the ABC signature sequences of CaCdr1p and other fungal transporters too appear to have diverged away from that of other ABC transporters. Whether this is so as to compensate for the substitutions in their N-NBDs or whether they have evolved a new mechanism for coming together for ATP hydrolysis and drug efflux is a question worth examining.

Signature motifs are other domains which are the hallmark sequences of NBDs of ABC transporters that display highly conserved sequences across the evolutionary scale; however, there are also instances of appearance of selective divergence within this motif. For example, human ABC transporters such as TAP [35] and CFTR [36] have degenerated Signature motifs (Figure 6). In contrast, all the family members of ABC transporters of fungi, particularly of PDR subfamily display divergence in their Signature motifs. Thus, the Signature motif of N-NBD of CaCdr1p is well conserved but has C-NBD with a degenerated Signature motif (Figure 6). Our recent analysis revealed that the conserved and degenerated Signature sequences of the CaCdr1p are functionally indispensable and cannot be exchanged. This emphasizes the uncompromised asymmetry that exists between the NBDs of CaCdr1p and in other yeast ABC transporters. Similar to other ABC transporters, the well-conserved serine (S304) and glycine (G306) residues present in conserved Signature motif of N-NBD are also critical for the functioning of CaCdr1p. For example, even the substitution at the equivalent position residues of degenerated Signature motif of C-NBD with the conserved ones and vice versa does not support the function of the transporter [25]. The well-conserved glycine present at fourth position of Signature motif (LSGGQ) is involved in the ATP catalysis

Table 1: Substrates and inhibitors of CaCDR1 substrates.

Substrates	Fluconazole, ketoconazole, voriconazole, Itraconazole, miconazole, lipids, steroids, R6G, cycloheximide, rhodamine 123, cerulenin, trifluoperazine, nigericin, tamoxifen, verapamil, cycloheximide, propanil, diuron, linuron, disulfiram, anisomycin, doxorubicin, 4-nitroquinoline –N-oxide, benomyl, yohimbine HCl, quinidine, etoposide, chlorobromuron, vinblastine, tamoxifen, gefitinib, fluphenazine, topotecan, daunorubicin, DM-11, AT-12 niguldipine, dexamethasone, berberine, terbinafine, tritylmazole	[7, 53]
Inhibitors/modulators	Milbemycins, enniatin, FK506, FK520, unnarmicins, curcumin, disulfiram	[54–57]

[25, 30, 36–39]. Biochemical analysis revealed that a small change at this position (G → A) results in steric hindrance between methyl group of alanine and γ-phosphate of ATP. If this glycine is exchanged with bulky, charged aspartate or glutamate, it leads to a complete loss of ATPase and protein activity [25, 40, 41]. The critical nature of serine and glycine in WT-CaCdr1p can also be compared with similar residue of those proteins whose crystal structures are known. The existing structural information suggests that the Signature motifs of ABC proteins; Rad50 of *Pyrococcus furiosus*, MJ1096 of *Methanocaldococcus jannaschii*, GlcV of *Sulfolobus solfataricus,* Sav1866 of *Staphylococcus aureus,* mouse CFTR, HlyB, and MalK of *E.coli*, are involved in the head to tail ATPase site formation with the Walker A and Walker B motifs of the opposite NBDs, sandwiched with ATP molecules wherein the Signature motif is a "sensor" for an ATP γ-phosphate in the opposing domain [24, 42–49]. Based on the conserved nature of these motifs, it is reasonable to speculate that in CaCdr1p, the conserved S304 and G306 of NBD1 probably fall within close proximity of the ATP binding site. In addition, divergent residues present in C-NBD Signature region are also equally important and may be part of the ATPase site as well. However, it still requires experimental validation.

Additionally, it is shown that in addition to highly conserved and critical S304 and G306 residues, the equipositional residues N1002 and E1004 of degenerated Signature motif of C-NBD of WT-CaCdr1p have also evolved to be functionally essential. Notably, pairs of residue like V303, G305 of N-NBD and L1001, V1003, Q1005 of C-NBD Signature motif though part of otherwise conserved Signature sequences has apparently no functional relevance. These residues when replaced with either alanines or with its equipositional substitutes continued to show phenotypes similar to cells expressing WT-CaCdr1p.

Functional nonequivalence in the NBDs of ABC proteins of yeast is the result of variations in the conserved motifs (Walker A, Walker B, H-loop and Signature motif). These variations in N-NBD may have evolved in response to degenerated Signature motif of C-NBD. Thus, in CaCdr1p, both canonical and noncanonical ATP binding sites are formed similar to TAP and CFTR proteins. Recently, Ernst et al. hypothesized that in Pdr5p of *S. cerevisiae*, one ATP molecule catalyzed at the canonical active site may be sufficient to reset the TMDs whereas the second non-canonical site (regulatory site) may be engaged to serve as platform for keeping domains in dimeric form (inward facing) [49, 50].

6. CaCDR1 Extrudes Structurally Unrelated Substrates

The range of CaCdr1p substrates varies enormously and includes structurally unrelated compounds such as azoles, lipids, and steroids (Table 1). This promiscuity towards substrates is a characteristic feature of most ABC-type drug transporters and, hence, makes their functionality all the more complex to understand. Expectedly, predicting the residues involved in substrate binding without high-resolution structural data is a challenge. Yet, using a combination of biochemical assays along with site-directed mutagenesis, it has been possible to partially dissect the substrate binding pockets of CaCdr1p wherein role of some of the TMS amino acids in drug extrusion is becoming apparent [17, 18, 21].

7. Nature of Substrate Binding

Experiments with purified CaCdr1p have conclusively shown that ATP binding to CaCdr1p is not a prerequisite for drug binding and both the mechanisms of drug and ATP binding result in specific conformational changes which take place independent of each other [51]. A direct link between the ability of CaCdr1p to translocate fluorescent glycerophospholipids and efflux drugs has also been demonstrated [51]. Considering chemically diverse substrates which are expelled by CaCdr1p, the exact number of residues involved in drug binding and transport is far from understood.

As mentioned earlier, the CaCdr1p was overexpressed as a GFP-tagged fusion protein in a heterologous hyperexpression system and was characterized for drugs and nucleotide binding [52]. Iodoarylazido prazosin (IAAP, a photoaffinity analogue of P-gp substrate, prazosine) and azidopine (a dihydropyridine photoaffinity analogue of P-gp modulator, verapamil) were shown specifically to bind with *CaCDR1-GFP*. Interestingly, IAAP binding with CaCdr1p-GFP was competed out by molar excess of nystatin while azidopine binding could only be competed out by miconazole, thus, highlighting the possibility of different drug binding sites for the two analogues [52]. Gauthier and coworkers [52] have also shown that membranes prepared form CaCdr1p and CaCdr2p expressing cells are capable of binding the photoaffinity analogue of rhodamine 123 (^{125}I) iodoaryl azido-rhodamine 123 (IAARh123) and that both N-terminal and C-terminal halves of CaCdr2p contribute to rhodamine binding [52].

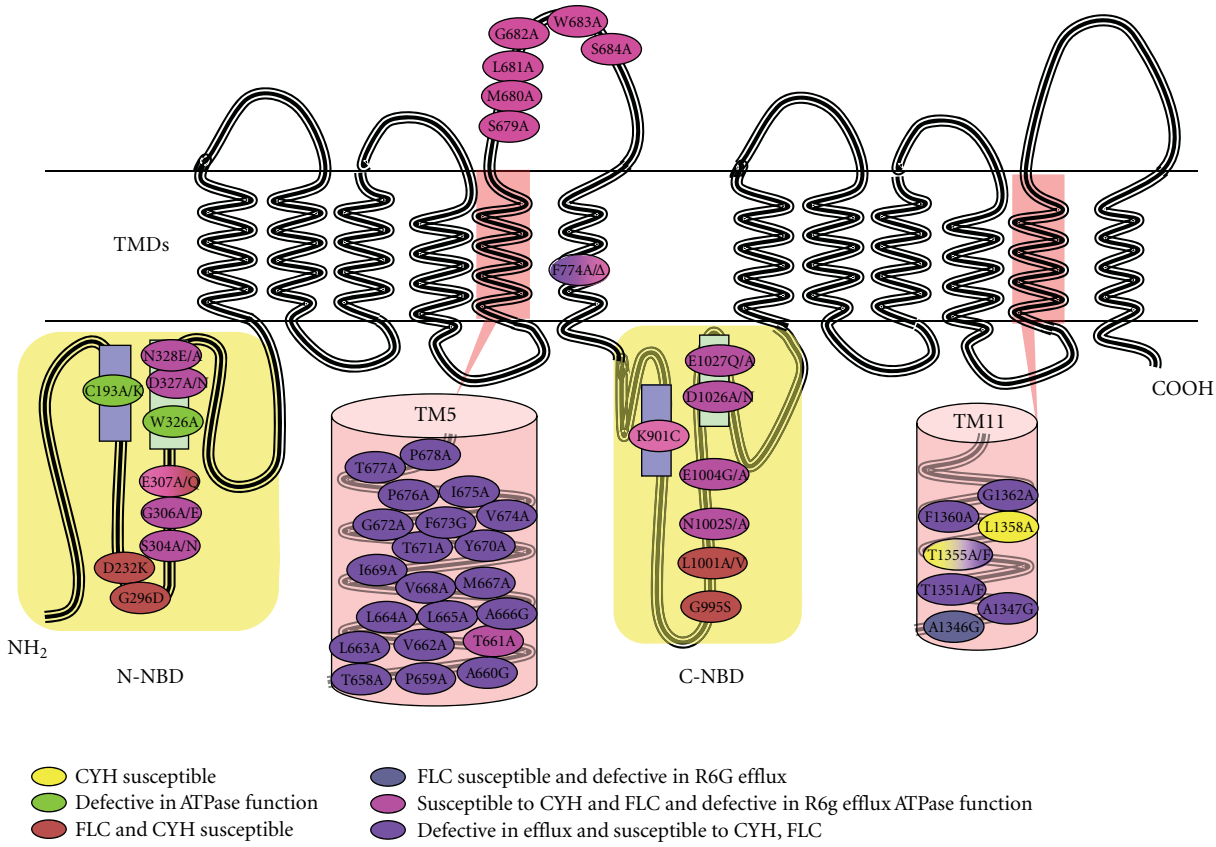


Figure 7: Cartoon of CaCDR1 protein depicting the location and phenotype of the mutated amino acids. Residues important for the resistance to CYH have been marked in yellow, defective in ATPase function in green, susceptible to FLC and CYH in blue, susceptible to FLC and defective in R6G efflux in grey, susceptible to CYH and FLC and defective ATPase function in pink, defective in efflux and susceptible to CYH, FLC in purple.

To understand the mechanism of drug transport mediated by CaCdr1p, a battery of its mutant variants that drastically affect various stages of drug extrusion have been generated (Figure 7). Amino acids of two of the twelve TMSs of CaCdr1p were subjected to alanine scanning wherein all the residues were replaced with alanines. The alanine scanning of TMS 11 of CaCdr1p showed that at least seven residues which were critical for determining substrate specificity and drug transport were clustered on the hydrophilic face of the α helical projection of TMS11 [18]. In contrast, alanine scanning of TMS 5 highlighted the importance of all 21 residues in drug transport and substrate specificity [17]. Based on the drug susceptibility pattern, the mutant variants of the TMS 5 could be grouped into two categories. The mutants belonging to first category exhibited sensitivity to all the tested drugs while the mutants placed in the other category showed intermediate level of resistance. While the ATPase activity and drug binding were largely unaffected, rhodamine 6G (R6G) and [^{3}H] fluconazole (FLC) efflux was abrogated in all the mutant variants. Based on the competition experiments with the molar excess of substrates during R6G efflux, we could identify residues which may be specific for interactions with miconazole (MCZ), itraconazole (ITR), and ketoconazole (KTC) and those which were common to all the three azoles. Notably, FLC which is also a substrate of CaCdr1p did not compete with R6G efflux; hence implying that CaCdr1p has independent binding sites for this azole. All the mutant variants display uncoupling between ATPase activity and drug transport, and thus TMS 5 of CaCdr1p not only appears to impart substrate specificity but probably also acts as a communication helix. What constitutes the substrate/drug binding pocket and how TMS 5 interacts with other helices of CaCdr1p are some of the issues that remain to be resolved (Figure 8).

Together, studies so far suggest that the drug binding sites in CaCdr1p are scattered throughout the protein and probably more than one residue of different helices are involved in binding and extrusion of drugs. However, there is still insufficient information available to predict where exactly the most common antifungals, such as azoles bind and how they are extruded. However, such studies should pave the way for future investigations related to the dynamics of substrate selection and may improve our approach in the design of new inhibitors/modulators of drug transporter for clinical applications.

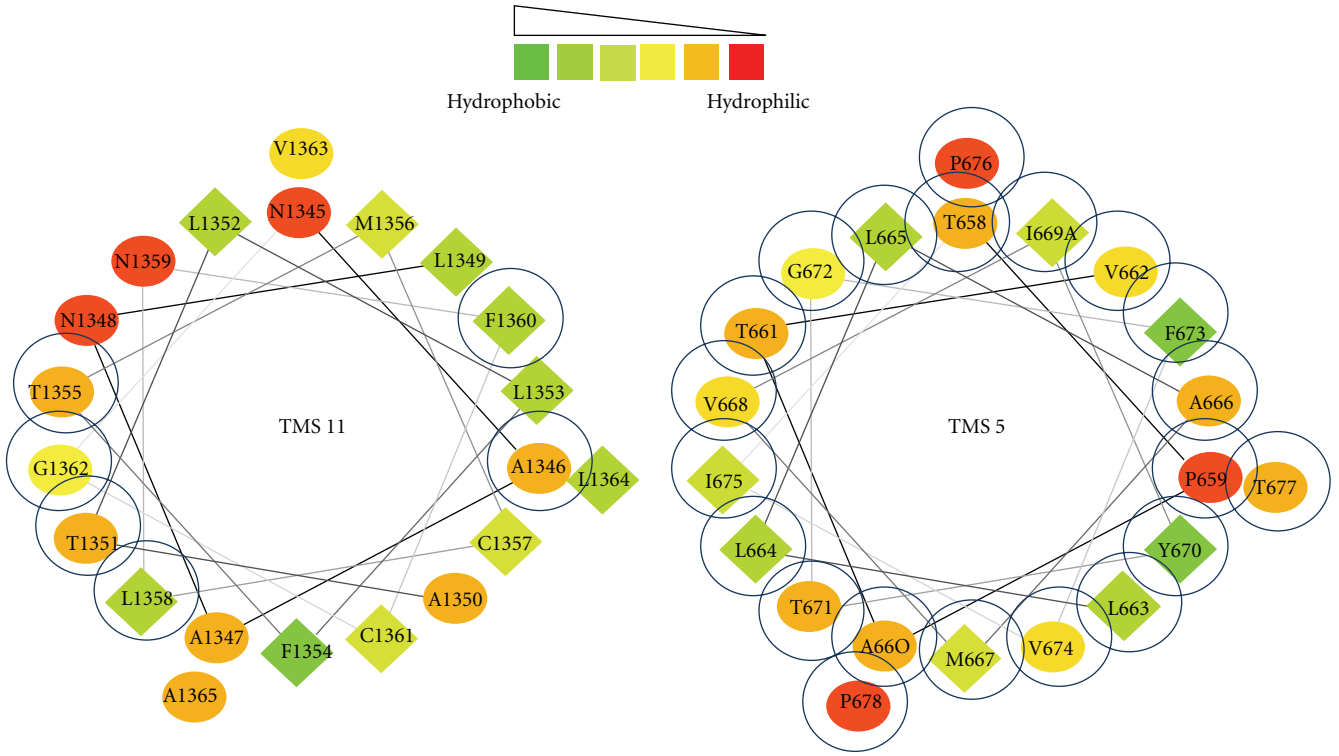


FIGURE 8: Helical wheel projection of TMS 5 and TMS11 of CaCdr1p. Helical wheel projection of the protein sequence was constructed by the EMBOSS PEPWHEEL program. This displays the sequence in a helical representation as if looking down the axis of the helix. The hydrophilic residues as circles, hydrophobic residues as diamonds. Hydrophobicity is color coded as well: the most hydrophobic residue is green, and the amount of green is decreasing proportionally to the hydrophobicity, with zero hydrophobicity coded as yellow. Hydrophilic residues are coded red with pure red being the most hydrophilic residue, and the amount of red decreasing proportionally to the hydrophilicity. The mutations that affected drug resistance are circled blue.

8. Concluding Remarks

The drug transporters belonging to either ABC or MFS superfamily of proteins are the main contributors of azole resistance in pathogenic *C. albicans*. In this regard, CaCdr1p, a major ABC multidrug transporter, has been widely studied. CaCdr1p like its sister fungal homologues is unique in terms of variant sequences present in otherwise conserved domains in NBDs. It is established that each unique substitution in CaCdr1p and by extension of other fungal ABC transporters have dedicated role in ATP binding and hydrolysis and, thus, are essential for drug efflux. Why fungal transporters alone have evolved and retained these divergent domain based amino acid substitution is not understood. But these unique residues also provide an opportunity to develop novel modulators or inhibitors of these efflux pump proteins.

Abbreviations

ABC: ATP binding cassette
MFS: Major facilitator superfamily
IAAP: Iodoarylazido prazosin
NBD: Nucleotide-binding domain
TMSs: Trans membrane segments
TMDs: Trans membrane domains
CDR: *Candida* drug resistance protein
GFP: Green fluorescence protein.

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Review Article

The Dynamic Structure of the Estrogen Receptor

Raj Kumar,[1] Mikhail N. Zakharov,[2] Shagufta H. Khan,[1] Rika Miki,[3] Hyeran Jang,[2] Gianluca Toraldo,[2] Rajan Singh,[4] Shalender Bhasin,[2] and Ravi Jasuja[2]

[1] *Department of Basic Sciences, The Commonwealth Medical College, Scranton, PA 18510, USA*
[2] *Section of Endocrinology, Boston University School of Medicine, Boston, MA 02118, USA*
[3] *Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA*
[4] *Division of Endocrinology and Metabolism, Charles Drew University of Medicine and Science, Los Angeles, CA 90059, USA*

Correspondence should be addressed to Ravi Jasuja, jasuja@bu.edu

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The estrogen receptor (ER) mediates most of the biological effects of estrogens at the level of gene regulation by interacting through its site-specific DNA and with other coregulatory proteins. In recent years, new information regarding the dynamic structural nature of ER has emerged. The physiological effects of estrogen are manifested through ER's two isoforms, ER_α and ER_β. These two isoforms (ER_α and ER_β) display distinct regions of sequence homology. The three-dimensional structures of the DNA-binding domain (DBD) and ligand-binding domain (LBD) have been solved, whereas no three-dimensional natively folded structure for the ER N-terminal domain (NTD) is available to date. However, insights about the structural and functional correlations regarding the ER NTD have recently emerged. In this paper, we discuss the knowledge about the structural characteristics of the ER in general and how the structural features of the two isoforms differ, and its subsequent role in gene regulation.

1. Introduction

The estrogen receptor (ER) is a ligand-inducible intracellular transcription factor that mediates most of the biological effects of estrogens at the level of gene regulation [1–3]. Estrogen biology is exceedingly complex and important in the development and function of numerous tissues and physiological phenomena [4–6]. In the nucleus, the ER up- or downregulates the expression of target genes by interacting through its site-specific DNA and with other coregulatory proteins that include coactivators and corepressors [1–3]. The ligand-bound ER binds as homodimer to specific DNA sequences termed estrogen response elements (EREs) and regulates transcription through interaction with transcription modulators and recruitment of the general transcription machinery [7]. In recent years, new information regarding the ER structures, intra- and intermolecular interactions, posttranslational modifications, and several other factors pertaining to the ER actions has emerged [8–10]. Like other members of the nuclear hormone receptor (NHR) family, the ER is composed of several functional domains that serve specific roles [11]. Starting from NH_2- to COO-terminus, the principal domains are (1) the N-terminal domain (NTD); (2) DNA-binding domain (DBD); (3) ligand-binding domain (LBD). Two activation function (AF) domains, AF1 and AF2, located within the NTD and LBD, respectively, are responsible for regulating the transcriptional activity of ER [12] (Figure 1(a)).

Full transcription activity of the ER is thought to be achieved by synergism between the two AFs, and their activities are promoter and cell specific [16]. AF1 functions as hormone independent, whereas AF2 function requires the presence of hormone/steroid [12, 17]. In this paper, we focus on the two isoforms of human ER (ER_α (NR3A1) and ER_β (NR3A2)), encoded by two different genes. Both have been cloned and characterized [18]. The physiological effects of estrogen are manifested through both ER_α and ER_β. The ER_α and ER_β receptor isoforms display distinct tissue distributions and signaling response [19–21]. ER_α and ER_β have also been shown to form hetero dimers on EREs [22]. In terms of sequence homology, the ER_β shows a high homology to ER_α in the DBD (more than 95% amino acid identity) and

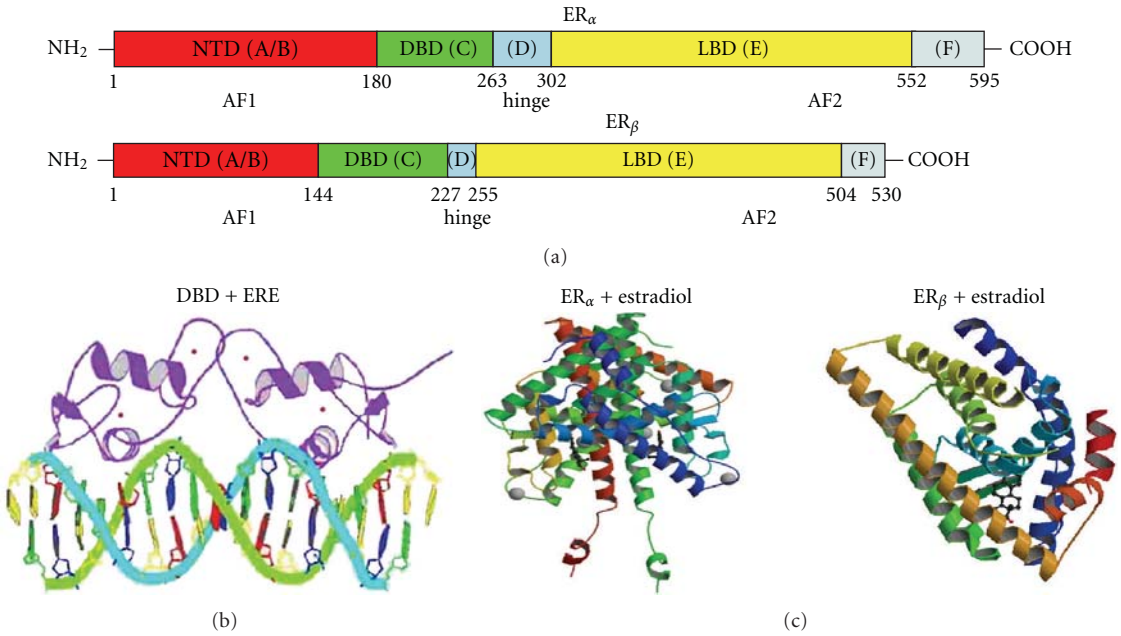


Figure 1: (a) shows the sequence organization of the two isoforms of estrogen receptors, ER_α and ER_β. Different domains are highlighted in different colors: NTD—amino terminal domain—in red; DBD—DNA binding domain—in green; hinge region—in blue; LBD—ligand-binding domain—in yellow; F region located towards the C-terminal end—in grey. Amino acid sequence position is given for each domain. (b) shows estrogen receptor DBD in complex with DNA-ERE (estrogen response element). Structure 1HCQ from PDB (protein databank) [13]. (c) shows 3-dimensional structures of ER_α (left) and ER_β (right) bound to estradiol (PDB structures 1A52 [14] and 3OLS [15]).

in the LBD (~55% amino acid identity) [19, 22]. However, the NTD of ER_β is shorter than that of ER_α with a very poor sequence homology of only ~15% compared to that of ER_α. The three-dimensional structures of the independently expressed DBD and LBD have been solved and show overall folds that represent globular proteins with natively ordered conformations [13, 23–25]. To date, no three-dimensional natively folded structure for the NTD is available not only for the ER but for the entire nuclear hormone receptor (NHR) superfamily. Even though the full length structure of the peroxisome proliferator-activated receptor-γ (PPAR-γ) has been solved, it failed to show any signature of structure formation in its NTD [26]. Warnmark et al. have previously provided insights about the structural and functional correlations regarding the ER NTD [27]. In this paper, we discuss the knowledge about the structural characteristics of the ER and its role in gene regulation.

2. The Hinge Region

The "D" domain which follows DBD is known as a hinge region (Figure 1(a)). It contains nuclear localization signal which gets unmasked upon ligand binding and serves as a flexible region connecting DBD and LBD. Hinge regions of ER_α and ER_β share only 36% homology [19].

3. The "F" Region

The LBD is followed by the C terminal "F" domain, which contains 42 amino acids. Its action was first characterized by Montano et al. by single-point mutations in the domain as well as by whole domain deletion [29]. The "F" domain was found to modulate gene transcription in a ligand-specific manner. The ligand, promoter, and tissue-specific modulation capabilities of the "F" domain were recently studied in detail by Koide et al. [30]. It is also known to impact receptor dimerization [31].

4. The Ligand-Binding Domain

Like other NHRs, the "E" domain of ER contains LBD (Figure 1(a)). It consists of 12 helices, contains hormone binding pocket, and is responsible for the most part of functions activated by ligand binding, such as coregulator binding to AF2 [32] and dimerization interface. While ER_α and ER_β have both overlapping and unique functions, the overall homology between the ER_α protein LBD and ER_β protein LBD does not exceed 55% [19]. However, the two proteins (ER_α and ER_β) display distinct regions of sequence homology [4, 19]. The amino acid residues 223–343 and 404–457 in ER_α and ER_β show a significantly higher homology than that of the sequence encompassing 223–457 and 344–403, respectively [33]. Interestingly, the stretch of the ER LBD amino acid residues 465–468, with lowest homology to ER_β, has been found to be most solvent accessible [34]. On the other hand, the conserved regions with greater homology are protected against degradation and are in direct contact with the ligand [34]. Despite low sequence homology in LBDs within the NHR superfamily, the three-dimensional structural organization of the LBD monomers is strikingly similar.

Both isoforms of ER-LBDs have been shown to form dimers with agonist and antagonist ligands. The dimer interface is primarily encompassed by helices 10 and 11.

As a member of the NHR superfamily of transcription factors, ER_α contains a globular LBD structure that harbors a hormone-binding site, a homo- or heterodimerization interface, and coregulator (activator and repressor) interaction sites [35–38]. The ER_α LBD structure contains 11 α-helices (H1–H12) [24, 39] (Figure 1(c)). The first crystal structure of an ER_α LBD bound to its natural ligand 17β-estradiol (E2) showed that in a compact ellipsoid cavity, E2 is buried in a highly hydrophobic environment [24]. Within this pocket (formed by 22 residues), hydroxyl groups in estradiol at positions 3 and 17 play a crucial role in orienting the steroid/hormone ligand. These hydroxyl groups of the A and D rings are hydrogen bonded to Glu353 from H3, Arg394 from H5, and a water molecule and His524 from H11. In an agonist-bound form, ER_α is spatially organized in a three-layered structure with helices 4, 5, 6, 8, and 9 lining up on one side by H1 and H3, and on the other side are helices 7, 10, and 11 [24]. Due to the central role of estrogen signaling in diverse diseases ranging from cancer to aging, several synthetic ligands to ER_α have been developed [40–43]. The crystal structure of the complex of ER_α LBD bound to the nonsteroidal ligand, diethylstilbestrol, also shows that the hydrophobic interactions primarily govern the accommodation of distinct LBD structures [44].

The crystal structures of the human ER_β bound to genistein [25], estradiol [14] (Figure 1(c)), and rat ER_β to raloxifene [25] assert the importance of hydrogen bond network on the opposite sides of the respective ligands [45]. The bicyclic moiety of genistein orients in a position similar to the C- and D-ring of E2, facilitating the formation of hydrogen bonds of hydroxyl moieties with histidine groups of the receptor [25]. The specificity of the ligand association between the ER_α and ER_β may stem from the distinction in the residues lining the binding pocket [46]. Quite diverse family of compounds (estrogens, some androgens, phytoestrogens, antiestrogens, and environmental estrogens) have been shown in the past to have estrogenizing activity, and to interact with the ER from rat uterus and human breast tumor cells. Interactions of these structurally diverse ligands highlight the intrinsic ER_α and ER_β LBD plasticity [47–49].

5. The DNA-Binding Domain

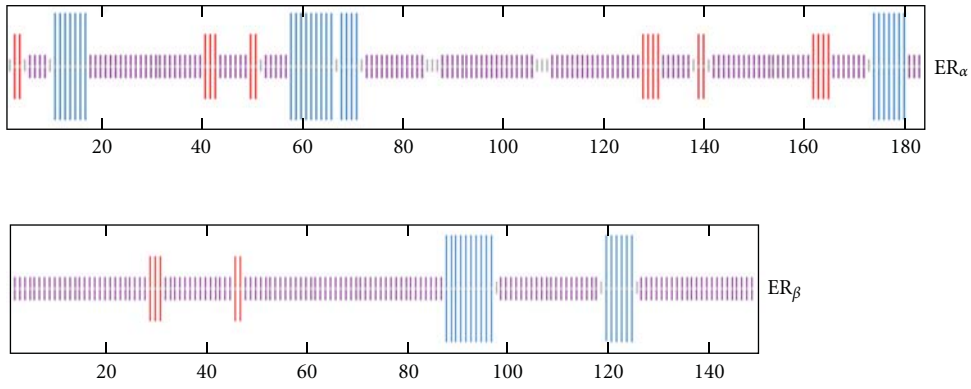
Adjacent to the N-terminal transactivation region (A/B domain), a conserved C domain encompasses the DNA-binding sequence [19]. This DNA-binding domain associates with the response elements which can either reside proximally to the promoter regions or enhancer regions located distant from the transcription initiation site [50]. ER DNA binding domain usually binds to the estrogen response element (ERE) composed of a palindromic hexanucleotide 5′AGGTCAnnnTGACCT3′ [51–53]. The DBD of both ER_α and ER_β isoforms shares the same DNA response elements. The ERE sequences play an important regulatory role [54, 55]. Not only does it dictate the binding affinity of the ER, but also it has been shown to modulate the recruitment of coactivators [56, 57]. The ER_α DBD : ERE structures have been studied extensively by several biophysical techniques [13, 23, 55, 58]. Three-dimensional structure of the ER_α has been solved using nuclear magnetic resonance as well as X-ray crystallographic techniques both alone and in complex with DNA (Figure 1(b)) [13, 23, 55, 58]. The DBD : ERE interactions and ERE-facilitated dimerization are in part mediated through the P box and D box sequences in the Zinc finger domains. These Zn finger subdomains are comprised of 8 cysteine residues that coordinate with the two Zn^{+2} ions. While P box actively interacts with the ERE nucleotides, the D box is present at the dimerization interface [29, 30, 54].

The specificity of ER recognition by ERE is exemplified by interesting studies describing its association with glucocorticoid response element (GRE). Three amino acids in the first Zn finger region or ER dictate its interaction with ERE and GRE [13]. Substitution of these three amino acids with the corresponding amino acids from the glucocorticoid receptor's DBD completely changes ER DBD's specificity for an ERE, and it strongly binds to a GRE sequence to initiate GRE-mediated transcriptional activity [13, 23, 54, 55, 58]. Transcriptional regulation at the ERE can be mediated via two separate mechanisms of ER action. Liganded ER can directly associate with specific response element sequences. In the other mode of action, the ER may participate in a multiprotein, preinitiation complex and regulate gene transcription without a direct interaction with any DNA sequence [59–61]. Together, these mechanisms highlight the complex role of coactivators and response elements in eliciting specificity in transcriptional output.

6. The N-Terminal Domain

To date relatively little information has been available on the structure of the N-terminal regions of the NHRs. Even though the full-length structure of the peroxisome proliferator-activated receptor-γ (PPAR-γ) has been solved it failed to show any signature of structure formation in its very short NTD [26]. We and others have shown that the glucocorticoid receptor's N-terminal transactivation AF1 region and a shorter core fragment of AF1, the AF1 core, are unstructured in aqueous solution [62–66]. In other words, the NTD amino acid sequences possess an intrinsically disordered (ID) conformation, a feature of activation domains of many transcription factors [27, 62, 65, 67, 68]. Similar results have been reported for the ER_α and ER_β, androgen-, and progesterone receptor [69–71]. Thus, activation domains of many signaling proteins including the ER's NTD/AF1 are known to exist in an ID state. One of the reasons for their existence as an ID region seems to be to help them in promoting molecular recognition by providing surfaces capable of binding specific target molecules [72–75].

The computational analyses have established that under physiological conditions, the combination of low mean hydrophobicity and relatively high net charge represent an important prerequisite for the lack of well-defined compact structure in proteins or protein regions/domains [75]. The ID nature of the ER NTD/AF1 has been confirmed by circular



A summary of predicted secondary structural elements in the ER NTD

ER isoform	α-helix	β-sheet	Random coil
ER$_\alpha$	27a.a. (14.7%)	17a.a. (9.2%)	125a.a. (67.9%)
ER$_\beta$	16a.a. (10.7%)	5a.a. (3.3%)	126a.a. (84.0%)

Figure 2: Secondary structural elements predictions of the ER NTD using network protein sequence analysis method as described [28]. Blue, red, and purple colors indicate helix, β-sheet, and random coil, respectively. The upper panel: ER$_\alpha$; the middle panel: ER$_\beta$. The table at the bottom summarizes the contents of different secondary structural elements in the NTD of both ERs.

dichroism method [27]. We performed secondary structural analyses of the ER$_\alpha$ and ER$_\beta$ NTD using network protein sequence analysis [28]. The analytical results show that more than 67% of ER$_\alpha$ NTD contains random coli conformation, whereas in case of ER$_\beta$, the amount of random coil is found to be more than 80% with only a small proportion as helix and sheet in both the cases (Figure 2). It has been proposed that the ID nature of an activation domain allows it to rapidly "sample" its environment until appropriate concentration and affinity of the binding partner proteins are found [65], meaning that they may not be structured until they have recruited and bound their proper interaction partners. Then, either by induced-fit or selective binding of a particular conformer, a high-affinity activation domain : binding partner protein interaction occurs [65, 73]. In case of NHRs' ID NTD/AF1 domains, it has been shown that they undergo a transition to a folded state upon interaction with either components of the general transcription machinery or with other comodulators [76].

Several coregulatory proteins are involved in the effect of the ER on target gene transcription. The TATA box-binding protein (TBP) has a central role in the basal transcription machinery and can directly bind to the NTD of the ER$_\alpha$ but fails to bind to ER$_\beta$ NTD and to potentiate ER-activated transcription [27]. This difference in TBP binding could imply differential recruitment of target proteins by the NTDs of ER$_\alpha$ and ER$_\beta$. The affinity of the ER$_\alpha$ NTD : TBP interaction was determined to be in the micromolar range, as assessed by surface plasmon resonance spectroscopy [27]. Based on these results, it has been proposed that the interaction between ER$_\alpha$ NTD and TBP may proceed in a two-step manner with initial very fast, low-affinity association, followed by a slow, folding event and tighter association [27]. The initial association may be occurring by electrostatic interactions between the acidic residues of highly negatively charged ER$_\alpha$ NTD and the positively charged TBP. However, this initial unstable protein complex subsequently may convert into a more stable form by the folding of the ID ER$_\alpha$ NTD and the formation of specific contacts between the two proteins. In this study, the secondary structures of the independently expressed NTDs of the ER$_\alpha$ and ER$_\beta$ were analyzed using NMR and circular dichroism spectroscopy [27].

Secondary structural analyses concluded that both ER$_\alpha$ and ER$_\beta$ NTDs are unstructured in solution [27]. Further, when ER$_\alpha$ NTD was bound to TBP, structural changes were induced in ER$_\alpha$ NTD [27]. These results support models of TBP as a target-protein for the N-terminal activation domain of ER$_\alpha$. Further, the dissociation of this binding suggests a complex behavior, with a rapid dissociation for ER$_\alpha$ NTD molecules that did not undergo proper folding and a slower dissociation for those molecules that did fold successfully upon physical interaction with the TBP [27]. Such a two-step binding mechanism is consistent with the change in protein conformation that accompanies the ER$_\alpha$ NTD : TBP interaction. Observed differences in binding of TBP to ER$_\alpha$ NTD and ER$_\beta$ NTD supports a model where the two receptors may be utilizing different sets of target binding proteins [65]. This is consistent with the reports of functional differences between ER$_\alpha$ NTD and ER$_\beta$ NTD where it has been shown that the ER$_\alpha$ AF1 domain can function in an autonomous manner, whereas the AF1 function of ER$_\beta$ cannot [27]. It has also been reported that under most conditions ER$_\beta$ possesses a weaker transactivational potency compared to ER$_\alpha$ [6], and these differences appear to be cell and promoter specific [6]. We have earlier shown that TBP binding induces secondary/tertiary structure formation in the ID AF1 domain of

the glucocorticoid receptor such that AF1's interaction with specific coregulatory proteins and subsequent AF1-mediated transcriptional activity is significantly enhanced [77, 78].

Based on the binding of TBP and consequent folding of these ID activation domains, it can be hypothesized that the interaction between NHRs' NTD/AF1 and TBP may be a unified mechanism, through which these ID AF1/NTD acquire a functionally active conformation under physiological conditions. In this conformation, the NTD/AF1 may be able to create favorable protein interaction surfaces for its interaction with specific coregulatory proteins. Of course, the exclusion of certain other binding partners cannot be ruled out. It could thus be hypothesized that a complex and dynamic binding pattern for the N-terminal activation domains of the NHRs occurs to achieve transcriptional activation, where the NTD/AF1 region must be able to obtain different conformations dependent on the binding partner(s). However, a clear picture will emerge only when the functionally folded three-dimensional structure of the NTD/AF1 is solved. At least for now, the differential effects observed in case of two ER isoforms (ER_α NTD and ER_β NTD) suggests that TBP may not be a common coregulator that must bind/fold all the NHRs' NTD/AF1. Thus, it is quite possible that other protein components from the basal transcription machinery may provide such interactions. In fact, we and others have observed that at least in case of the androgen receptor, its ID NTD/AF1 undergoes disorder/order transition through its interaction with RAP74, a subunit of TFIIF, an important component of basal transcription machinery [70, 79].

7. Summary and Perspectives

Recent observations have led to the conclusion that in cells, ER and several other NHRs behave very dynamically such that their kinetic behavior in cells allows them to rapidly interact with various coregulatory proteins, and with chromatin and DNA [80]. Further, the ER moves to various sites in cells to function, and the local concentrations and various other constellations of potential coregulatory proteins are required to associate with the ER to activate or repress the expression of target genes [80]. The LBD crystal structures have clearly demonstrated that differing sets of coactivators/corepressors come together in response to agonist or antagonist ligand binding, such that agonist in one cell type can be an antagonist in another cell type. The overall picture is one of a complex, dynamic network controlled by the ER. It is not yet clear whether unique tissue/cell-specific coregulatory protein interactions can fully explain the tissue/cell-specific actions of the ER and other NHRs. When the clear picture will emerge, it is certain that other dynamic considerations will prove to be the dominant underlying mechanism.

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Review Article

Catalytic Site Cysteines of Thiol Enzyme: Sulfurtransferases

Noriyuki Nagahara

Department of Environmental Medicine, Nippon Medical School, 1-1-5 Sendagi Bunkyo-ku, Tokyo 113-8602, Japan

Correspondence should be addressed to Noriyuki Nagahara, noriyuki@nms.ac.jp

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Thiol enzymes have single- or double-catalytic site cysteine residues and are redox active. Oxidoreductases and isomerases contain double-catalytic site cysteine residues, which are oxidized to a disulfide via a sulfenyl intermediate and reduced to a thiol or a thiolate. The redox changes of these enzymes are involved in their catalytic processes. On the other hand, transferases, and also some phosphatases and hydrolases, have a single-catalytic site cysteine residue. The cysteines are redox active, but their sulfenyl forms, which are inactive, are not well explained biologically. In particular, oxidized forms of sulfurtransferases, such as mercaptopyruvate sulfurtransferase and thiosulfate sulfurtransferase, are not reduced by reduced glutathione but by reduced thioredoxin. This paper focuses on why the catalytic site cysteine of sulfurtransferase is redox active.

1. Introduction

Cysteine residues in proteins maintain the protein conformation, coordinate metal(s), and regulate protein function [1–3]. Enzymes with catalytic site cysteines (Table 1) [4–42] have critical roles in biologic processes such as cell cycle regulation, apoptosis, and signal transduction [43].

A cysteine residue that easily accepts and donates (an) electron(s) is referred to as a redox-active cysteine, and has a lower pK_a value than an unperturbed cysteine residue. Clairborne and colleagues extensively and successfully studied redox changes of cysteine residues and reviewed the biologic importance of redox-active cysteine [44, 45]; a redox-active cysteine is generally a thiolate at physiologic pH and is easily oxidized to a sulfenic acid. Cysteine-related enzymes are generally inhibited by mild oxidation and are reversibly reduced by thioredoxin or glutathione. The sulfenyl form is a reaction intermediate for peroxiredoxin to form disulfide [46] or protein tyrosine phosphatase 1B to form sulfenyl amide [47, 48].

The sulfenyl form is further oxidized to the sulfinyl form and/or sulfonyl form. It is noteworthy that cysteine sulfinate desulfinase catalyzes the desulfination of cysteine sulfinic acid [49, 50], which is not a reversible reaction. On the other hand, cysteine sulfinic acid reductase (sulfiredoxin) catalyzes the reduction of cysteine sulfinic acid [51, 52], although neither thioredoxin nor glutathione can reduce sulfinic acid. Thus, sulfination of cysteine residues is a reversible oxidative process under the conditions that cysteine sulfinic acid reductase can access the catalytic site cysteine of an enzyme. When the reductase cannot access the catalytic site cysteine, sulfination is as irreversible as sulfonation. Recent studies in redox biology indicate that sulfenic acid is a molecular switch [53].

2. The Catalytic Site Cysteine Residue of Sulfurtransferase Is Redox Active

The catalytic site cysteine of a thiol enzyme is generally redox active: a cysteine residue with a low pK_a value easily accepts and donates (an) electron(s). The catalytic site cysteine is essential for oxidoreductase to form a (intramolecular) disulfide and/or sulfenyl intermediate, and its high reactivity of the nucleophilic cysteine is advantageous for the catalysis of transferase (desulfurase, phosphatase, and sulfurtransferase), hydrolase (cysteine protease), and isomerase (protein disulfide isomerase) (Table 1).

The effects of perturbing the pK_a of a cysteine residue in a protein are not well explained. It is generally considered that a decrease in the pK_a of a cysteine residue is caused by positively charged groups of neighboring amino acid residues and/or strengthening of electrostatic interactions between the group and the sulfur atom due to an increase in the electron density of the sulfur atom of the cysteine

Table 1: Typical thiol enzymes.

Classification		Enzyme name defined as a thiol enzyme	Oxidative inactivation
Oxidoreductase		Glutathione family [4]	Not defined
		Glutaredoxin family [5]	Not defined
		Glyceraldehyde-3-phosphate dehydrogenase [6]	Yes [7, 8]
		Peptide-methionine (S)-S-oxide reductase [9]	Not defined
		Peroxiredoxin [10, 11]	Yes [12]
		Sulphiredoxin [13]	Not defined
		Thioredoxin family [14]	Not defined
Transferase	Desulfurase	Cysteine desulfurase[1] [15]	Not defined
	Phosphatase	Cdc[2] 25 family [16]	Yes [17, 18]
		Protein-tyrosine phosphatases [19]	Yes [20]
	Sulfurtransferase	Mercaptopyruvate sulfurtransferase [21, 22]	Yes [23]
		Thiosulfate sulfurtransferase [24]	Yes [25, 26]
Hydrolase	Cysteine protease	Actinidain family [27]	Not defined
		Bromelain family [28]	Yes [29]
		Calpain family [30]	Yes [31]
		Caspase family [32]	Yes [33]
		Cathepsin family [34]	Yes [35]
		Chymopapain family [36]	Yes [37]
		Ficin family [38]	Not defined
		Mir1-CP[3] [39]	Not defined
		Papain family [40]	Yes [41]
Isomerase		Protein disulfide isomerase [42]	Not defined

[1] pyridoxal 5′-phosphate-dependent enzyme
[2] cdc, cell division cycle
[3] Mir1-CP. Maize insect resistance-cysteine protease

residue. Further, hydrogen bonding stabilizes the proton-dissociated state of the cysteine residue to maintain the pK_a perturbation. Hol and colleagues proposed the interesting notion that the alpha-helix macropole in a protein structure contributes to lowering the pK_a of a cysteine residue [54, 55].

Comparative studies of primary structures of sulfurtransferases (mercaptopyruvate sulfurtransferase [MST] and evolutionarily related rhodanese [TST] [22, 55, 56]) revealed that the consensus sequences around the catalytic cysteine of MST and TST are CG(S/T)G and C(R/Y)(K/H)G, respectively (Figure 1) [22, 55, 56].

The tertiary structures of MST and TST are persulfurated enzymes and stable catalytic intermediates (and also free-TST) [57–60]. In X-ray structural studies of bovine TST by Ploegman and colleagues [58–60] and Hol et al. [61], persulfide was stabilized by a ring of persulfide-stabilizing NH groups; Arg[248], Lys[249], Val[251], and Thr[252] (Figure 1) contributed to hydrogen bonding with an outer sulfur atom of a persulfide at the catalytic site Cys[247], and in addition, Gly[254] and Ser[274] with the Sγ of Cys[247]. Further, two helix-dipoles (α9 and α10) (Figure 2(a)) contribute to lowering the pK_a of the catalytic cysteine residue to approximately 6.5 [54, 62].

Similar to TST, an X-ray structural study of *Leishmania major* persulfurated MST by Alphey et al. [57] revealed that

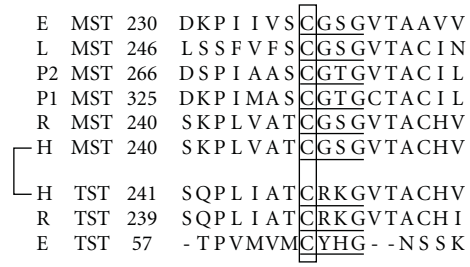
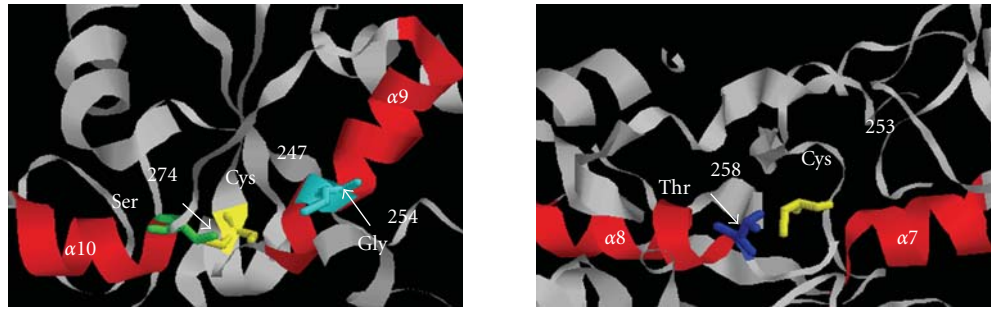


Figure 1: Comparison of the amino acid sequences around a catalytic site cysteine residue between MST and TST Sequence identity was analyzed using GENETYX (GENETYX CORPORATION). Box, a catalytic site. E, *E. coli* (D10496 for MST, NP_417883 for TST); H, *Homo sapiens* (BC009450 for MST, D87292 for TST); L. *Leishmania* (CAC85741); P1 and P2, *Arabidopsis thaliana* (AB032864 and AB032865 for MSTs); R, *Rattus norvegicus* (D50564 for MST, BC088449 for TST). Underlined amino acids, consensus sequences for MST or TST.

Gly[254], Ser[255], Gly[256], Val[257], Thr[258], and Ala[259] (Figure 1) contribute to hydrogen binding with an outer sulfur atom of a persulfide at the catalytic site Cys[253], and further, Thr[258] with the S$\gamma$of Cys[253]. Two helix-dipoles (α8 and α9)



(a) (b)

Figure 2: Model for the two α-helix dipoles of TST and MST, each structure is represented using RasMol. (a) bovine liver TST from 1DP2, red ribbon structure represents two helix-dipoles (α9 and α10) and ball-and-stick model in yellow represents a catalytic site Cys247. (b) *Leishmania major* MST from 1CKG red ribbon structure represents two helix-dipoles (α8 and α9), red ribbon structure represents two helix-dipoles (α8 and α9), and ball-and-stick model in yellow represents a catalytic site Cys253.

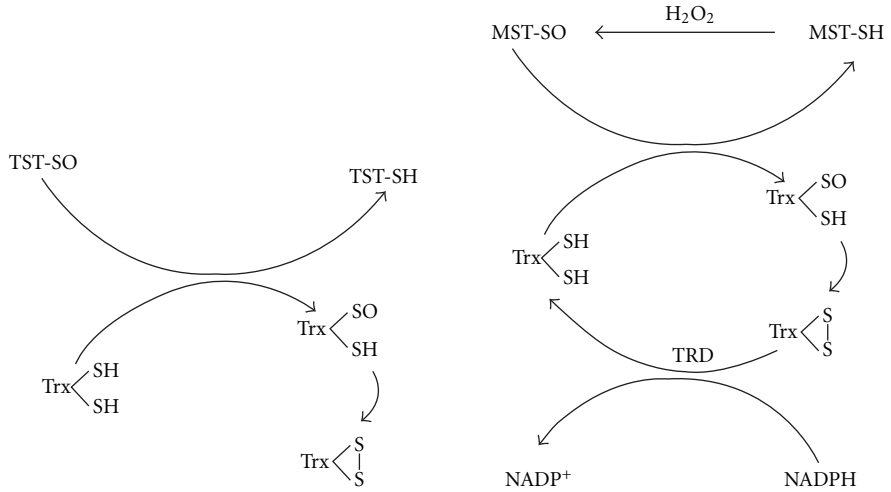


Figure 3: Thioredoxin oxidase activity of TST and thioredoxin peroxidase activity of MST, Proposed thioredoxin oxidase activity of TST (a) reported by Nandi and colleagues [63], which is same as thioredoxin peroxidase activity of MST (b) (from Figure 10 of Nagahara et al. Current Medical Chemistry 2009. 16: 4422). Trx: thioredoxin; TRD: thioredoxin reductase.

(Figure 2(b)) also contribute to lowering the pK_a of the catalytic cysteine.

The Cdc25 phosphatase family is a rhodanese superfamily [64, 65], and the catalytic subunit contains an alpha-helix macropole like MST and TST [66], which could contribute to lowering the pK_a of the catalytic cysteine. A member of the pyridoxal 5′-phosphate-dependent enzyme family, cysteine desulfurase (*E. coli* NifS CsdB), has an alpha-helix macropole like MST and TST [63, 67].

In sulfurtransferases, alpha-helix macropoles surrounding a catalytic cysteine characterize the cysteine as redox active, indicating that hydrogen bonding between an outer sulfur atom of a persulfide at the catalytic site cysteine with surrounding amino acids is important for stabilizing catalytic intermediates.

3. Sulfenate Formation at a Catalytic Site in Sulfurtransferase

When MST and TST are oxidized, catalytic site cysteines are reversibly sulfenated [23, 68] and are stable, probably due to hydrogen bonding. Sulfenyl TST was confirmed by the observation of thioredoxin oxidase activity and was reduced by reduced thioredoxin (Figure 3(a)) [68]. On the other hand, sulfenyl MST was confirmed by the observation of thioredoxin peroxidase activity (Figure 3(b)) and mass spectrometric data, and was reduced not by reduced glutathione but rather by reduced thioredoxin [23]. These findings indicate that the half-redox potential of sulfenate is lower than that of glutathione and higher than that of thioredoxin ("low redox potential sulfenate" [23]). The redox potential of

the cysteine residue is pH-dependent due to pH-dependent perturbation of the electric field strength surrounding the cysteine residue via interactions of the cysteine residue with basic amino acids. In fact, the pH-dependent perturbation of the redox potential of the cysteine residue was demonstrated in the thioredoxin superfamily [69]. The active-site loop of TSTs contains two basic residues whereas no charged residues are observed in MSTs [65], suggesting that the electric field strength surrounding cysteine residue of mitochondrial TST is larger than that of MST. This hypothesis, however, has not been tested experimentally.

4. Possible Biologic Function of Catalytic Site Sulfenate of Sulfurtransferase

Sulfenyl sulfurtransferase is neither a reaction intermediate nor an active form whereas the sulfenyl form is a reaction intermediate of a thiol-oxidoreductase. Therefore, the biologic relevance of a conversion between sulfenate and thiolate at a catalytic cysteine is not clear. There are two possibilities: first, the molecular feature was accidentally acquired during the molecular evolution of the thiol enzyme family, and second, some molecular entity, such as an antioxidant protein, has evolved under oxidizing atmospheric conditions.

MST and TST are widely distributed in eukaryotes and prokaryotes [56, 70], and in eukaryotic cells, MST is distributed in the cytoplasm, mitochondria, and in chloroplasts (in plants) [71, 72]. On the other hand, TST distribution is restricted to the mitochondria and chloroplasts (in plants) [72–74]. Thus, both MST and TST are located in mitochondria and chloroplasts (in plants). Based on the minor catalytic contributions, the latter possibility is likely: MST and TST could locally serve as antioxidant proteins.

Unlike sulfurtransferases, a sulfenyl amide is found at the catalytic site cysteine in protein tyrosine phosphatase IB in an unusual oxidized form. This enzyme is oxidized to form sulfenate at the catalytic site cysteine, and the Sγ atom of the cysteine covalently binds to the main chain nitrogen atom of an adjacent serine to form sulfenyl amide [47, 48]. This sulfenyl amide enzyme is inactive. Reduced glutathione cleaves (reduces) the ring structure of sulfenyl amide to completely restore activity [44, 45]. The redox regulation of the enzymatic activity correlates with signal transduction [75–77] via the regulation of protein dephosphorylation [78–81].

Cdc25C, a member of the phosphatase family, has two redox active cysteines (Cys^{330} and Cys^{377}). Mild oxidation forms sulfenate at one of the two redoxactive cysteines (Cys^{377}) resulting in the formation of an intramolecular disulfide between them, which produces an inactive form of the enzyme [17, 18]. Further, the oxidized form is reduced not by reduced glutathione but rather by reduced thioredoxin [18], meaning that the cdc25 family forms a low redox potential disulfide. The redox regulation of the enzymatic activity correlates with the regulation of the cell cycle via the regulation of protein dephosphorylation [17, 18]. Further oxidation forms sulfinate at Cys^{377}, which is an inactivated form, resulting in degradation of the protein [17].

The cysteine protease caspase, which regulates apoptosis, is also inactivated by mild oxidation, probably due to sulfenate formation at the catalytic site cysteine, and can be reduced by reduced glutathione *in vitro* [33]. Physiologic levels of glutathione, however, are unable to restore activity [33], and other cellular reductants such as thioredoxin have not been examined. The biologic importance of redox regulation of the caspase activity remains unknown.

5. Summary

Both MST and TST are localized in mitochondria and chloroplasts, and probably serve as antioxidant proteins.

The catalytic site cysteine residue of MST and TST is redox active, probably due to helix dipoles.

Stable and low redox sulfenate is formed at the catalytic site cysteine of MST and TST, and is reduced by thioredoxin.

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Review Article

The Catalytic Machinery of a Key Enzyme in Amino Acid Biosynthesis

Ronald E. Viola,[1] Christopher R. Faehnle,[2] Julio Blanco,[1] Roger A. Moore,[3] Xuying Liu,[4] Buenafe T. Arachea,[1] and Alexander G. Pavlovsky[1]

[1] *Department of Chemistry, University of Toledo, Toledo, OH 43606, USA*
[2] *Structural Biology Lab, Cold Spring Harbor Labs, Cold Spring Harbor, NY 11724, USA*
[3] *Rocky Mountain Labs, National Institutes of Health, Hamilton, MT 59840, USA*
[4] *Department of Pharmacology, Yale University, New Haven, CT 06520, USA*

Correspondence should be addressed to Ronald E. Viola, ron.viola@utoledo.edu

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The aspartate pathway of amino acid biosynthesis is essential for all microbial life but is absent in mammals. Characterizing the enzyme-catalyzed reactions in this pathway can identify new protein targets for the development of antibiotics with unique modes of action. The enzyme aspartate β-semialdehyde dehydrogenase (ASADH) catalyzes an early branch point reaction in the aspartate pathway. Kinetic, mutagenic, and structural studies of ASADH from various microbial species have been used to elucidate mechanistic details and to identify essential amino acids involved in substrate binding, catalysis, and enzyme regulation. Important structural and functional differences have been found between ASADHs isolated from these bacterial and fungal organisms, opening the possibility for developing species-specific antimicrobial agents that target this family of enzymes.

1. Introduction

Enzyme-catalyzed reactions organized into sequential pathways are responsible for producing the molecules needed to sustain life. While many essential metabolic pathways are present in all known life forms, there are also significant differences between microbial and mammalian metabolism. Most microbial species can synthesize all of their important metabolic building block molecules, while mammals must acquire many of these from dietary sources. These metabolic differences provide a substantial number of potential protein targets to be examined for the development of selective antimicrobial agents. There is a growing need to identify effective new antibiotics that function against new targets to combat the expanding threat from pathogenic species that are becoming increasingly resistant to existing antibiotics [1, 2]. There are, however, fundamental problems that must be resolved for effective biocides to be developed against metabolic enzyme targets. Infectious microorganisms often use their host as a source of essential metabolites, thus bypassing inhibitors designed to block key steps in microbial metabolism. In many instances microorganisms have developed alternative routes for the production of important metabolites that can readily bypass inhibited enzyme reactions [3]. Essential enzymatic pathways can also be altered in response to an antibiotic threat. Although these microbial pathways may not exist in mammals, related enzymes with similar active site geometries or substrate binding motifs can potentially interact with even the most carefully designed inhibitors. The structure and mechanism of a bacterial metabolic enzyme must be thoroughly characterized before a potential drug target can be fully evaluated. Aspartate β-semialdehyde dehydrogenase (ASADH) is a key enzyme in an essential amino acid biosynthetic pathway that is not present

in mammals. This enzyme has been thoroughly investigated and is now being examined as a target for the development of new antimicrobial agents.

2. The Aspartate Biosynthetic Pathway in Microbes

The aspartate biosynthetic pathway is present only in plants and microbes. The commitment step to this pathway is the phosphorylation of aspartic acid catalyzed by a family of aspartokinases (AK). The next enzyme in the pathway, ASADH, catalyzes the production of aspartate semialdehyde (ASA) that is located at a critical junction in this pathway. From this point one pathway branch leads to the production of lysine through the metabolite diaminopimelate, while the alternative route leads to the synthesis of methionine, threonine, and isoleucine with homoserine serving as their common intermediate (Figure 1). Thus, one quarter of the amino acids required for protein synthesis in all organisms are linked through and synthesized by this pathway [4]. The aspartate pathway is exquisitely regulated to control the total amino acid output as well as the relative levels of each amino acid. This regulatory scheme must also maintain the levels of several essential metabolic intermediates during periods of low protein synthesis. This is accomplished by coordinated regulation by the end product amino acids, both through feedback inhibition and by selective gene repression [5]. This regulatory scheme allows each end product to modulate the flux through the initial pathway steps, with branch point allosteric enzymes providing further control over the product levels.

In addition to the essential amino acids produced in the aspartate pathway, several important metabolites are synthesized that play crucial roles in important developmental processes, such as cell wall biosynthesis, protective dormancy, and virulence factor production. For example, dihydrodipicolinate is a precursor of dipicolinate, the major component of bacterial spores [6], and diaminopimelate (DAP) is required for cross-linking of the peptidoglycan polymers [7] in bacterial cell wall synthesis (Figure 1). Another product of this pathway, *S*-adenosylmethionine, is an essential methyl group donor that also serves as a precursor for quorum sensing signaling molecules with critical roles in triggering virulence factors in infectious microorganisms [8, 9]. Homoserine lactone signals, for example, are essential for both virulence and biofilm development in the human pathogen *Acinetobacter baumannii* [10].

Because this pathway produces many essential compounds that are involved in a range of critical functions, disruptions to the aspartate pathway are fatal to those microorganisms. In particular, selective perturbations of the *asd* gene that encodes for ASADH are lethal to numerous infectious microorganisms. For example, *asd* mutants of *Salmonella typhimurium* develop an absolute growth requirement for diaminopimelate (DAP), a critical cell wall cross-linking component (Figure 1) in Gram-negative bacteria [11]. This mutated organism undergoes cell lysis when DAP is not supplied, and, since this metabolite is not produced in mammals it cannot be supplied by the host organism. A similar loss of viability is observed in *asd*-deficient *E. coli* strains. During amino acid starvation microorganisms often use specific transport systems to import exogenous amino acids available from the host environment [12]. However, *de novo* biosynthesis of lysine is essential for the survival of *Mycobacterium tuberculosis* during infection in mice, despite the presence of lysine in the host [13]. Even if an organism could mutate to improve lysine transport capacity in response to aspartate pathway inhibition, reversal of the decarboxylation that produces lysine from DAP is neither kinetically nor thermodynamically feasible. Both of these end products and several additional intermediates of this pathway are thus critical for microbial cell viability, both in culture and during host infection. Blockage of the aspartate pathway is fatal to microorganisms. Therefore the identification of effective inhibitors of key aspartate pathway enzymes should provide lead compounds for the development of new biocides. To achieve this aim we have focused on the functional and structural characterization of the microbial ASADH family of enzymes.

3. Sequence and Structural Comparisons among the Aspartate-β-Semialdehyde Dehydrogenases

Aspartate-β-semialdehyde dehydrogenase (ASADH) catalyzes the second reaction in the aspartate pathway, the reductive dephosphorylation of β-aspartyl phosphate to aspartate-β-semialdehyde (ASA) (Scheme 1), at a critical branch point in this pathway.

The ASADHs from a variety of organisms encompass a considerable diversity of sequence homologies, ranging from less than 10% to as high as 95% sequence identity when compared to the *Escherichia coli* enzyme (*ec*ASADH). The ASADH enzymes in microorganisms can be divided into three branches consisting of the enzymes from Gram-negative bacteria, Gram-positive bacteria, and archaea/fungi. These branches were initially identified and partitioned through sequence alignments and now, with representative high resolution structures available from each branch, have been compared by structural alignments. The earliest structures of ASADHs are from enzymes that were isolated and purified from Gram-negative bacteria. These enzyme forms share significant sequence and structural homology and include structures of the ASADHs from *E. coli* [14, 15], *Vibrio cholerae* [16], and *Haemophilus influenzae* [17]. The overall structure of these ASADHs is a homodimer with an extensive contact surface between the subunits. Each monomer is composed of a carboxy-terminal domain primarily involved in hydrophobic intersubunit contacts, and a more hydrophilic amino-terminal domain that forms the active site and NADP binding site (Figure 2).

The ASADH from *Streptococcus pneumoniae* (*sp*ASADH) is the first member of the Gram-positive bacterial branch that was structurally characterized [18]. *sp*ASADH is a good representative of the other Gram-positive bacterial ASADHs with greater than 40% sequence identity to these enzymes, while having less than 25% identity with any of

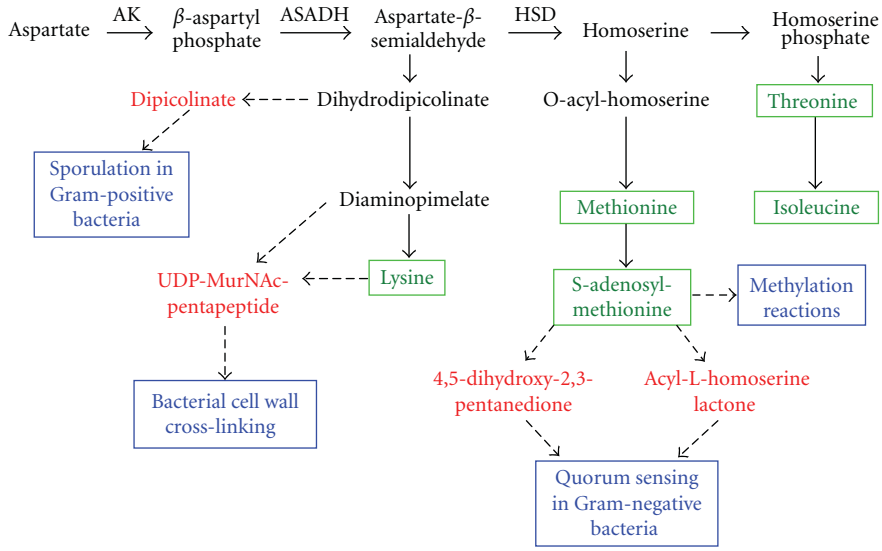
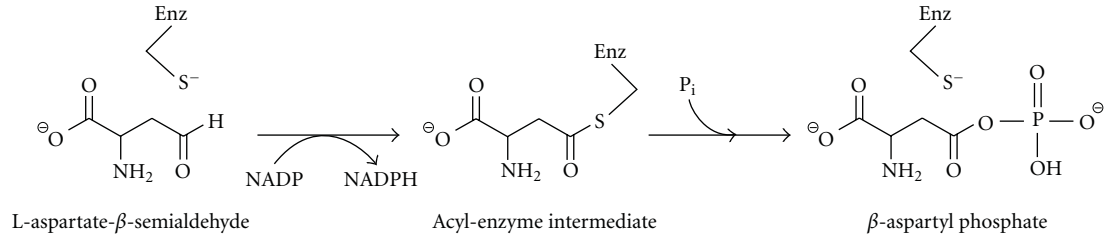


Figure 1: The aspartate biosynthetic pathway in microorganisms. The end product amino acids produced by this pathway are shown in green. Pathway-specific metabolites (shown in red) play crucial roles in microbial life cycle functions (shown in blue).



Scheme 1: The conversion of aspartate β-semialdehyde to β-aspartyl phosphate catalyzed by aspartate β-semialdehyde dehydrogenase in the reverse (nonphysiological) reaction.

the Gram-negative enzymes. Unexpectedly, this *sp*ASADH has lower sequence homology to the Gram-negative enzymes than to those of the archaeal/fungal branch, with sequence identities from this comparison ranging from 18 to 30%. The structure of the ASADH from the archaeal hyperthermophile *Methanococcus jannaschii* (*mj*ASADH) has a similar overall fold and domain arrangement as the Gram-negative enzyme forms [19] despite being less than 10% sequence identity. But the complete set of functionally important active site amino acids has been conserved in *mj*ASADH, suggesting an identical mechanism in the enzyme from this ancient organism despite its lower catalytic efficiency. The first structure of an ASADH from a fungal species, *Candida albicans* (*ca*ASADH), was recently determined, and it also possesses a similar overall fold, domain organization, and active site structure as the other members of this enzyme family [20]. This fungal enzyme has less than 30% sequence identity to any of the bacterial ASADHs and only 40% identity to its closest homologue. Alignment of the structures that have been determined from each branch of the ASADH family shows that both *sp*ASADH and *ca*ASADH are most similar to the archaeal ASADH from *M. jannaschii* and more distantly related to the Gram-negative enzymes.

Despite the overall sequence and structural similarities, there are a number of insertions and deletions which serve to differentiate the three ASADH branches. Structural variations between these branches include a sequence of residues on the enzyme surface that contribute to the binding pocket for the coenzyme NADP. The *E. coli* ASADH is representative of the Gram-negative enzymes, with a highly flexible coenzyme binding loop in the absence of NADP that becomes ordered in response to NADP binding [15]. The archaeal *mj*ASADH has three conserved insertions totalling 30 residues when aligned against the Gram-negative bacterial *ec*ASADH [19], with each insertion located on the surface of the structure. A 13-residue insertion in *mj*ASADH leads to an alternative orientation of the coenzyme binding loop, differing from that in the Gram-negative enzymes by about 90°, causing it to drape over and occlude the coenzyme binding pocket [19]. In addition, the helical subdomain that comprises a significant fraction of the dimer interface (Figure 2) is absent in the archaeal *mj*ASADH. The Gram-positive *sp*ASADH has the same number of residues in this region as the Gram-negative bacterial enzymes, but two short β-strands replace the helix-turn-helix structure observed in the helical subdomain of *ec*ASADH (Figure 2). The fungal enzyme from *C. albicans* is also missing the helical

subdomain [20] and contains most of the insertions and deletions observed in the archaeal enzyme. These structural changes suggest differences in how each branch of this enzyme family can carry out its catalytic role, even though each possesses an identical repertoire of highly conserved active site functional groups.

4. Role of Active Site Functional Groups

In spite of the overall sequence diversity between the different branches of the ASADH family the identity of the core active site functional groups has been preserved throughout evolution (Figure 3). A set of active site mutants of ASADH from *H. influenzae* (*hi*ASADH) was examined kinetically and structurally with the goal of more precisely establishing the role for each functional group in substrate recognition and binding. A cysteine had been previously identified from biochemical studies as the likely active site nucleophile [21]. Replacement of a single sulfur atom with an oxygen in the C136S mutant virtually eliminates catalysis (Table 1), supporting the essential role of this residue as the catalytic nucleophile. Some decrease in catalytic activity would be expected because the hydroxyl group of serine is a weaker nucleophile than a cysteine sulfhydryl group. Only minor changes are observed in the active site structure of this mutant despite the nearly complete loss of catalytic activity. But a change in orientation of the introduced hydroxyl group is likely responsible for the very low activity of this mutant. The side chain hydroxyl group of the introduced serine rotates by about 90 degrees, with this new orientation stabilized by a hydrogen-bond to an adjacent backbone carbonyl group [22]. This reorientation not only moves this functional group out of the position needed to act as a nucleophile, but also competes with the involvement of this backbone carbonyl in intermediate stabilization and alters the position of the bound phosphate group.

Mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes a similar reaction, an oxidative phosphorylation, to the reverse reaction catalyzed by ASADH and appears to do so by the same mechanism [23]. Before the first structure of ASADH was available, sequence alignments to the mechanistically related enzyme (GAPDH) family were used to identify and then test the roles of possible active site functional groups. Both enzymes were proposed to contain a cysteine-histidine catalytic dyad; however the corresponding position of the catalytic histidine in GAPDH is occupied by a conserved glutamine in ASADH [24]. Replacement of this functional amino acid causes a loss in catalytic activity that is not fully recovered even when a histidine is introduced at this position in ASADH. This mystery was solved when the first ASADH structure revealed that the essential histidine residue actually comes from a completely different position in the primary sequence, with a loop containing H277 folded into the enzyme active site [14]. The H277N mutant of *hi*ASADH has significantly impaired activity (Table 1), highlighting the key role for this residue. However a ternary complex structure with NADP and the active site-directed inactivator, S-methyl-L-cysteine sulfoxide (SMCS), showed continuous density extending from the Cys136 nucleophile [22]. This

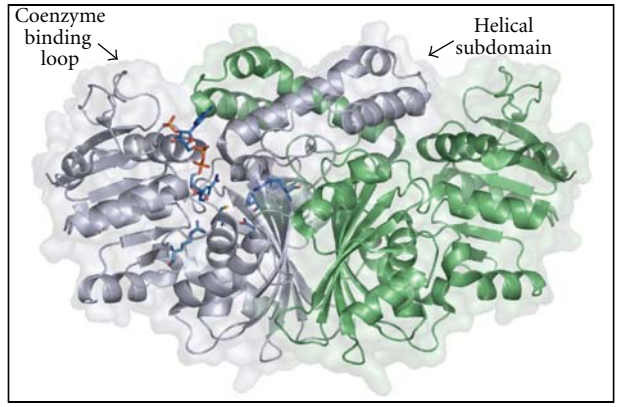


Figure 2: Ribbon drawing and surface rendering of the ASADH from *Escherichia coli* showing the overall structure. Each of the ASADH structures shares a conserved domain organization and exists as a functional homodimer. The largest structural differences between the different branches of this enzyme family manifest themselves in the coenzyme binding loop and in the helical subdomain bridging the dimerization interface. The bound coenzyme NADP and the active site amino acids are shown in one subunit as blue sticks.

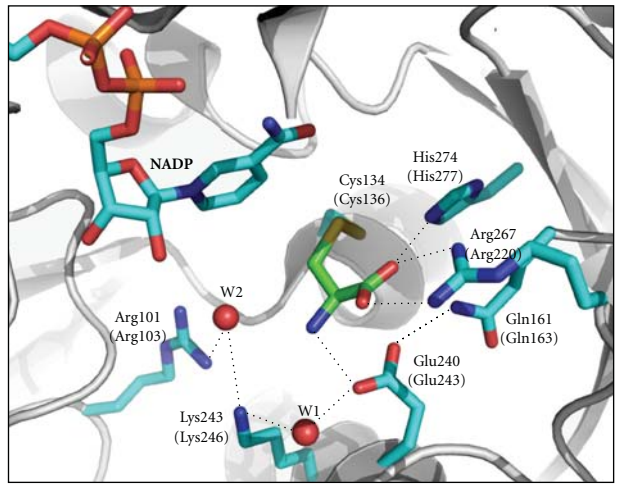


Figure 3: The active site structure of *Vibrio cholerae* ASADH with bound NADP and the covalent inactivator SMCS (shown in green). Cys134 is the active site nucleophile, and His274 is the acid-base catalyst. Glu240 and Arg267 are substrate binding groups, with Arg101 and Lys243 comprising part of the phosphate binding site that is occupied in this structure by a water molecule (W2). *H. influenzae* numbering is in parentheses (figure adapted from reference [16]).

structure is consistent with the covalent attachment of SMCS to the active site nucleophile. The position of this bound inactivator is shifted in the H277N mutant relative to its position when bound in the wild-type enzyme [15]. This reorientation moves this compound further from the bound phosphate that must attack the carbonyl carbon of the acyl-enzyme intermediate to generate β-aspartyl phosphate in the reverse reaction (Scheme 1).

Table 1: Kinetic and structural consequences of ASA dehydrogenase active site mutants.

Active site group[a]	Proposed function	Mutant	Catalytic activity[b]	Structural effect of the mutation	PDB code
Cys136 (Cys134)	Active site nucleophile	C136S	<0.01%	Rotation of S136 shifts the amino group of ASA and moves the bound phosphate by 1 Å	1PQP
His277 (His274)	Acid-base catalyst	H277N	1.0%	Breakdown of the initial tetrahedral intermediate is slowed by a shift in the phosphate position	1PQU
Arg270 (Arg267)	Binds the substrate carboxyl group	R270K R267L	0.1% 9.5%	The introduced lysine moves about 2 Å away from the original arginine position	1PS8
Glu243 (Glu240)	Binds the substrate amino group	E243D	1.2%	The bound intermediate shifts position by about 0.5 Å towards the shorter side chain of D243	1Q2X
Lys246	Helps orient bound phosphate	K246R	3.3%	The introduced arginine rotates by about 90° to form new interactions with S99 and K242	1PU2
Arg103	Binds phosphate	R103K R103L	0.4% 0.07%	Displaces the active site loop (N135 to S139) which shifts C136 away from H277	1PR3 1OZA

[a]*H. influenzae* sequence numbering, with the numbers in parenthesis referring to the *V. cholerae* sequence
[b]k_{cat} determined by varying ASA at fixed NADP levels and expressed as a percent of wild type enzyme activity

So in summary for these catalytic mutants, the C136S mutant still allows substrate binding but formation of the covalently bound intermediate is slowed by a less efficient and misoriented nucleophile. In contrast, the H277N mutant appears to form the initial tetrahedral intermediate efficiently, but its subsequent breakdown is hindered by a shift away from the bound phosphate nucleophile. In each case, small perturbations in the positioning of essential catalytic groups or reactive intermediates are shown to have dramatic effects on enzyme catalysis.

Additional mutant structures of *hi*ASADH have been determined, with each substitution replacing a putative substrate binding group in order to assess their function. Each of these mutants displayed significantly impaired catalytic activity, ranging from ~10% to less than 0.1% that of the wild-type enzyme [25]. However, the structural basis for the diminished activities is different for each mutation (Table 1). A conserved arginine (Arg270) in the ASADH family aligns with a conserved Arg231 in the GAPDH family, a residue that had previously been assigned a role in binding the substrate phosphate group [26]. Based on kinetic studies this arginine residue was proposed to have a comparable role in *hi*ASADH, namely, binding the carboxyl group of the substrate ASA [24]. The structures of substrate complexes of the wild-type enzyme [17] and of the inactivator SMCS covalently bound to *Vibrio cholerae* ASADH (*vc*ASADH) [16] support this assignment by clearly establishing the presence of a bidentate interaction between Arg270 and the carboxylic group of the substrate ASA.

Arginine270 was replaced by lysine in *hi*ASADH to assess its relative importance in substrate binding by disrupting the bidentate interaction that is formed with ASA. Maintaining this interaction between the introduced lysyl amino group and the ASA carboxyl group will require a shift in the orientation of both the lysyl side chain and the resulting tetrahedral intermediate relative to their positions in the wild-type enzyme. The dramatic loss of activity in R270K can be explained by a shift of the intermediate away from the position that is necessary to form and maintain a productive interaction between its carboxyl group and the altered substrate binding residue during the catalytic cycle. A neutral side chain introduced at this position in *ec*ASADH (R267L) cannot form any binding interaction with the substrate carboxyl group, leading to a 30-fold increase in the K_m for ASA [24] while also permitting greater flexibility for positioning of the covalent intermediate. This flexibility increases the likelihood that the intermediate can adopt a catalytically viable conformation relative to that imposed in the *hi*ASADH R270K mutant, and this conformational flexibility is manifest in the 100-fold greater activity seen in the R267L *ec*ASADH enzyme form (Table 1).

Glutamate243 provides a side chain carboxyl group in the active site of ASADH whose role in the catalytic cycle had not been definitively established. This group is highly conserved among ASADHs from different organisms, and structural studies have shown that it is in position to potentially interact with the amino group of the tetrahedral intermediate in the *hi*ASADH complex [17]. An E243D mutant was produced to assess the possible role of this residue in substrate and intermediate binding. Kinetic studies of E243D failed to show the expected detrimental effect on substrate interactions, with the K_m for ASA unchanged from that of the wild-type enzyme. Instead the catalytic efficiency of this mutant is significantly compromised, reducing the k_{cat} value to about 1% of that of wild-type *hi*ASADH (Table 1). In this case the position of the bound intermediate (and presumably

that of the bound substrate) shifts towards the shorter side chain of the introduced aspartate at this position. This shift allows this mutant to maintain substrate binding affinity, but compromises the positioning between the intermediate and the bound cofactor thereby leading to impaired catalytic efficiency.

Each of these mutations was prepared with the aim of removing and testing critical substrate binding groups. However the structural rearrangements that occur as a consequence of these replacements manifest themselves not in a loss in substrate binding affinity, but in a loss of catalytic activity. Structural characterization of these mutants shows that only subtle changes in key active site residues, such as rotation of a side chain to form a new hydrogen-bond or a shift in position relative to a catalytic intermediate, are sufficient to adversely affect catalysis.

The phosphate-binding site of ASADH is capable of accommodating different tetrahedral oxyanion analogues [27], leading to different functional consequences. Both oxyanion substrates and oxyanion inhibitors bind in the same position by using the same set of ligands (Figure 4), raising the question of what distinguishes a substrate from an inhibitor? In the apoenzyme the side chain hydroxyl of Thr137 forms a hydrogen-bond with Asn135, and this pairing prevents any interactions with the active site Glu243. Upon binding of either oxyanion substrate, phosphate or arsenate, Thr137 switches hydrogen-bonding partners through a 60° rotation that disrupts its interaction with Asn135. This new conformation moves the threonine hydroxyl group into position to interact with and orient Glu243, an important substrate binding group. Glu243 remains in this substrate binding position in the arsenate structure, and this hydrogen-bond with the Thr137 hydroxyl group persists even in the absence of bound ASA. So, the presence of an oxyanion substrate in the active site helps to position Glu243 to interact with the substrate. However, in the presence of the inhibitor periodate this threonine does not change position and switch partners. In this inhibitor-bound structure Thr137 remains hydrogen-bonded to Asn135, which is now oriented away from Glu243 and cannot stabilize the position of this side chain. This single change in hydrogen-bonding partners is apparently sufficient to interfere with the binding of ASA. Thus, subtle shifts in the position of side chains, even those not directly involved in substrate binding or catalysis, can lead to a sequence of events that result in loss of function for a finely tuned enzyme catalyst.

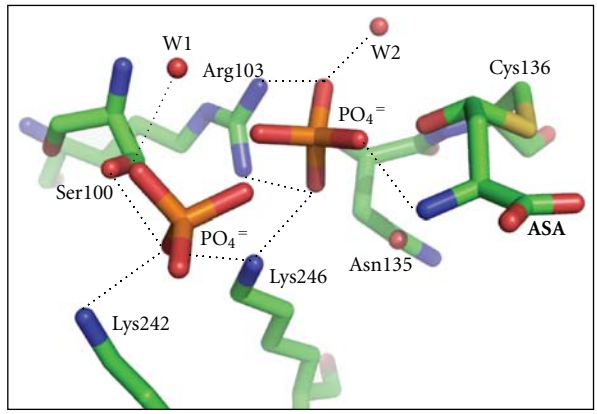


Figure 4: The oxyanion binding site in *H. influenzae* ASADH. Each of the oxyanion substrates and inhibitors interacts with the same protein ligands, Arg103 and Lys246, and is bound within attacking distance of the covalent acyl-enzyme intermediate. In the substrate structures with either phosphate or arsenate a second oxyanion molecule is bound to Ser100, Lys242, and Lys246 (figure adapted from reference [28]).

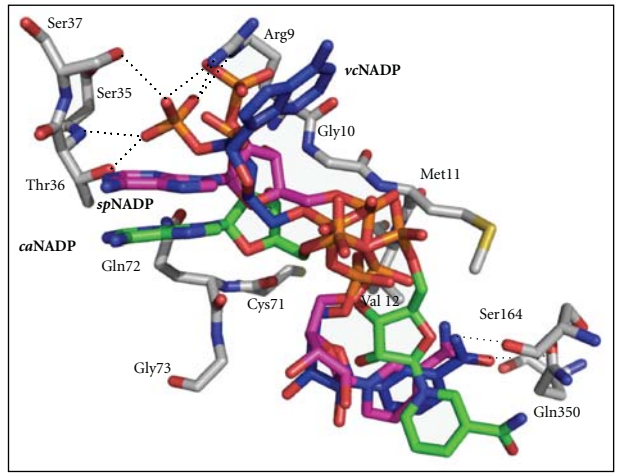


Figure 5: Differences in coenzyme binding in ASADHs. An overlay of the NADP binding orientations in *V. cholerae* ASADH (blue), *S. pneumonia* ASADH (red), and *C. albicans* ASADH (green). The center of the adenine ring in the Gram-positive and fungal enzymes has shifted by about 8.5 Å with respect to its position in *vc*ASADH, and the position of the exocyclic amine on C6 has moved by nearly 14 Å (figure adapted from reference [20]).

5. Differences in Coenzyme Binding and Specificity

The active site functional groups of ASADH are already poised to accommodate amino acid substrate binding in the apoenzyme. However, the binding of NADP is required to induce a domain closure that sets up the active site for catalysis. NADP binding and the coupled domain closure are driven by numerous interactions between the enzyme and the molecular features that are distributed throughout the coenzyme. In *vc*ASADH, Arg9 is repositioned during NADP binding to form an electrostatic interaction with the 2′-phosphate, with additional hydrogen-bonds to phosphate from Thr36 and Ser37 (Figure 5). A consensus sequence, **SG**x**G**, present in each branch of the ASADH family interacts *via* backbone carbonyl hydrogen-bonds to the amide nitrogen of the nicotinamide, while a conserved glutamine (Gln350) in the bacterial enzymes and a corresponding asparagine in the archaeal enzyme are in position to hydrogen-bond to the amide oxygen (Figure 5).

Binding interactions to the adenine ring of NADP are less conserved between the Gram-negative and Gram-positive bacterial enzymes, leading to some drastic differences in

coenzyme binding. The adenine base in *vc*ASADH is oriented by a cation-π interaction with Arg9 (Figure 5) in the consensus **G**xx**G**xx**G** sequence which is part of the Rossmann nucleotide fold [29]. A surface loop spanning from Leu189 to Ser195 closes around NADP in these Gram-negative enzymes, with the exocyclic N6 of the adenine base forming a hydrogen-bond with the backbone carbonyl of a proline (Pro192) located on the helical subdomain from the opposite subunit of the dimer (Figure 2). This interaction plays a critical role in the change from an open to a closed enzyme conformation upon coenzyme binding [16]. The overall domain movements encountered in the transition from the apoenzyme to the NADP complex in Gram-positive *sp*ASADH are similar to those observed for *vc*ASADH [15]. Rotation of the N-terminal domain toward the active site in response to NADP binding appears to be a universal mechanism utilized throughout the ASADH family. However, none of the interactions that drive domain closure in both *ec*ASADH and *vc*ASADH are observed in *sp*ASADH. Thus, facilitation of the commonly observed domain closure in *sp*ASADH must be driven by a different set of interactions than those that drive the same closure in the Gram-negative ASADHs.

A completely new binding pocket for the adenine base is found in *sp*ASADH, along with an altered 2′-phosphate binding site, from that previously observed in the Gram-negative enzymes. The adenine binding pocket in *sp*ASADH is forged between an α-helix and the coenzyme binding loop of the N-terminal domain. Interactions between the adenine ring and the side chains of Thr76 and Ser37, along with a cation-π interaction with Arg39, serve to anchor the adenine ring in this pocket [18]. These new interactions cause the adenine ring to adopt an altered position in *sp*ASADH formed by rotation around the bonds linking the nicotinamide and adenine ribose with the bridging diphosphates. As a consequence the center of the adenine ring is shifted by about 8.5 Å with respect to its position in NADP bound in *vc*ASADH, and the position of the exocyclic amine on C6 that formerly interacted with Pro192 from the adjacent subunit has been displaced by nearly 14 Å (Figure 5). However, the 2′-phosphate of NADP bound in the *sp*ASADH structure moves less than 5 Å relative to its position in *vc*ASADH, and the phosphate binding groups move to accommodate this shift.

Comparison of the coenzyme binding site between archaeal *mj*ASADH and the bacterial enzymes also shows some conserved similarities. In bacterial ASADHs the backbone amino groups from a methionine and valine are hydrogen-bond donors to the pyrophosphate of NADP (Figure 5), and a change from methionine to serine in the *mj*ASADH structure does not alter these pyrophosphate interactions. A similar reorientation of the adenine ring to that observed in *sp*ASADH is seen in the binding of NADP to the ASADH from *M. jannaschii* [19]. An extended surface loop in *mj*ASADH (from residues 40 to 77) forms the binding pocket for this conformation of the bound NADP. Specific contacts between the adenine ring and the enzyme include two hydrophobic interactions to Leu91 and Leu95 that are within 4.0 Å of the plane of the adenine ring and a cation-π interaction with Arg39. Similar interactions and a closely related coenzyme orientation are also found in the binding of the adenine ring in the fungal enzyme structure (*ca*ASADH) [20] (Figure 5).

These changes in coenzyme conformation and in the nature of the interactions with the 2′-phosphate of NADP in the *mj*ASADH structure suggest the possibility of altered coenzyme specificity relative to that seen for the well-studied Gram-negative bacterial enzymes. Preference for NADP *versus* NAD binding in enzymes is mediated through specific interactions with either the 2′-phosphate of NADP or the 2′-hydroxyl of NAD. The ASADHs from *E. coli*, *V. cholera*e, and *H. influenzae* are each highly specific for NADP. The structure of the NADP complex in *vc*ASADH shows that the 2′-phosphate group is bound by the same arginine that stacks against the adenine base, along with three hydrogen bonds that are formed with two serines and a threonine. This arginine in *vc*ASADH is not conserved in *mj*ASADH but instead is replaced by a threonine that is still in the correct position to form a potential hydrogen-bond with the 2′-phosphate of NADP but, unlike in *vc*ASADH, is not capable of binding to the adenine base.

The possibility of expanded coenzyme specificity for the archaeal enzyme was examined kinetically by testing both NADP and NAD as substrates near the physiological temperatures for this thermophilic organism. *mj*ASADH is found to have comparable catalytic rates with either NAD or NADP when examined at 70°C [19]. However, this enzyme still shows a preference for NADP over NAD as its coenzyme, with a Michaelis constant that is 130-fold lower for NADP ($K_{\mathrm{NADP}} = 13\,\mu\mathrm{M}$) compared to that for NAD ($K_{\mathrm{NAD}} = 1700\,\mu\mathrm{M}$). It appears that the relaxed specificity for NADP in *mj*ASADH is primarily due to absence of this critical arginine residue, since the positively charged guanidinium group of this arginine interacts directly with the negatively charged 2′-phosphate of NADP. An additional explanation for this altered coenzyme specificity comes from the structure of the NADP complex in which the adenine and ribose end of the NADP is bound in a completely different binding pocket in archaeal *mj*ASADH compared to the Gram-negative bacterial enzyme family.

6. The Presence of an Intersubunit Communication Channel

The subunit interface in the homodimer for both the bacterial and archaeal ASADHs is composed primarily of hydrophobic β-sheets. The Gram-negative bacterial ASADHs have the largest dimer interface, formed by a conserved hydrophobic β-sheet and complemented by the helical subdomain to create over 3400 Å^2 of buried surface area (Figure 2). This subdomain is also present in the Gram-positive bacterial *sp*ASADH, but a 16-residue deletion results in a single α-helix followed by an unstructured loop forming the top portion of the dimer interface with about 2600 Å^2 of total buried surface area. A 48-amino acid deletion in *mj*ASADH results in the complete removal of the helical subdomain that makes a significant contribution to the

dimer interface in *ec*ASADH. As a consequence the thermophilic *mj*ASADH structure has a much smaller dimer interface of about 2000 Å^2. The enzyme from *C. albicans* (*ca*ASADH) is also missing the helical subdomain, with the 44 amino acids that constitute this helix-turn-helix motif in the Gram-negative enzyme forms replaced by an unstructured 3 amino acid loop [20]. As a consequence this fungal enzyme has the smallest dimer interface (~1800 Å^2) and the lowest percentage of buried surface area among the structurally characterized ASADHs. Interestingly, there is good correlation between the dimer interface area and enzymatic activity, with the enzyme forms possessing the highest buried dimer surface having the highest catalytic activity [30]. This correlation suggests that intersubunit communication in the ASADH dimer is critical to promote highly efficient catalysis.

A network of hydrogen bonds has been identified across the dimerization interface that is proposed to allow active site to active site communication in the functional ASADH dimer from the bacterial enzyme branch [16]. These structural observations support the earlier kinetic experiments that had suggested an alternating site reactivity model for ASADH catalysis [31]. The active site Glu240 in subunit A of *vc*ASADH is positioned through a hydrogen-bond to Gln161 (Figure 3). Gln161 also forms a hydrogen-bond with the backbone amide of Thr159, which in turn is hydrogen-bonded through its side chain hydroxyl group to the hydroxyl group of Tyr160 across the dimer interface in subunit B (Figure 6). A complementary series of interactions links the active site Glu240 in subunit B to Tyr160 in subunit A, such that each active site is linked to an amino acid in the adjacent subunit through a network of three hydrogen-bonds. The orientation of the Tyr160 residues at the domain interface is crucial to maintaining this network. The tyrosine side chain position is stabilized by π-stacking to the tyrosine from the other subunit and also by a perpendicular π-stacking interaction with Phe345 in the same subunit (Figure 6).

The structure of *mj*ASADH does not allow the intersubunit communication route that is seen in the bacterial enzymes. Two methionines are present within the dimerization interface region of *mj*ASADH that contribute to the hydrophobic subunit interactions, but replace the conserved tyrosines in each subunit that forms the heart of the hydrogen-bonding network bridging between the two active sites (Figure 6). The phenylalanines that stabilize these tyrosines through π-stacking have also been replaced in the archaeal ASADH branch with a conserved threonine (Figure 6). These amino acids that are crucial for intersubunit communication have also been replaced in the *ca*ASADH enzyme. The base-stacked tyrosine is substituted by either leucine or methionine at this position in all fungal ASADHs, while the stabilizing phenylalanine is now a conserved valine in this branch of the enzyme family [20]. These amino acid replacements disrupt the intersubunit hydrogen-bonding network observed in Gram-negative ASADHs, leading to the loss of this communication channel. The alternating sites reactivity observed in the bacterial forms of ASADH is likely absent in both the archaeal and fungal enzymes and could explain the very low catalytic activity in these enzyme forms despite the presence of an identical constellation of active site functional groups throughout the ASADH enzyme family.

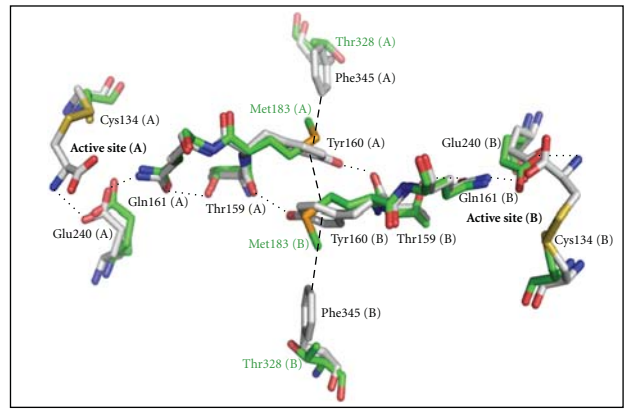


FIGURE 6: Proposed intersubunit communication channel in bacterial ASADHs. The subunit interface of *vc*ASADH (gray) is bridged by a hydrogen-bonding network (dotted lines) connecting the active sites of each subunit and stabilized by π-stacking interactions (dashed lines). In the subunit interface of *mj*ASADH (green) replacement of Tyr160 with Met183 interrupts the hydrogen-bonding network, and replacement of Phe345 with Thr328 disrupts the π-stacking stabilization.

7. Catalytic Mechanism and Covalent Intermediates

The proposed catalytic mechanism of ASADH in the reverse (nonphysiological) direction involves the initial attack of the active site cysteine nucleophile (Cys136) on the carbonyl carbon of the substrate ASA. The enzyme-bound tetrahedral intermediate produced by this reaction (Figure 7, structure a) is set up to transfer a hydride ion to the NADP coenzyme bound at an adjacent site, leading to an acyl-enzyme intermediate (Figure 7, structure b). Attack of bound phosphate at the carbonyl carbon of this intermediate produces a second enzyme-bound tetrahedral intermediate (Figure 7, structure c). Collapse of this intermediate with expulsion of the enzyme thiolate group yields the β-aspartyl phosphate product and leaves the enzyme ready to bind another molecule of ASA and repeat the catalytic cycle.

The chance to characterize a true intermediate in an enzyme-catalyzed reaction is a rare opportunity to bridge a mechanistic gap that is generally only hypothesized between the substrate and the product complexes, or extrapolated from intermediate-analogue structures. For the ASADHs this feat has been accomplished twice, capturing and structurally characterizing two different reactive intermediates in the catalytic cycle of this enzyme and lending structural support to this proposed mechanism.

A ternary complex produced by diffusing the substrates ASA and phosphate into crystals of *hi*ASADH was examined with the aim of determining the role of different active

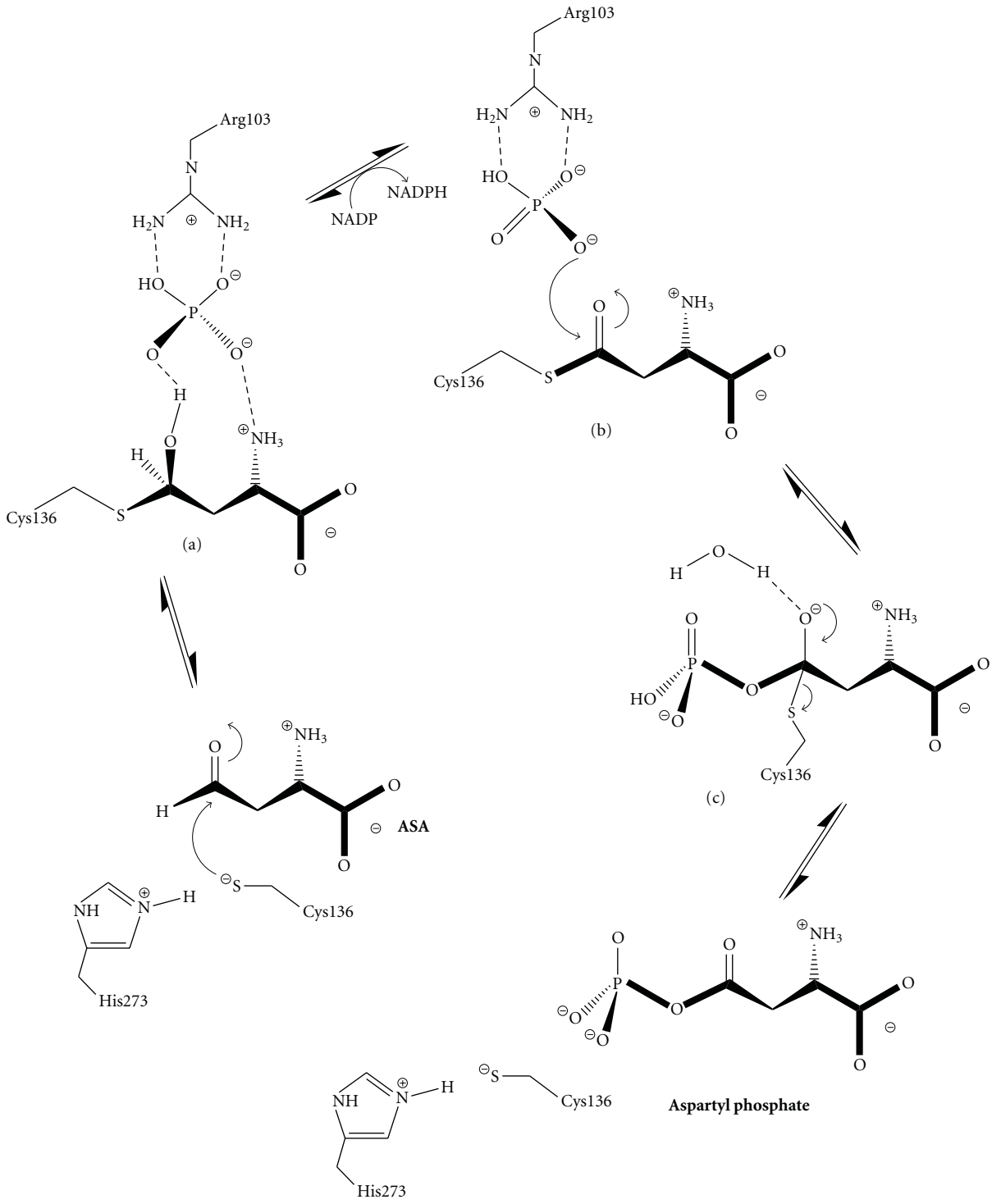


Figure 7: Catalytic mechanism of the reversible aspartate β-semialdehyde dehydrogenase-catalyzed conversion of ASA to aspartyl phosphate. (a) Tetrahedral intermediate derived from nucleophilic attack on ASA. (b) Acyl-enzyme intermediate produced by hydride transfer to NADP. (c) Proposed tetrahedral intermediate obtained from phosphate attack on the acyl-enzyme.

site groups in the catalytic mechanism. However, what was observed was not the structure of the enzyme-substrate complex, but instead the structure of the actual tetrahedral intermediate in the catalytic cycle of ASADH (structure a in Figure 7). The bound aspartyl intermediate was modeled into the continuous density emanating from the side chain of the cysteine nucleophile [17], and this structure clearly shows the tetrahedral geometry around the carbon that is covalently attached to the active site cysteine thiolate group (Figure 8). Subsequent hydride transfer from this tetrahedral

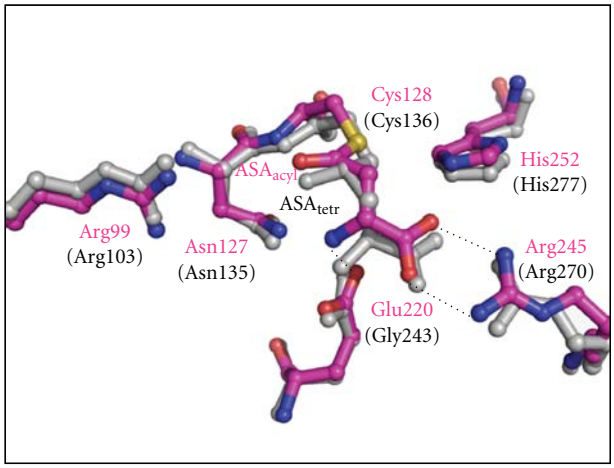


FIGURE 8: Overlay of the tetrahedral intermediate (ASA$_{tetr}$, gray bonds and black labels) produced in *hi*ASADH and the acyl-enzyme intermediate (ASA$_{acyl}$, magenta bonds and labels) obtained in *sp*ASADH. The change in geometry of the acyl carbon of this bound intermediate is tracked by shifts in the substrate binding groups, Asn127, Glu220, and Arg245. *H. influenzae* numbering is in parentheses.

intermediate to NADP would lead to formation of the acyl-enzyme intermediate. However, exclusion of the coenzyme from the crystallization conditions eliminates the hydride transfer step that is required for the reaction to proceed, thus stopping the catalytic cycle at this stage and allowing the determination of this important structure.

The structure of the acyl-enzyme intermediate (structure b in Figure 7) was also determined by running the reaction in the nonphysiological direction. Soaking the *sp*ASADH-NADP complex crystals with the substrate ASA allows the catalytic cycle to proceed up to formation of the acyl-enzyme intermediate [18]. Exclusion of phosphate eliminates the nucleophile required to complete the catalytic cycle. Kinetic studies had previously shown that the acyl-enzyme can be quite stable in the absence of phosphate [32]. The refined structures show the key differences between the tetrahedral intermediate and the acyl-enzyme intermediate. The position of the substrate binding groups that interact with these intermediates shift only slightly between these two structures, with the largest movements caused by the conversion from a tetrahedral carbon geometry in the previous intermediate structure to a planar carbon geometry in the acyl-enzyme (Figure 8).

8. Concluding Remarks

Through a combination of kinetic, mutagenic, and structural studies the detailed catalytic mechanism of the aspartate β-semialdehyde dehydrogenases has been defined, and the essential functional groups that play a role in substrate binding, catalysis, and regulation have each been identified. Subtle changes in any of these critical functional groups are sufficient to alter the finely tuned catalytic machinery of this enzyme, leading to enzyme forms with significantly impaired activity. Differences have been observed between the members of this enzyme family, isolated from bacterial, archaeal, and fungal species, regarding their catalytic efficiency, their coenzyme selectivity, and their mode of catalysis. This extensive body of work on the different members of the ASADH family now allows us to evaluate this enzyme as a drug target. Compounds are now being screened [33] that can potentially recognize these structural and functional differences to produce initial inhibitors that can be developed into species-specific antimicrobials against this key enzyme in an essential metabolic pathway.

Abbreviations

AK:	Aspartokinases
ASA:	Aspartate β-semialdehyde
ASADH:	Aspartate β-semialdehyde dehydrogenase
DAP:	Diaminopimelate
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
SMCS:	S-methyl-L-cysteine sulfoxide
ca:	*Candida albicans*
ec:	*Escherichia coli*
hi:	*Haemophilus influenzae*
mj:	*Methanococcus jannaschii*
sp:	*Streptococcus pneumoniae*
vc:	*Vibrio cholerae.*

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Review Article

Metal Preferences of Zinc-Binding Motif on Metalloproteases

Kayoko M. Fukasawa,[1] Toshiyuki Hata,[2] Yukio Ono,[2] and Junzo Hirose[2]

[1] *Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan*
[2] *Faculty of Pharmacy and Pharmaceutical Science, Fukuyama University, Gakuen-cho, Fukuyama 729-0292, Japan*

Correspondence should be addressed to Kayoko M. Fukasawa, kmf@po.mdu.ac.jp

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Almost all naturally occurring metalloproteases are monozinc enzymes. The zinc in any number of zinc metalloproteases has been substituted by some other divalent cation. Almost all Co(II)- or Mn(II)-substituted enzymes maintain the catalytic activity of their zinc counterparts. However, in the case of Cu(II) substitution of zinc proteases, a great number of enzymes are not active, for example, thermolysin, carboxypeptidase A, endopeptidase from *Lactococcus lactis*, or aminopeptidase B, while some do have catalytic activity, for example, astacin (37%) and DPP III (100%). Based on structural studies of various metal-substituted enzymes, for example, thermolysin, astacin, aminopeptidase B, dipeptidyl peptidase (DPP) III, and del-DPP III, the metal coordination geometries of both active and inactive Cu(II)-substituted enzymes are shown to be the same as those of the wild-type Zn(II) enzymes. Therefore, the enzyme activity of a copper-ion-substituted zinc metalloprotease may depend on the flexibility of catalytic domain.

1. Introduction

Proteolytic enzymes are recognized by their catalytic type, that is, aspartic, cysteine, metallo, serine, threonine, and others as yet unclassified. The largest number of proteolytic enzymes are classified as metalloproteases [1]. Almost all metalloproteases contain one or two zinc ions, and several enzymes contain one or two cobalt or manganese ions. The HExxH motif forming an α-helix is well conserved in many monozinc enzymes as the active site in which the two histidine residues coordinate with the zinc ion [2]. Some other monozinc proteases have different zinc-binding motifs, for example, HxxE(D)-aa_n-H in the carboxypeptidase family or HxD-aa_{12}-H-aa_{12}-H in the matrix metalloprotease family [2]. Dipeptidyl peptidase (DPP) III also has a unique zinc-binding motif, which was classified as family M49 in 1999 by MEROPS (peptidase database) after rat DPP III had been cloned and the HELLGH motif of DPP III was identified as an active site coordinated with a zinc ion [3, 4]. Although the motif HELLGH could not be found in any other metalloproteases, it exists in three kinds of monooxygenases (tyrosine, phenylalanine, and tryptophan hydroxylases) as an iron-binding site, as revealed by a search of the NBRF-PIR protein sequence database.

Zinc atoms in several zinc metalloproteases, for example, astacin [5], carboxypeptidase A [6], and thermolysin [7, 8], have been substituted by other divalent cations to probe the role of the metal for catalysis and structure. Some of these enzymes, for example, DPP III and astacin, were shown to have high metal substitution tolerance by metal substitution studies [9]. However, it is difficult to determine the relationship between the metal tolerance and the metal coordination structure of zinc metalloproteases.

Here, we show the metal coordination structure of the unique zinc-binding motif of DPP III, in which the zinc-binding motif is stabilized by several hydrogen bonds with acidic amino acid residues surrounding the zinc-binding motif, in order to clarify the relationship between the metal tolerance and the structure of the zinc-binding domain. The metal tolerances of both DPP III and del-DPP III, whose active site converts into a normal zinc-binding motif (HExxH), are shown here and compared with those

Table 1: The zinc coordination residues and the residues that fix the coordination with hydrogen bonds.

Zinc metalloprotease	Coordination residues	Residues that form the hydrogen bond with the coordination residues	PDB no.
(1) Thermolysin type	(HExxH- aa_n-E)	α-helix-aa_n-α-helix	
Thermolysin	His^{142}, His^{146} Glu^{166}	Asp^{170}-2.8 Å-His^{142} Asn^{165}-2.8 Å-His^{146}	1KEI
Vibriolysin	His^{345}, His^{349} Glu^{369}	Asp^{373}-2.8 Å-His^{345} Asn^{368}-2.8 Å-His^{349}	3NQX
Staphylococcus aureus metalloproteinase	His^{144}, His^{148} Glu^{168}	Asn^{167}-2.8 Å-His^{148} Asp^{172}-2.8 Å-His^{144}	1BQB
Zinc aminopeptidase	His^{265}, His^{269} Glu^{288}	Phe^{272}(C=O)-2.9 Å-His^{269}	1Z1W
Leukotriene A4 hydrolase	His^{295}, His^{299} Glu^{318}	Glu^{325}-2.8 Å-His^{295} Gly^{303}(C=O)-2.6 Å-His^{299}	1SQM
Human thimet oligopeptidase	His^{473}, His^{477} Glu^{502}	Glu^{509}-2.6 Å-His^{473}	1SQM
Human neutral endopeptidase (Neprilysin)	His^{583}, His^{587} Glu^{646}	Asp^{650}-2.9 Å-His^{583} Asp^{590}-2.7 Å-His^{587}	1DMT
(2) Endopeptidase type		(HExxH-aa_n-E or D) α-helix-aa_n-random coil	
Peptidyl-Lys metalloendopeptidase	His^{117}, His^{121} Asp^{130}	Asp^{154}-2.7 Å-His^{117} Thr^{128}(C=O)-2.8 Å-His^{121}	1GE6
(3) Carboxypeptidase A type		β-sheet-aa_n-random coil	
Carboxypeptidase A	His^{69}, His^{196} Glu^{72}	Asp^{142}-2.7 Å-His^{69}	1YME
Putative lysostaphin peptidase	His^{232}, His^{311} Asp^{236}	Glu^{315}-2.6 Å-His^{311} Gly^{216}(C=O)-2.8 Å-His^{232}	2GU1

reported for other metalloproteases [10]. Finally, we discuss the relationship between the catalytic activities and metal coordination structures of metal-substituted enzymes.

2. Identification of a Zinc-Binding Motif in DPP III

We start with the identification of the zinc-binding motif in DPP III, which will be used for further investigation of the relationship between the metal tolerance and the metal coordination structures of DPP III. The deduced amino acid sequences from cDNA for human, rat, and fruit fly DPP IIIs are 723–738 amino acids long and conserve the amino acid sequence HELLGH-aa_{52}-E [3, 11, 12], which resembles the HExxH-aa_n-E zinc-binding motif conserved in many metalloproteases, such as thermolysin [13] and leukotriene A_4 hydrolase [14]. Site-directed mutagenesis was performed on rat DPP III in order to testify that the HELLGH-aa_{52}-E is a zinc-binding domain. Site-directed mutagenesis studies have clearly shown that the H450Y, H455Y, and E508A mutants, which lack zinc ions, lose their catalytic activity [4]. Replacement of Glu^{451} in these mutants with an alanine or an aspartic acid restores a mol of zinc ion per mol of protein but does not restore catalytic activity [4]. These results show that the H^{450}ELLGH-aa_{52}-E^{508} motif is a catalytic domain of which His^{450}, His^{455}, and Glu^{508} are ligands of a zinc ion and of which Glu^{451} is a catalytic amino acid residue, in the same way that the H^{142}ExxH-aa_{19}-E^{166} motif of thermolysin is a catalytic domain of which His^{142}, His^{146}, and Glu^{166} are ligands of a zinc ion and Glu^{143} is a catalytic amino acid residue.

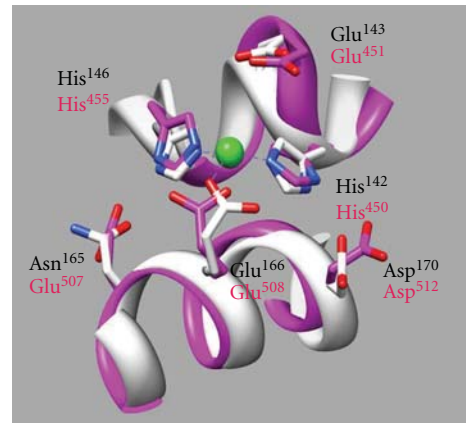
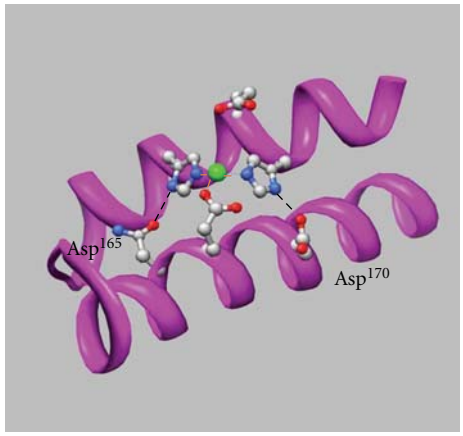
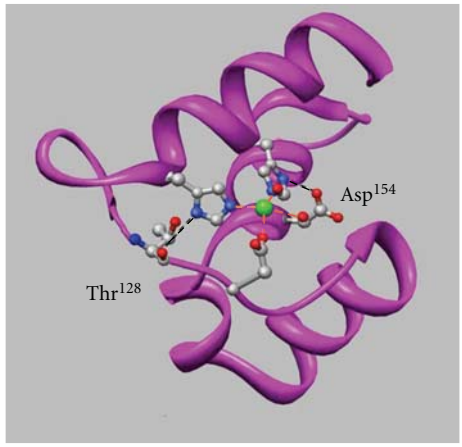


Figure 1: The superimposition of the active sites of rat DPP III and thermolysin. Zinc ion is shown as a green sphere, and amino acid side chains are shown as sticks colored red for oxygen and blue for nitrogen. Metal coordinates in light blue and hydrogen bonds in yellow are indicated by dashed lines. Carbon atom and amino acid chain are shown colored white for thermolysin and magenta for DPP III. Metal coordination bonds are indicated by light blue dashed lines.

The 1.95-Å crystal structure of yeast DPP III representing a prototype for the M49 family of metalloproteases was resolved by Baral et al. [15]. It shows a novel protein fold with two domains forming a wide cleft containing the catalytic metal ion. However, the three-dimensional structure of zinc coordination (His^{460}, His^{465}, and Glu^{517}) and the catalytic active (Glu^{461}) residues are structurally conserved,



(a)



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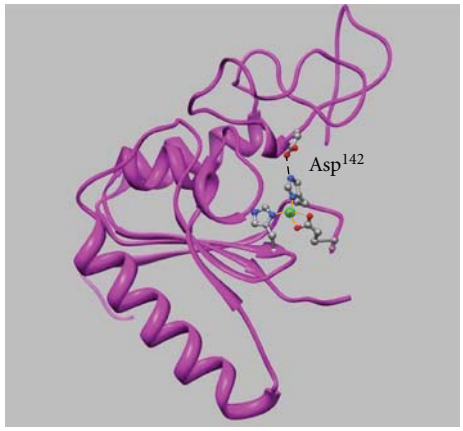

(c)

Figure 2: Three-dimensional structures of the catalytic domain models for thermolysin ((a): PDB 1KEI), peptidyl-Lys metallopeptidase ((b): PDB 1GE6), and carboxypeptidase A ((c): PDB 1YME). The zinc ion is shown as a green sphere, and amino acid side chains are shown as sticks colored red for oxygen, blue for nitrogen, and silver for carbon. Hydrogen bonds are indicated by dashed lines.

Table 2: Kinetic parameters for the hydrolysis of Arg-Arg-NA, zinc contents, and zinc dissociation constants of wild-type and mutated rat DPP IIIs.

Enzymes	$k_{cat}/K_M \times 10^{-4}$ (M^{-1} s^{-1})	Zinc content (mol/mol of protein[a])	Zinc dissociation constant (M) (K_d)
Wild-type	73.6 ± 6.9	1.02 ± 0.15	$(4.5 \pm 0.1) \times 10^{-13}$
E507D	22.8 ± 1.9	0.65 ± 0.07	$(1.0 \pm 0.2) \times 10^{-11}$
E507A	4.43 ± 0.41	0.29 ± 0.04	$(1.0 \pm 0.2) \times 10^{-8}$
E512D	21.0 ± 0.19	0.45 ± 0.06	$(1.4 \pm 0.1) \times 10^{-12}$
E512A	2.45 ± 0.28	0.08 ± 0.01	$(2.6 \pm 0.7) \times 10^{-9}$

[a]Values are means ± SD of two separately prepared enzymes with duplicate determinations.

similar to those presented in many metalloproteases, such as thermolysin [13]. The HELLGH motif and the third ligand (Glu^{517}) of DPP III construct a helix α14 and a helix α16, respectively [15]. The 3D structure of DPP III is similar to that of thermolysin [13] or leukotriene A_4 hydrolase [16], the zinc-binding domain of which is constructed of two α-helixes, for HExxH (containing two zinc ligands) and xNEx (third ligand).

Figure 1 shows the superimposition of the active sites of rat DPP III and thermolysin. The helix α14 of DPP III has a slightly larger loop than that of thermolysin, and the glutamic acid on the motif comes close to zinc ion comparing with the glutamic acid on the normal helix of thermolysin [13, 17].

3. Stabilization of the Coordination between Ligands and Metal

In the 3D structural model of the zinc-binding domain of many zinc enzymes—neprilysin [18], thermolysin [13], carboxypeptidase A [19], leukotriene A_4 hydrolase [16], aspzincin [20], and DPP III [17]—the His, His, and Glu residues that coordinate with the zinc ion are engaged in hydrogen bonds with one or two acidic amino acid residues (Glu or Asp) or other carbonyl oxygen atoms (Table 1). 3D structural models of catalytic domains of thermolysin (PDB: 1KEI), peptidyl-Lys metallopeptidase (PDB: 1GE6), and carboxypeptidase A (PDB: 1YME) are shown in Figure 2. In thermolysin (a), the oxygen atoms of Asp^{165} and Asp^{170} are engaged in hydrogen bonding with the nitrogen atoms of His^{146} and His^{142}, respectively. Asp^{154} and Thr^{128} of peptidyl-Lys metalloendopeptidase (b) and Asp^{142} of carboxypeptidase A (c) are also engaged in hydrogen bonding with His^{117}, His^{121}, and His^{69}, respectively. It was proved through the mutational studies of rat DPP III that this network of hydrogen bonds close to the zinc-binding motif plays an important role in stabilizing the coordination of the zinc ion to the protein [17]. The hydrogen bonds surrounding the zinc-binding motif of rat DPP III are shown in Figure 3, and the kinetic parameters, zinc contents and zinc dissociation constants of the several mutants are shown in Table 2. The replacement of Glu^{507} and Glu^{512}, the oxygen atoms of which bind with the nitrogen atoms of His^{455} and

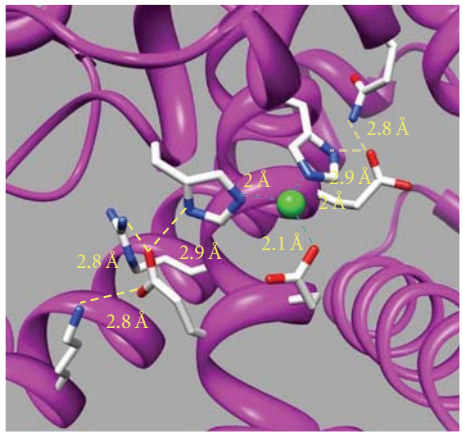

Figure 3: Molecular modeling of the catalytic site of rat DPP III. The model was generated as a template of the human DPP III crystal structure [39]. The zinc ion is shown as a green sphere, and amino acid side chains are shown as sticks colored red for oxygen, blue for nitrogen, and white for carbon. Metal coordinates in light blue and hydrogen bonds in yellow are indicated by dashed lines.

His^{450}, respectively, increases the dissociation constants by factors of $10\sim10^5$ and correlatively reduces the zinc contents and enzyme activities. The hydrogen bonds between acidic amino acid residues and zinc ligands (His, His, and Glu) may stabilize the coordination of the zinc ion with the protein of the metalloprotease.

4. Metal Substitutions of Monozinc Metalloproteases

Almost all metalloproteases are monozinc enzymes. Some enzymes contain two zinc ions for catalytic domains, for example, human renal dipeptidase [36], and a few are dicobalt or dimanganese enzymes, for example, *Pyrococcus furiosus* methionine aminopeptidase [37] or *Escherichia coli* proline aminopeptidase [38], respectively.

The zinc in numerous zinc metalloproteases has been substituted by several divalent cations. The cobalt(II)- or manganese(II)-substituted enzymes showed nearly restored catalytic activity or even excess activity from apoenzyme, as seen in Table 3.

Gomis-Rüth et al. [5] demonstrated in the metal substitution studies of astacin that Cu(II)-astacin displays enzyme activity of about 37%, while Ni(II)- and Hg(II)-astacin were almost inactive. In the crystal structure of Cu(II)-astacin, the metal ion is pentacoordinated with His^{92}, His^{96}, His^{102}, Tyr^{149}, and H_2O, as in native Zn(II)-astacin or Co(II)-astacin; however, in the Ni(II)-astacin or Hg(II)-astacin, the metal ion is hexacoordinated with an additional solvent molecule or tetracoordinated with no ordered solvent molecule, respectively [5]. The restoration of catalytic activity in these substituted astacins was shown to be dependent on the metal coordination structure [5].

Meanwhile, almost all Cu(II)-substituted enzymes, such as thermolysin [7, 8], carboxypeptidase A [6], aminopeptidase B [22], or endopeptidase from *Lactococcus lactis* [30], show only partial activation or very low activities. The reason why these Cu(II) enzymes do not demonstrate catalytic activities may be that the coordination geometry of Cu(II) is more rigid than that of Zn(II) or Co(II).

In the case of DPP III, Co^{2+}-, Ni^{2+}- and Cu^{2+}-DPP IIIs showed comparable catalytic activities to Zn^{2+}-DPP III; the kinetic parameters are shown in Table 4 [9]. DPP III shows high flexibility of the metal ion for the catalytic activity compared with thermolysin or aminopeptidase B. Thermolysin or aminopeptidase B is a subclan MA (E) metalloprotease containing an HExxH-aa_n-E motif, and the 3D structure of the active domain is very similar to that of DPP III described above. The zinc ion in a subclan MA (E) metalloprotease or DPP III is tetracoordinated with three ligands (His, His, and Glu) and a water molecule. The metal-substituted (Co^{2+}, Cu^{2+}, or Ni^{2+}) DPP III may have the same tetrahedral coordination structure as Zn^{2+}-DPP III, so these enzymes are able to maintain the catalytic activity. The zinc in del-DPP III, whose active site converted into HExxH, was substituted with Co^{2+}, Ni^{2+}, or $Cu^{2+}$to investigate the grounds for activation of the Cu^{2+}-DPP III [10]. The Co^{2+}-del-DPP III and Ni^{2+}-del-DPP III showed comparable catalytic activity to that of Zn^{2+}-del-DPP III, while the Cu^{2+}-del-DPP III showed no catalytic activity, as in the case of thermolysin or aminopeptidase B [8–10].

The EPR (electron paramagnetic resonance) parameters of various Cu^{2+}-substituted metalloproteases are shown in Table 5. Each parameter is exactly alike between DPP III and thermolysin, aminopeptidase B, or del-DPP III [8–10, 22]. The results show that the Cu(II) coordination structures of the HExxH-aa_n-E and HExxxH-aa_{52}-E motifs are very similar.

In the superimposition of the 3D structure models of active sites of DPP III and del-DPP III, the α-helix of DPP III, which is abnormally composed of 5 amino acid residues per one turn of the α-helix, is a larger loop than that of del-DPP III, the same as the case for the superimposition of the active sites of DPP III and thermolysin (Figure 1). The coordination geometries of the two enzymes are similar, while the position of Glu^{451}, which is essential for the enzyme activity, is slightly closer to the copper ion in DPP III than in del-DPP III. The distances of oxygen atoms of the Glu^{451} residues of del-DPP III and wild-type DPP III are 4.8 Å and 3.2 Å from the zinc ion, respectively. Zn(II) coordination geometry is flexible, so both wild-type and del-DPP IIIs could have catalytic activity. However, the oxygen atom of Glu^{451} in Cu(II)-del-DPP III is not able to bind to the oxygen atom of the water molecule that is coordinated with the copper ion because the Cu(II) coordination geometry is very rigid. Therefore, the catalytic activity of Cu(II)-del-DPP III was diminished.

Some other Cu(II)-substituted enzymes, for example, aminopeptidase Ey [21], vibriolysin [40], hyicolysin [32], and *Legionella* metalloendopeptidase [33], were shown to have enzyme activities. These enzymes are all classified in subclan MA (E), the same as thermolysin or aminopeptidase B. The metal coordination structures of these enzymes have not been shown in detail; however, the catalytic domain may be more flexible than that of thermolysin or aminopeptidase B, in the same way as Cu(II)-substituted DPP III.

Table 3: Reactivated Co(II) and Mn(II) enzymes substituted from apo-metalloproteases.

Clan	Subclan	Name of enzyme	Replaced Ion	Reference
MA	E	Aminopeptidase Ey	Co^{2+}, Mn^{2+}	Tanaka and Ichishima [21]
MA	E	Aminopeptidase B	Co^{2+}	Hirose et al. [22]
MA	E	Saccharolysin	Co^{2+}, Mn^{2+}	Achstetter et al. [23] and Büchler et al. [24]
MA	E	Lysyl aminopeptidase	Co^{2+}, Mn^{2+}	Klein et al. [25]
MA	E	Oligopeptidase F	Co^{2+}, Mn^{2+}	Yan et al. [26] and Monnet et al. [27]
MA	E	Mycolysin (Thermolysin)	Co^{2+}, Mn^{2+} Co^{2+} (200%), Mn^{2+} (10%)	Chang and Lee [28], and Holmquist and Vallee [29]
MA	E	Oligopeptidase O	Co^{2+} Mn^{2+} (50%)	Tan et al. [30] and Baankreis et al. [31]
MA	E	Hyicolysin	Co^{2+}	Ayora and Götz [32]
ME	E	Legionella metalloendopeptidase	Mn^{2+} (69%)	Dreyfus and Iglewski [33]
MA	A	Epralysin	Co^{2+} (58%)	Diermayr et al. [34]
MA	M	Astacin	Co^{2+} (140%)	Gomis-Rüth et al. [5]
MA	M	MEP[a] (*Gf*[b] MEP)	Co^{2+} Mn^{2+} (200%)	Nonaka et al. [35]
MA	M	*Po*[c] MEP	Co^{2+} (80%) Mn^{2+} (30%)	Nonaka et al. [35]

[a] Peptidyl-Lys metallopeptidase; [b] *Grifola frondosa*; [c] *Pleurotus ostreatus.*

Table 4: Kinetic parameters for the hydrolysis of Arg-Arg-NA and metal contents of various metal-DPP IIIs.

Enzyme	K_M ($\times 10^{-5}$ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($\times 10^4$ M^{-1} s^{-1})	Metal content (mol/mol of protein)
Zn^{2+}-DPP III	8.1 (±1.0)	7.1 (±0.2)	8.8	0.8 (±0.1)
Co^{2+}-DPP III	8.2 (±0.9)	7.0 (±0.1)	8.5	1.0 (±0.1)
Cu^{2+}-DPP III	9.9 (±1.1)	10.1 (±0.3)	10.2	1.1 (±0.1)

Table 5: EPR parameters of Cu^{2+} proteases.

	$g_\perp$	g_{II}	A_{II} ($\times 10^{-4}$ cm^{-1})
Cu^{2+}-DPP III[a]	2.06	2.27	167
Cu^{2+}-del-DPP III[b]	2.06	2.27	161
Cu^{2+}-thermolysin[c]	2.06	2.26	163
Cu^{2+}-aminopeptidase B[d]	2.06	2.27	157
Cu^{2+}-carboxypeptidase A[e]	2.05	2.33	115

References [a][9], [b][10], [c][8], [d][22], and [e][6].

5. Conclusions

In this paper, we compared metal flexibility with the geometry of metal coordination of metalloproteases, to investigate why DPP III shows metal tolerance. Metal substitution of Zn(II) by Co(II) or Mn(II) on metalloproteases generally maintains catalytic activity, because the metal coordination geometries of Zn(II), Co(II), and Mn(II) are flexible. Most Cu(II)-substituted enzymes could not restore the catalytic activities, because the Cu(II) coordination geometry is very rigid. However, Cu(II)-substituted DPP III showed the same catalytic activity as that of Zn(II)-DPP III. We then studied the metal flexibilities and metal coordination geometries of many metallopeptidases, especially DPP III and del-DPP III, but we could not prove a relation between the metal flexibility and the metal coordination geometry. The metal tolerance of DPP III might depend on the flexibility of the metal-binding motif, not on the metal coordination geometry. By comparison of the 3D structure of active sites of DPP III and del-DPP III, both coordination geometries are seen to be similar, while the positions of catalytic amino acid residues (Glu) on those zinc-binding motifs are slightly different. We conclude that the catalytic site of Cu(II)-DPP III could be flexible enough to form the catalytic complex, with substrate and H_2O.

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Review Article

Serpin Inhibition Mechanism: A Delicate Balance between Native Metastable State and Polymerization

Mohammad Sazzad Khan,[1] Poonam Singh,[1] Asim Azhar,[1] Asma Naseem,[1] Qudsia Rashid,[1] Mohammad Anaul Kabir,[2] and Mohamad Aman Jairajpuri[1]

[1] *Department of Biosciences, Jamia Millia Islamia University, Jamia Nagar, New Delhi 110025, India*
[2] *Department of Biotechnology, National Institute of Technology Calicut (NITC), NIT Campus P.O., Calicut, Kerala 673601, India*

Correspondence should be addressed to Mohamad Aman Jairajpuri, m_jairajpuri.bi@jmi.ac.in

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The serpins (**ser**ine **p**roteinase **in**hibitor**s**) are structurally similar but functionally diverse proteins that fold into a conserved structure and employ a unique suicide substrate-like inhibitory mechanism. Serpins play absolutely critical role in the control of proteases involved in the inflammatory, complement, coagulation and fibrinolytic pathways and are associated with many conformational diseases. Serpin's native state is a metastable state which transforms to a more stable state during its inhibitory mechanism. Serpin in the native form is in the stressed (S) conformation that undergoes a transition to a relaxed (R) conformation for the protease inhibition. During this transition the region called as reactive center loop which interacts with target proteases, inserts itself into the center of β-sheet A to form an extra strand. Serpin is delicately balanced to perform its function with many critical residues involved in maintaining metastability. However due to its typical mechanism of inhibition, naturally occurring serpin variants produces conformational instability that allows insertion of RCL of one molecule into the β-sheet A of another to form a loop-sheet linkage leading to its polymerization and aggregation. Thus understanding the molecular basis and amino acid involved in serpin polymerization mechanism is critical to devising strategies for its cure.

1. Introduction

Serpins (**ser**ine **p**roteinase **in**hibitor**s**) are the largest super family of protease inhibitors involved in many critical biological processes like blood coagulation, fibrinolysis, programmed cell death, development and inflammation [1–3]. Serpins are structurally heterogeneous and functionally diverse proteins found in the organisms ranging from viruses to vertebrates [4–6]. Over 400 different serpins have been identified in organisms including viruses, plants, insects, animals and prokaryotes [7]. Serpins like α-antitrypsin, α-antichymotrypsin, C1-inhibitors, antithrombin, and plasminogen activator inhibitor-1 (PAI), play absolutely critical role in the control of proteases involved in the inflammatory, complement, coagulation and fibrinolytic pathways, respectively, and are associated with diseases like emphysema/cirrhosis, angioedema, familial dementia, chronic obstructive bronchitis and thrombosis [8]. Not all serpins act as proteinase inhibitors some are inhibitors of other types of proteinases, while others are noninhibitors. For example, the viral serpin crmA inhibits interleukin-1 converting enzyme and squamous cell carcinoma, antigen-1 (SCCA-1) inhibits cysteinyl proteinases of the papain family. Noninhibitory serpins perform diverse functions, including roles as chaperones, for example, the 47-kD heat shock protein [HSP47] and hormone transportation like cortisol-binding globulin [7].

2. Serpin Structure and Common Mechanism of Action

There is a high rate of conservation in the structure among the members of serpin family in which the average size of

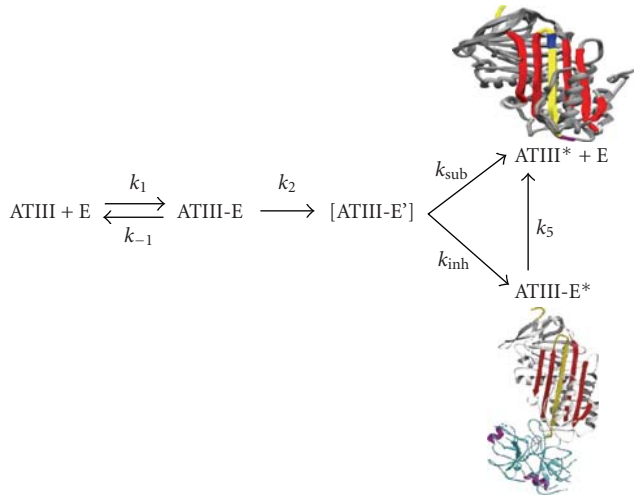

Figure 1: The scheme shown here (taking antithrombin as an example) represents the suicide substrate inhibition mechanism common to all inhibitory serpins. The scheme represents the interaction between the serpin (antithrombin, ATIII) and protease (E); ATIII-E is the noncovalent Michaelis complex; ATIII-E' is the proposed intermediate before partitioning; ATIII-E* is the stable protease-inhibitor complex; ATIII* is the cleaved ATIII. The outcome of the reaction is dependent on the partitioning between the inhibitory (k_{inh}) and substrate pathways (k_{sub}). The figures represent the cleaved and factor Xa bound ternary complexes of antithrombin [3].

protein is 350–400 amino acids and the molecular weight of 40–50 kDa [4]. The serpin fold is comprised of 3-β sheets (A, B, C) and 7–9 alpha helices. A solvent exposed stretch of amino acids termed as reactive centre loop (RCL) contains the protein recognition site which forms a flexible stretch of ~17 residues between β sheets A and C [8]. The mechanism of inhibition of serpin has been demonstrated biophysically and structurally as suicide substrate-like inhibitory mechanism (as shown in Figure 1) where after binding to protease it is partitioned between cleaved serpin and serpin-protease complex [9]. Initially serpin binds to protease through a noncovalent Michaelis-like complex by interactions with residues flanking the scissile bond (P1-P1′) [6]. Attack of the active site serine on the scissile bond leads to a covalent ester linkage between Ser-195 of the protease and the backbone carbonyl of the P1 residue resulting in the cleavage of the peptide bond. Protease specificity is determined by the P1-P1′ bond which is positioned such that it is readily accessible to proteases, Table 1 shows the residues involved in P1-P1′ in various serpins. RCL inserts into the β sheet A and transports the covalently bound protease with it. As a result protease gets translocated by over 70 Å (Figure 1) and its active site gets distorted [6, 8]. Distortion of the active site prevents the final hydrolysis events and the result is an irreversible covalent serpin-enzyme complex. When active serpins are proteolytically inactivated in a substrate-like reaction, they undergo an important structural transition with a resultant increase in their conformation stability [9]. The P4-P4′ sequence of RCL is highly conserved in all inhibitory serpins and mutations in this region (P2 Gly to Pro mutation in antithrombin) result in loss of inhibitory activity (Table 1). Furthermore, the amino acids of the hinge region have small side chain that allows loop flexibility necessary for complex formation. In contrast, the RCL of ovalbumin is in a fully extended, rigid alpha-helical conformation that is unable to conform to the active site of a protease which explains its lack of inhibitory activity. The driving force for this conformational change is thought to be the energy loss associated with the increased loop insertion in the complexed serpin.

3. Domains Involved in Serpin Inhibition Mechanism

Several regions are important in controlling and modulating serpin conformational changes; Figure 2 shows functionally and structurally important regions of serpin [10]. A portion of RCL from P15–P9 is called the hinge region [3, 11]. In S → R transition it provides mobility which is vital for the conformational change of the RCL. Hinge region contains many conserved residues between P15–P10, towards the N-terminal of the RCL. Out of these the amino acid at P14 (Ser-380) is of critical importance as its insertion in the β-sheet A is a prerequisite for inhibitory activity [12]. In antithrombin the replacement of P12 Ala by the Thr causes loss of inhibitory activity which is due to polymerization of antithrombin [13]. The breach region represents the point of initial insertion of the RCL which is located at the top of the A β-sheet [14]. Near the centre of A β-sheet is the shutter domain [15]. The breach and shutter are two major regions that assist sheet opening and accept the conserved hinge of the RCL when it inserts [8]. Highly conserved residues located in the shutter region of the β-sheet A of the serpin fold are Ser-53 and Ser-56 which play an important role in the serpin conformational transitions [16]. The gate region is composed of s3C and s4C strands which has been primarily observed by studies of the transition of active PAI-1 into latency [17]. In order to insert fully into the A β-sheet

Table 1: Represents the Scissile bond in various serpins and their protease targets. Antithrombin is an efficient inhibitor of factor Xa, thrombin and factor IXa. Residues flanking the P1-P1′ are also critical for protease multispecificity.

Clade; Serpin	P4	P3	P2	P1-P1′	P2′	P3′	P4′	Primary target
A1; α1-PI	A	I	P	M- -S	I	P	P	Neutrophil elastase
A3; α1-ACT	I	T	L	L- -S	A	L	V	Cathepsin G
A4; kallistatin	I	K	F	F- -S	A	Q	T	Tissue kallikrein
A5; PCI	F	T	F	R- -S	A	R	L	Activated protein C
A10; PZ	I	T	A	Y- -S	M	P	P	fXa & fXIa
B1; MNEI	A	T	F	C- -M	L	M	P	Neutrophil elastase
B2; PAI-2	M	T	G	R- -T	G	H	G	plasminogen activator
B3; SCCA1	G	F	G	S- -S	P	A	S	Cathepsin K, L & S
B6; PI6	M	M	M	R- -C	A	R	F	plasmin
C1; antithrombin III	I	A	G	R- -S	L	N	P	IIa & fXa
D1; heparin cofactor II	F	M	P	L- -S	T	Q	V	IIa
E1; PAI-1	V	S	A	R- -M	A	P	E	Plasminogen activator
E2; protease nexin 1	L	I	A	R- -S	S	P	P	Plasminogen activator
F2; α2-antiplasmin	A	M	S	R- -M	S	L	S	plasmin
G1; C1-inhibitor	S	V	A	R- -T	L	L	V	C1
I1; neuroserpin	A	I	S	R- -M	A	V	L	Plasmin & plasminogen activator

Scissile bond

without cleavage, the RCL has to pass around the β-turn linking strand s3C and s4C [10, 18].

4. Ligand-Dependent Serpins

One of the important features of serpins is their ability to bind various protease and nonprotease ligand (Table 2) and some of the examples of ligand-dependent serpins are antithrombin, heparin cofactor II, protein C inhibitor, plasminogen-activator inhibitor-1 (PAI-1), protease nexin-1 and kallistatin [19]. Binding of ligand to these serpins regulate their activity, like PAI-1 binds to vitronectin or ZPI binds to protein Z [20]. Several serpins which interact with glycosaminoglycan ligands have their reactions with proteases improved by such ligands in accordance with a ternary complex bridging mechanism. Vitronectin also seems to help the reaction of PAI-1 with thrombin [21]. A subfamily of serpins exists whose inhibitory activity is greatly accelerated upon binding of heparin and other negatively charged polyanions, such as heparan sulfate and dermatan sulphate. The members of this group include heparin cofactor II (HCII), antithrombin, protein C inhibitor, protease nexin 1 (PN-1) and PAI-1. Heparin is a highly negatively charged glycosaminoglycan consisting of alternating glucosamine and iduronic acid monomers. Studies have demonstrated that upon binding to heparin antithrombin gets activated. Binding of heparin pentasaccharide to the D helix of antithrombin causes a series of large conformational shifts (as shown in Figure 3). Extension of D-helix at both the ends leads to an interdomain rotation of the bottom half of the antithrombin relative to the top half, which leads to RCL expulsion from the β-sheet A and exposure of the P1 arginine (Arg-393) residue [18, 22]. The basic residues in this site that interact with the pentasaccharide are Lys-11 and Arg-13 in the N-terminal end; Arg-46 and Arg-47 in the A-helix; and Lys-114, Lys-125, and Arg-129 in the region of the D-helix (Figure 3). Two nonbasic residues, Phe-121 and Phe-122, reside near these positively charged amino acids of the heparin-binding domain but make minimal direct contact with the pentasaccharide sequence [23]. It has been shown that mutation of either Phe-121 or Phe-122 leads to decline in antithrombin-heparin binding affinity. Cofactor binding serpins are critically balanced to maintain its native metastability during its binding and transformation of conformational change (Figure 3).

5. Serpin Metastable Form

The metastable native state of serpins is thought to be like a kinetically trapped folding intermediate that is blocked by a very high kinetic barrier [24, 25]. The study of this kinetic trap provided an important link to understand structure-function relationships of serpins but the molecular basis by which this kinetic trap prevents the native serpin to form a more stable state is unknown [26]. It has been suggested that there are certain native interactions in the metastable form which drags it to get converted into a more stable form [24]. Studies have shown that the other stable conformation in the native serpin chain is the latent RCL-inserted form [17, 27]. For example the native form of a serpin, plasminogen activator inhibitor-1, readily gets transitioned into the latent form under physiological conditions with a half-life of 1-2 hours [28] and same happens in other serpins under mild denaturing conditions [29].

In serpins, when RCL is inserted into the A β-sheet, there occur a gradual decline in the free energy of the serpin and an increase in its stability [30–32]. Surprisingly, serpins also undergo a rise in thermostability when complexed with

Table 2: Serpins and their known protease and nonprotease ligands.

Serpin	Non protease ligands	Protease target
Antithrombin	Heparin, heparin sulfate	Thrombin, factor Xa, factor IXa
Antichymotrypsin	DNA, $A\beta_{1-42\,\text{peptide}}$	Cathepsin G
Alpha-1 Proteinase inhibitor	—	Neutrophil elastase
C1-Inhibitor	Heparin, collagen	Cls of complement system
Headpin	—	Lysosomal cathepsin
Heparin CofactorII	Heparin, dermatan sulfate	Thrombin
HSP47	Collagen	—
Kallistatin	Heparin	Tissue Kallikerin
Maspin	Collagen	—
MENT	DNA	Nuclear cysteine proteinase
Plasminogen Activator inhibitor-1	Heparin, heparin sulfate, vitronectin	tPA, uPA, thrombin, aPC
Protein C inhibitor	Heparin, retinoic acid	uPA, thrombin, aPC
Protease nexin-1	Heparin, collagen	Thrombin uPA
Thyroxine Binding Globulin	Thyroxine, triiodothyronine	—
Protein Z dependent protease inhibitors (ZPI)	Protein Z	Factor Xa

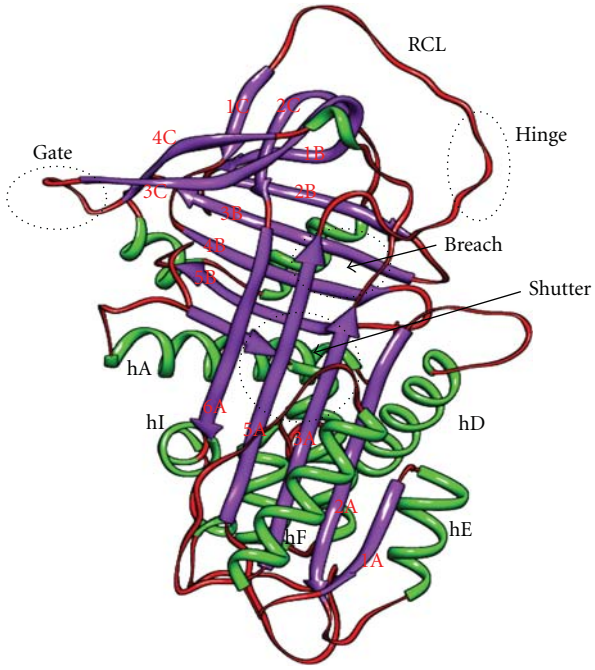

Figure 2: Important domains in serpin conformations. Several regions are important in controlling and modulating serpin conformational changes. The *Reactive Centre Loop* is involved in protease recognition and conformational transformation as strand 4A after inhibition. The P15–P9 portion of the RCL is called the *hinge* region. The point of initial insertion of the RCL which is the *breach* region, located at the top of the A β-sheet. Near the center of A β-sheet is the *shutter domain*. The breach and shutter are two major regions that assist sheet opening and accept the conserved hinge of the RCL when it inserts. The *gate region* is composed of s3C and s4C strands which has been primarily observed by studies of the transition latency. The image was drawn in chimera using the PDB file of native antitrypsin conformation.

peptides analogous to the RCL [33]. This suggests that increased stability is a product of hydrogen bond formation and hydrophobic interactions made by the addition of a sixth strand to the A β-sheet as s4A. But the negative consequence of obtaining metastability is that serpins are vulnerable to mutations that can result in aberrant structural rearrangement into dysfunctional states with increased stability [34].

6. The Molecular Basis of Serpin Metastability

Native state of the serpin is dependent on nonideal interactions (Table 3) which imparts strain within the native fold that can be further alleviated by adopting alternative conformations [35]. Nonideal interactions include the presence of hydrophobic pockets [36, 37], overpacking of side-chains [38], and the burial of polar groups [39] and cavities in the hydrophobic core of the protein [40]. Molecular details of how such structural defects control the protein functions are yet to be elucidated. Native conformation regulates the inhibitory function of α-1 antitrypsin by controlling the rate of the conformational switch during complex formation with a target protease. Hence the conformational switch is driven by mobilization of unfavourable interactions in the native state into more favourable ones, such interactions seem to have control over conformational switch [41]. From fluorescence studies it has been identified that nonnative interactions were residing around the top of the A β-sheet and the F-helix [42, 43]. Some unfavourable interactions that are involved in maintaining the metastable state of antitrypsin are summarized in Table 3. The unfavourable interactions made by residues of the F-helix might play a crucial role in preventing incorrect folding in serpin [35, 44]. Lys-335 is suitably situated near the F-helix to assist in the sheet A opening where it forms critical interaction with Ile-169 and Leu-172, a reduction in the side chain of these residues

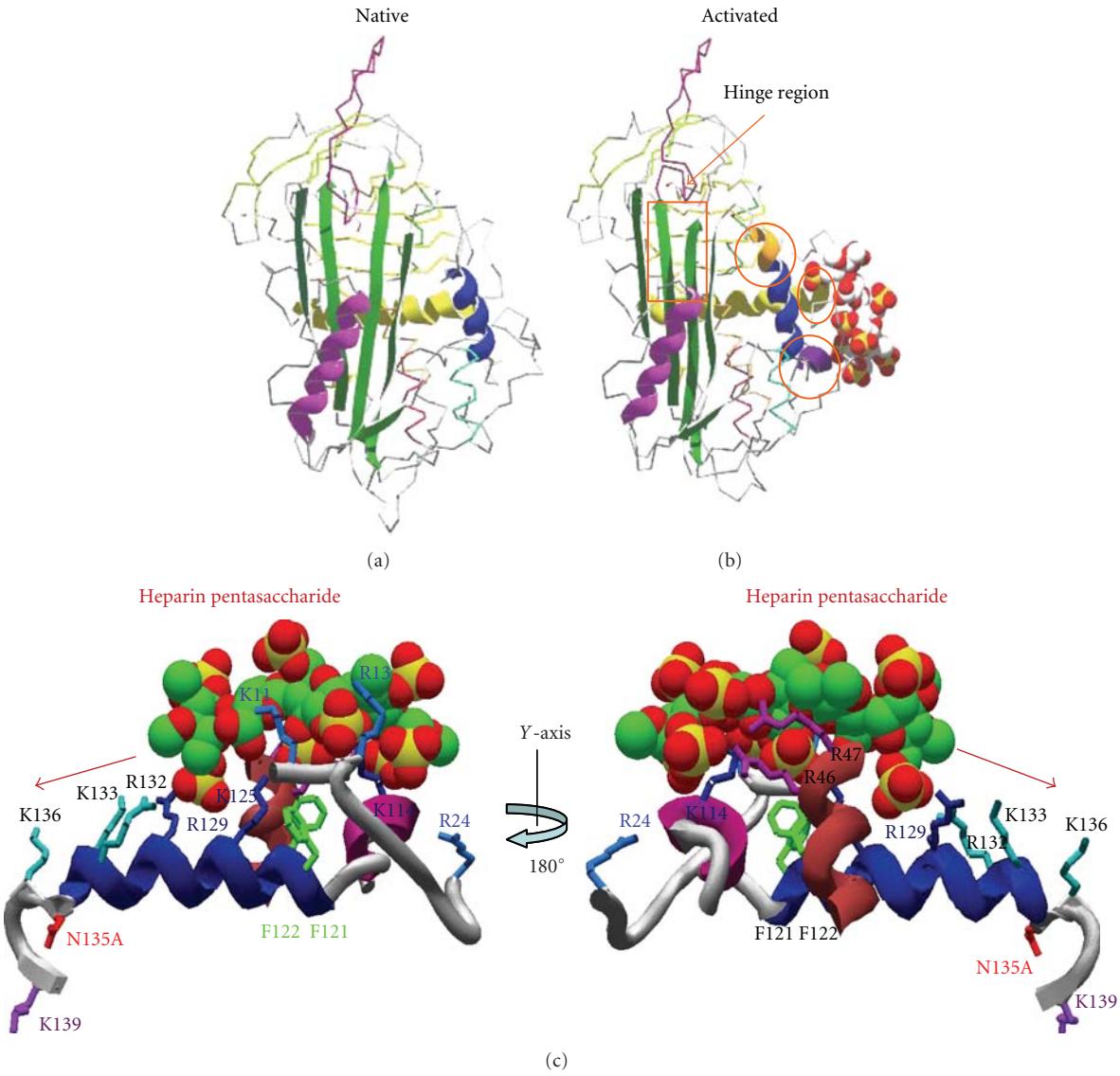

Figure 3: Conformational changes in cofactor (heparin) bound serpin (antithrombin) and residues involved in cofactor interaction. Heparin binding produces a series of conformational changes in antithrombin; extension of helix D by forming a 2 turn helix (P-helix) at the N-terminal end and a 1.5 turn extension of D-helix towards the C-terminal end. Moving of strand 3A and strand 5A and expulsion of reactive center loop leads to activated antithrombin. Given below are the basic residues in the heparin binding site that interact with the pentasaccharide are Lys-11 and Arg-13 in the N-terminal end; Arg-46 and Arg-47 in the A-helix; and Lys-114, Phe-121, Phe-122, Lys-125, and Arg-129 in the region of the D-helix. The figures were made by using antithrombin PDB (native 1E05; activated 1E03) files and swiss-prot PDB viewer.

results in increase in stability which contributes in the native strain. Polar-nonpolar interactions also contribute to native stability, Phe-189 interacts with Gly-164 and Thr-165 in the F-helix providing unfavourable interaction which contributes in native metastability [40].

Serpins make use of many conserved interactions which are distributed around the molecule to stay in the kinetic trap until a protease comes along and initiates further thermodynamically favourable conformational changes [44]. Decreasing the size of side chains in overpacked regions releases the native strain, and increasing the size of side chains in cavities stabilizes the native form, probably by providing better interactions with nearby residues. Likewise, filling exposed hydrophobic pockets by substituting larger residues also stabilizes the native state in antitrypsin [40]. Such unfavourable structural features are also found in other serpin proteins, like antichymotrypsin and antithrombin III, and the native strain in these serpins can also be relieved by compensating substitutions [45]. During protease inhibitor complex formation, unfavourable interactions in the native form are mobilized and transformed into more favourable ones [3]. These conformational changes result in the conversion of the metastable native form into a more stable conformation [46].

Table 3: Unfavourable interactions that contribute to the metastability of the native antitrypsin. Nonideal interactions include the presence of hydrophobic pockets, overpacking of side-chains, the burial of polar groups, cavities in the hydrophobic core of the protein and polar nonpolar interactions [40]. Lys-335 is one of the residues in antitrypsin that has been shown to play a crucial role in conformational switch during the process of inhibition. Local strain due to Lys-335 interactions in the native state is critical for the inhibitory activity.

Over packing of side chains	Polar-nonpolar interactions	Cavity filling mutations	Favourable interactions
Lys-335	Phe-189—Gly-164	Gly164Val	Native (Lys-335 is buried)
Ile-169	Thr-165-Val-161	Ala183Val	Cleaved
	Thr-165-Ile-169		Lys-335 forms salt bridge with Asp-171
Leu-172	Leu-172-Asn-186	Thr114Phe	Ile-169
	Lys-331-Val-333	Gly117Phe	Lys-168-Glu-346 (salt bridge)

7. Serpin Polymerization

The tendency of the RCL of serpins to become an additional strand in a pre-existing β-sheet also makes them prone to other types of loop-sheet interactions [47]. As a protein family, serpins are particularly prone to the formation of stable polymers owing to the metastability of their native fold and the thermodynamically driven β sheet opening out mechanism required for their role as protease inhibitors [3, 6]. Approximately -32 kcal /mol energy is released by the incorporation of the reactive centre loop (RCL) of the serpin into the centre of its main β-sheet [48]. Figure 4 shows point mutation in some representative serpins that leads to dimerization, aggregation and polymerization.

Severe deficiency of the Z variant of α-1-antitrypsin (Glu342Lys) results from a conformational switch and forms a unique linkage between the reactive centre loop of one molecule and A β sheet of a second [49]. This process of polymer formation was dependent on temperature and concentration, and the polymers that were formed had the appearance of "beads on a string" when seen by electron microscopy [49, 50]. Since this initial report, investigations have been concerned with the characterization and classification of clinically relevant mutations. Neuroserpin is a member of the serine proteinase inhibitor (serpin) superfamily that is secreted from the growth cones of neurons and inhibits the enzyme tissue type plasminogen activator (tPA) [51]. Aberrant protein linkage in mutants of neuroserpin causes intracerebral inclusions and dementia. Four different mutations of neuroserpin (Ser49Pro, Ser52Arg, His338Arg and Gly392Glu) have been described in humans [52–54]. All four mutations cause the spontaneous formation of neuroserpin polymers that are retained as inclusion (or Collins) bodies within neurons in the deeper layers of the cortex and the substantia nigra [35, 55]. It can be responsible for the occurrence of dementia, tremor, seizures, epilepsy and dysarthria that is present in different degrees depending on the harshness of the mutation involved. In heparin cofactor-II, there are two known polymerization variants which are Glu428Lys and Pro433Leu [56].

8. Polymeric Conformations in Serpin

Loop A sheet polymeric structure is characterised by the insertion of RCL of one serpin molecule into A β-sheet of another between strand three and five [57]. Initial evidence of this intermolecular linkage came from studies in which α-1 antitrypsin polymerization was hindered by incubation with peptides analogous to the RCL [49, 58] that was further supported by fluorescence energy transfer data [59]. In loop C sheet polymer, one of the strand of C β-sheet moves away to allow full insertion of the RCL and the position left vacant by s1C is filled by the RCL of second molecule [35]. This polymeric form is found in Mmalton variant of antitrypsin, antithrombin can also forms similar type of polymer when heated in the presence of citrate [60]. s7A polymer has only been shown in PAI-1, which is characterised by hydrogen bonding of the RCL of one molecule with six strand of the β-sheet A of the other, where donor RCL become the seventh stand of the acceptor molecule's A β-sheet [61]. In disulfide linked polymeric form the RCL is not involved as a polymer formation domain as it does in other polymeric forms. Dimer α-1 antitrypsin has single cysteine residue located on the B β-sheet and this dimeric species goes on to form high order polymer. However, this disulfide-linked polymer has similar linear morphology to both loop A and C sheet as supported by electron microscopy [62].

9. Helix B Variants in the Shutter Region

The shutter region of serpins is comprised of a number of well-conserved residues that play a significant role in the maintenance of stability and function [35]. Mutations in shutter region allow the aberrant opening of the A-sheet, with the likelihood of the insertion into its lower half of the reactive loop of another molecule to give intermolecular linkage and polymerization of the serpin. Shutter region includes F-helix, B-helix, strand s3A and s5A of β-sheet A that plays an important role in stability and function in serpins [55]. Conserved helix B residues interact with the β-sheet A at the upper portion of the shutter region where RCL inserts as s4A. Helix B mutations in α-1-antichymotrypsin (Leu55Pro) and α-1-antitrypsin (Phe51Leu, Ser53Phe and Val55Pro) can cause lung (emphysema) and liver diseases (cirrhosis). Protein C-inhibitor (Ser52Phe and Ser54Leu) and antithrombin (Pro80Ser/Thr, Thr85Met/Lys, Cys95Arg and Leu99Phe) have mutations which can result in angioedema and thrombosis [5, 25, 35, 56]. Importance of strand 6B deformation and exposure of helix B in smooth insertion of the reactive centre loop during serpin inhibition was hypothesized recently and indicated that helix B exposure in variants may increase its polymerization propensity [63].

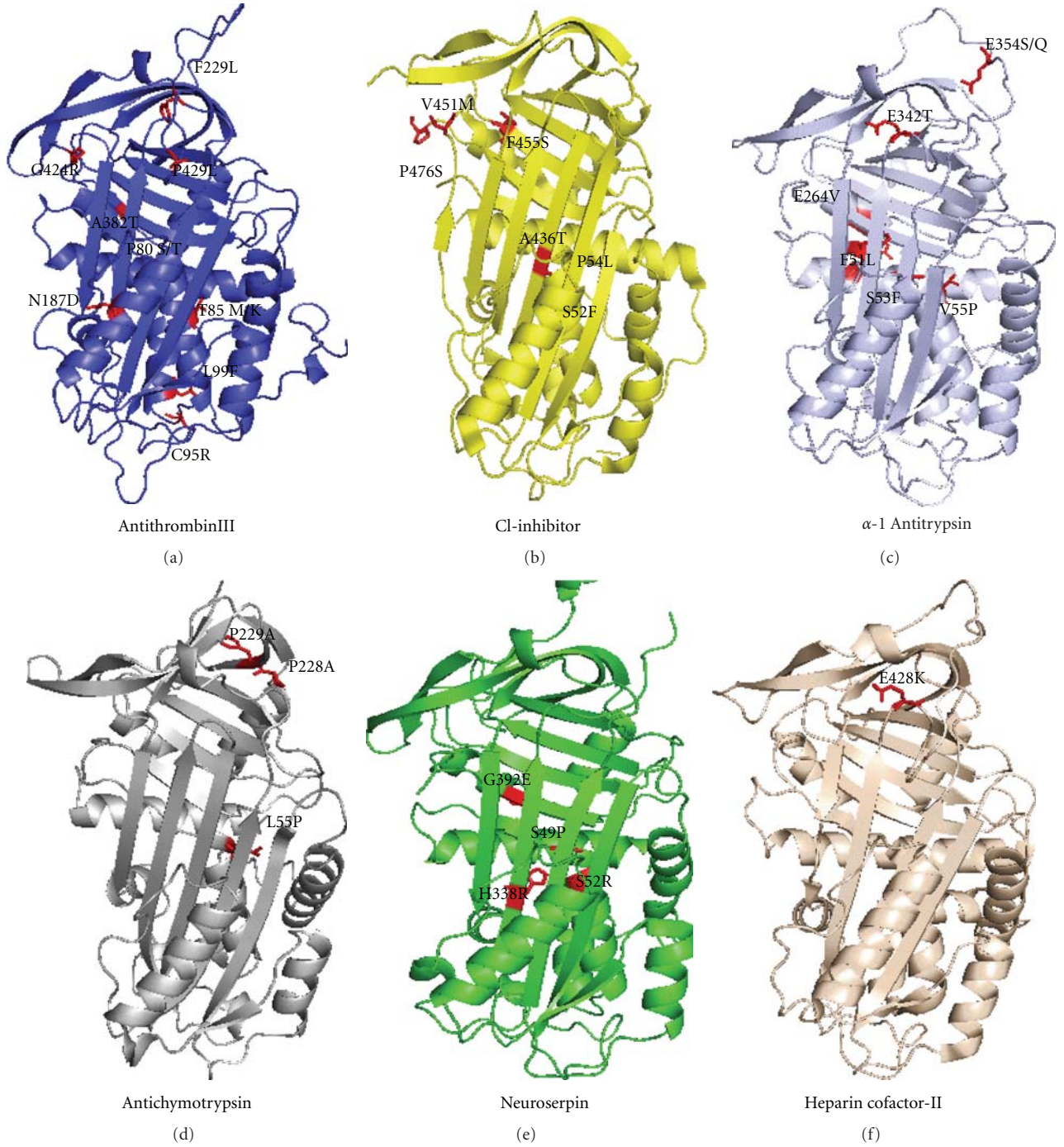

Figure 4: Point mutation in representative serpins that leads to latency, dimerization, aggregation and polymerization. Figure shows different serpin members like antithrombin (1e05), α-1 antitrypsin (2qug), neuroserpin (1jjo), antichymotrypsin (4caa), heparin cofactor-II (1jmj) and C1-inhibitor along with corresponding natural variant that gives rise to polymerization. Images were prepared by using Pymol visualization tool.

10. Accessible Surface Area and Stability Analysis of Serpin Variants

It is possible that the variants that contribute to serpin polymerization are deeply buried and introduce local destabilization. We took antithrombin, antitrypsin, antichymotrypsin, neuroserpin and heparin cofactor II as representative inhibitory serpins in which details of polymerization variants are known and did an analysis of burial and stability. Table 4 shows the positions of the serpin variants along with their ASA values. Analysis shows that in most cases the amino acid involved in the polymerization was completely buried in the native conformation. In the native state, antithrombin Pro-80, Thr-85, Leu-99, antitrypsin Phe-51, Ser-53 and Val-55

Table 4: Analysis of the residue burial and stability of natural variant of serpin involved in polymerization.

Serpins	ASA[b] (Native)	ΔΔG[c] (kcal/mol)
Antithrombin-P80 S/T[a]	0.0	−0.97, −0.72
Antithrombin-T85 M/K[a]	2.9	−0.73, −3.04
Antithrombin-C95R	9.3	−1.33
Antithrombin-L99F	1.1	−1.03
Antithrombin-N187D	9.6	−1.92
Antithrombin-F229L	1.5	−0.92
Antithrombin-A382T	28.0	−0.33
Antithrombin-G424R	2.5	−1.30
Antithrombin-P429L	12.7	−0.18
Antitrypsin-F51L	0.0	−1.42
Antitrypsin-S53F	0.0	−0.42
Antitrypsin-V55P	2.6	−2.28
Antitrypsin-E264V	5.2	0.47
Antitrypsin-E342T	6.9	1.02
Neuroserpin-S49P	0.0	−1.19
Neuroserpin-S52R	0.0	−1.06
Neuroserpin-H338R	0.0	−1.37
Neuroserpin-G392E	0.0	−0.30
Antichymotrypsin-L55P	1.6	−2.17
Antichymotrypsin-P228A	4.9	−1.45
Heparin Cofactor-II-E428K	18.0	−0.22

[a]Two different variant at the same position.
[b]Accessible Surface Area (ASA) values were determined from DSSP algorithm. The pdb codes used for the analysis are as follows: **a**ntithrombin (1t1f), antitrypsin (1qlp), neuroserpin (1jjo and 3fgq), antichymotrypsin (1yxa), and heparin cofactor-II (1jmj).
[c]ΔΔG were determined for the variants by using Imutant 2.0 at pH 7.0 and 25°C, the values were determined by using the difference of ΔG between the wild-type and the polymerization variants mentioned in the table.

and neuroserpin Ser-49 and Ser-52 residues showed the ASA value of zero, these residues are part of helix B. Helix B residues tend to show the maximum burial especially the residues that are conserved in the serpin.

To test the effect of deeply buried helix B variant of serpin on the overall stability point mutations were done using I mutant 2.0 program at temp 25°C and pH 7.0 and the results are summarized in Table 4. The results showed that all the polymerization variants of the helix B were destabilizing with ΔΔG values ranging from −0.4 kcal/mole to −3.0 kcal/mole. No apparent correlation was found between the relative burial and the magnitude of destabilization. Most destabilized variants were not necessarily the most buried ones. However the helix B residues like Thr-85 in antithrombin, Val-55 in antitrypsin and Leu-55 in antichymotrypsin tend to be more destabilized in comparison to other polymerization variants in helix B. The result clearly indicates that the deeply buried residues can cause conformational flexibility which results in the global destabilization in the polymerizing variants of serpins.

11. Conclusion

Serpins fold into structures that are metastable and employ a unique suicide substrate-like inhibitory mechanism to achieve stable state. Serpins make use of many conserved interactions like decreasing the size of side chains in overpacked regions, and increasing the size of cavities and filling of exposed hydrophobic pockets to release the native strain to convert from the native form to a cleaved inhibitory stable conformation. Due to its inhibition mechanism serpin are prone to conformational defects resulting in severe pathological disorders. Understanding the mechanism of polymer formation in serpins is confusing due to availability of several models and also due to the lack of crystal structures of natural variants. Identifying specific domains and interactions in serpin that contribute to inhibition and polymerization mechanism is important. Helix B region is a mutation hotspot in serpin that leads to polymerization due to deep burial, decreased stability and its involvement in RCL translocation.

Acknowledgments

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Review Article

Functional Subunits of Eukaryotic Chaperonin CCT/TRiC in Protein Folding

M. Anaul Kabir,[1] Wasim Uddin,[1] Aswathy Narayanan,[1] Praveen Kumar Reddy,[1] M. Aman Jairajpuri,[2] Fred Sherman,[3] and Zulfiqar Ahmad[4]

[1] *Molecular Genetics Laboratory, School of Biotechnology, National Institute of Technology Calicut, Kerala 673601, India*
[2] *Department of Biosciences, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India*
[3] *Department of Biochemistry and Biophysics, University of Rochester Medical Center, NY 14642, USA*
[4] *Department of Biology, Alabama A&M University, Normal, AL 35762, USA*

Correspondence should be addressed to M. Anaul Kabir, anaulk@nitc.ac.in

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Molecular chaperones are a class of proteins responsible for proper folding of a large number of polypeptides in both prokaryotic and eukaryotic cells. Newly synthesized polypeptides are prone to nonspecific interactions, and many of them make toxic aggregates in absence of chaperones. The eukaryotic chaperonin CCT is a large, multisubunit, cylindrical structure having two identical rings stacked back to back. Each ring is composed of eight different but similar subunits and each subunit has three distinct domains. CCT assists folding of actin, tubulin, and numerous other cellular proteins in an ATP-dependent manner. The catalytic cooperativity of ATP binding/hydrolysis in CCT occurs in a sequential manner different from concerted cooperativity as shown for GroEL. Unlike GroEL, CCT does not have GroES-like cofactor, rather it has a built-in lid structure responsible for closing the central cavity. The CCT complex recognizes its substrates through diverse mechanisms involving hydrophobic or electrostatic interactions. Upstream factors like Hsp70 and Hsp90 also work in a concerted manner to transfer the substrate to CCT. Moreover, prefoldin, phosducin-like proteins, and Bag3 protein interact with CCT and modulate its function for the fine-tuning of protein folding process. Any misregulation of protein folding process leads to the formation of misfolded proteins or toxic aggregates which are linked to multiple pathological disorders.

1. Introduction

The primary amino acid sequence of a protein contains all the information necessary for protein folding and its biological activity [1]. However, in a normal cellular condition, a nascent polypeptide chain faces a crowded environment and there is a good possibility that protein will be misfolded and will form aggregates that make the protein inactive, and in certain cases it becomes toxic for the cell. Both the prokaryotic and eukaryotic cells possess a family of proteins responsible for binding to nascent polypeptide chains and help them fold into biologically functional three-dimensional structures, they are known as molecular chaperones, and they vary in size and complexity [2–6]. Many of the molecular chaperones are induced in response to stress or heat, and so they got the name Hsp (heat shock protein). Molecular chaperones like Hsp90, Hsp70, Hsp40, and Hsp104 bind to nascent polypeptide chain at hydrophobic regions which are exposed to the crowded environment otherwise buried inside in a completely folded protein [7–10]. Molecular chaperones have developed multiple and diverse tertiary and quaternary structures to bind nonnative protein substrates. Though, there is a lack of sequence similarity among different families of chaperones and only a few of them are represented in all three domains of life (bacteria, archaea, and eukaryote), generally, they use convergent strategies to bind the substrates. Crystallographic and other evidence show that many chaperones including prefoldin, trigger factor, hsp40, and hsp90 have clamp-like structures, possibly responsible for the binding of nonnative substrates [11]. Another class of

cylindrical-shaped chaperones, known as chaperonins, is found to be conserved in all three domains of life and assist the folding of many cytosolic proteins [12, 13]. In some cases, the transient binding of nascent polypeptide chain is sufficient for protecting its hydrophobic regions and promoting its proper folding. However, for the folding of a multidomain protein, more than one class of chaperones might be involved, and they work in a concerted manner to generate a protective passage. For example, nascent polypeptide chain coming out from ribosome will first bind to Hsp70/Hsp90 which will help attain a quasinative structure and then will be transferred to chaperonin CCT for its final folding [14, 15]. Here we review the current status of understanding of protein folding by the chaperonin CCT complex in eukaryotes.

The chaperonins are large, multimeric, cylindrical protein complexes consisting of two stacked rings and each ring has 7–9 subunits [2, 4, 16–18]. On the basis of amino acid sequence homology, chaperonins have been categorized into two groups, group I and group II [19–24]. Group I is found in all eubacteria and endosymbiotic organelles like mitochondria, chloroplasts, and related organelles like hydrogenosomes and mitosomes whereas group II chaperonins are present in archaebacteria and in the cytosol of all eukaryotes [2, 17, 25–27]. Here we will give brief introduction to group I chaperonin and then will discuss group II chaperonin, CCT.

2. Group I Chaperonin

The function of this group has been well studied using GroEL/GroES from *Escherichia coli*. The genes for GroEL and GroES were discovered in a mutagenic screen for genes required for the growth of bacteriophage lambda and later found to be essential for the survival of *E. coli* itself in all conditions [28–30]. GroEL/GroES has been the subject of extensive structural and functional analysis for understanding protein folding *in vitro* and *in vivo* [31–33]. GroEL is about 800 kDa homooligomeric protein complex with ATPase activity and is composed of two seven-membered rings of 57 kDa subunits. Each ring has a central cavity aligned with hydrophobic surfaces for the binding of unfolded or denatured proteins [34–37]. The cochaperonin GroES is a heptameric ring complex composed of 10 kDa subunits and caps the GroEL folding chamber [38–40]. The folding cage generated by GroEL/GroES plays dual functions in protein folding. First, confinement of substrate protein in the GroEL/GroES complex is necessary for protecting them from aggregation. Second, the folding process in the chaperonin would be much faster than that of in a free solution [41]. X-ray studies of GroEL have revealed three distinct domains: equatorial domain, apical domain, and intermediate domain [42]. The equatorial domain is responsible for most of the intra- and intersubunit interactions as well as for the binding of ATP and its hydrolysis. The apical domain encompasses the entrance of central cavity and holds all the hydrophobic residues required for substrate binding. The intermediate domain connects both the domains and acts as a hinge for the movements of apical domain which is induced upon binding of ATP and its hydrolysis [43–45]. The substrate binding residues present in the apical domain is also responsible for binding of co-chaperonin, GroES, which is essential for GroEL-mediated protein folding. The mechanism of GroEL-mediated protein folding has been extensively studied using different approaches [16, 24, 46, 47]. Briefly, protein folding cycle starts with the binding of unfolded or denatured proteins and ATP at one end (the cis end) of GroEL, followed by the binding of GroES to the same end. The binding of GroES caps the entrance and releases the substrate to the central cavity. The binding of GroES also promotes ATP hydrolysis and the protein substrate gets folded in the "Anfinsen cage" of the GroEL-GroES-ADP complex. The binding of protein substrate and ATP to trans ring causes the release of GroES and substrate [48].

3. Group II Chaperonin

In the group II chaperonins, both archaeal thermosome and eukaryotic chaperonin containing TCP-1 (CCT; also known as TCP-1 ring complex, TRiC) are being studied using different techniques [49–52]. However, here we will confine our discussion to CCT complex only. The subunit TCP-1 of CCT complex was first isolated from murine testes and subsequently it was found to be constitutively expressed in other mammalian cells, insects, and yeasts [53–57]. The compelling evidence for CCT complex as a chaperonin came from the observations of its involvement in the assembly of actin and tubulin filaments [58–61]. Though, initially, it was discovered as a folding machine for actin and tubulin, later, it was found to be involved in the folding of 5–10% of newly synthesized cytosolic protein substrates [12, 52, 62, 63]. Like GroEL system, CCT also binds ATP and hydrolyses it during protein folding cycle, the CCT does not have detachable GroES-like cochaperonin, rather, a flexible protrusions located in the apical domain in each CCT subunit acts as a lid and is responsible for closing the central cavity [17, 64, 65].

3.1. Structure and Ring Arrangement of CCT. The chaperonin CCT is a large, cylindrical, multimeric complex having central cavity for binding unfolded or denatured polypeptides. The structure of CCT is much more complex compared to GroEL because each ring of CCT is composed of eight different but related subunits ranging between 52 and 65 kDa [58–60]. Moreover, it has been observed that all the eight subunits of CCT are essential in yeast [52]. Now the question is when homooligomeric GroEL is sufficient to fold an array of substrates, why did CCT complex evolve eight different subunits? Perhaps, one possible explanation could be that certain specific combinations of subunits interact with specific structural features or motifs of the protein substrates and hence eight different subunits give large number of combinations to accommodate a broad variety of substrates for their folding [66, 67]. Furthermore, the presence of eight different subunits encoded by eight paralogous genes indicates that every subunit might have some specialized role

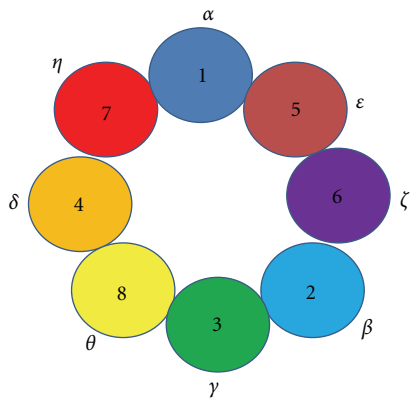

Figure 1: The intra-ring subunit arrangement of CCT. Both Greek alphabets and Arabic numbering system have been used to denote each of the subunits (this was made on the basis of [71]).

in the overall functioning of CCT. Phylogenetic analysis of these eight subunits suggested that functional specialization of the individual subunits took place in the early phase of eukaryotic evolution and associated with its cellular functions and became essential for its survival [68, 69]. The electron microscopy and single particle analyses show that the domain structures of CCT are similar to those of GroEL and archaeal thermosome, and three domains (equatorial, apical, and intermediate domains) are present in a single subunit [68]. The mammalian subunits of CCT are designated as CCTα, CCTβ, CCTγ, CCTδ, CCTε, CCTζ, CCTη, and CCTθ which are corresponding to Cct1p-Cct8p in yeast. Using biochemical and genetic approaches, it has been shown that CCT is a single heterooligomer of eight subunits and the arrangement of the subunits has a unique pattern as shown in Figure 1 [70, 71]. The subunits in a ring are arranged as Cct1p → Cct5p → Cct6p → Cct2p → Cct3p → Cct8p → Cct4p → Cct7p in a clockwise orientation. The arrangement of subunits in CCT ring also addressed by modeling using existing data, and four possible models have been proposed with clockwise and anticlockwise orientations; however, the relative positions of the subunits in a ring remain same [71–74]. The phasing between two rings of CCT has been addressed by three-dimensional reconstructions generated by electron microscopy and using monoclonal antibodies against Cct4p and Cct5p. It has been shown that interring communications take place through two different subunits in all the eight positions [75]. Moreover, the subunits associated with initiation and completion of the folding cycle cluster together in both inter- and intraring arrangements [75].

However, recent cryo-EM analysis of closed CCT suggested new positions in a ring for three subunits-Cct1p, Cct5p, and Cct7p, and the subunits in a ring are arranged as Cct1p → Cct7p → Cct5p → Cct4p → Cct8p → Cct3p → Cct2p → Cct6p in a clockwise orientation [76]. This observation identifies the existence of two-fold axis between two rings. It reveals a unique pattern of interring arrangement that generates three heterotypic interring contacts (Cct3p-Cct4p, Cct2p-Cct5p and Cct6p-Cct7p) and two homotypic interring contacts (Cct1p-Cct1p and Cct8p-Cct8p). As the relative positions of other five subunits remain same in both the models and only three are having deviation in each of these models, fine-tuning of biochemical and EM analyses will be required to fix their positions. Recently, crystal structure analysis has been done for CCT purified from bovine testis along with its natural substrate, tubulin in the open conformation [77]. This structure showed that substrate interacts with CCT in the loops of apical and equatorial domains. The organization of ATP binding sites indicates that the substrate is stretched inside the cavity. Moreover, the fold of each domain (equatorial, intermediate and apical) of the eight subunits of CCT found to be similar to those of α- and β-subunit of thermosome. The thermosome equatorial domains can be superimposed on their CCT homologues, though small rearrangements in the orientation of helices and loops along the equatorial domain were observed. In contrast to the crystal structures of GroEL and thermosome, apical domains of CCT showed a wide range of conformations, both in the central cavity and in the aperture of the lid domains [42, 78, 79]. This supports the existence of sequential, hierarchical mechanism of conformational changes induced by ATP.

3.2. Nucleotide Binding to CCT and Protein Folding. The binding of ATP to the subunits of CCT and its hydrolysis is absolutely required for the CCT-mediated folding of newly synthesized polypeptide or misfolded protein substrates [58, 60, 80]. In the absence of ATP or in presence of ADP, CCT remains in an open conformation in which substrate binding sites in the apical domains are exposed and bind the substrates. Addition of ATP induces the conformational changes and subsequently the central cavity is closed by built-in lid structure to confine the protein substrate inside the cavity and provides a secluded environment for its folding. However, just addition of ATP to CCT does not promote the lid closure as shown using non-hydrolyzable ATP analogs [65, 81]. Using non-hydrolyzable ATP analog (AMP-PNP), it has been demonstrated that AMP-PNP-CCT binds to actin and tubulin on one CCT ring [66, 82]. On the other hand, when CCT is blocked in its ADP-P_i state, no CCT-protein target complex was formed. This suggests that binding sites in the apical domain of CCT will be available for target proteins in ATP-CCT state but not in ADP-P_i-CCT state. Therefore, nucleotide exchange and hydrolysis might be working as a regulatory switch for the binding of target proteins to CCT [83].

3.3. Lid Structure of CCT. The bacterial chaperonin GroEL has a cofactor GroES which is used as a "lid" for the closure of central cavity essential for protein folding [84]. However, the eukaryotic cytosolic chaperonin CCT/TRiC does not have any homologue of GroES to cap the central cavity [17, 78]. The crystal structure of archaeal chaperonin thermosome has shown the presence of protrusions emerging from the apical domain and arranged in an iris-like β sheet which is responsible for closing the central cavity [78]. These apical protrusions are unique to group II chaperonins, like

thermosome and CCT, and they have been proposed to have GroES-like activity and act as a built-in lid that might open and close in an ATP-dependent manner [17, 85]. Compelling evidence for the requirement of lid responsible for encapsulating the unfolded polypeptides comes from the observations that lidless chaperonins lose the ability to fold stringent substrates [65, 86, 87]. The question is how does this "built-in lid" functions for the closing and opening of the central cavity for substrate binding and releasing? Though there are striking similarities between group I and group II chaperonins, the lid closure mechanism seems to be quite different from each other. The binding of GroES to GroEL occurs upon ATP binding to equatorial domain of the GroEL subunits whereas the lid formation in eukaryotic and archaeal chaperonins is triggered by the transition state of ATP hydrolysis, suggesting nucleotide cycle dependent mechanistic difference of lid closure [88]. In one study, using the *Thermococcus* chaperonin, it has been suggested that ATP binding/hydrolysis causes independent conformational changes in the subunits. However, complete closure of the lid is induced and stabilized by the interactions of the helical protrusions of different subunits [89].

Table 1: Mutations in the conserved ATP binding/hydrolysis domain and phenotypes in GroEL and three subunits of CCT complex.

Subunit	Amino acid replacement	Phenotype	References
GroEL	G35E	Normal	[96]
GroEL	D87N	Lethal	[96]
GroEL	D87K	Lethal	[96]
Cct1p	G45S	CS	[93]
Cct1p	G48E	HS	[92]
Cct1p	D96E	CS	[93]
Cct1p	G423D	HS	[93]
Cct2p	G41E	Lethal	[94]
Cct6p	G38S	Normal	[95]
Cct6p	G41E	TBZ^S, $NaCl^S$	[95]
Cct6p	G59R	TBZ^{SS}, $NaCl^S$	[95]
Cct6p	D89E	TBZ^S	[95]
Cct6p	G90E	TBZ^{SS}, $NaCl^S$	[95]
Cct6p	G414E	CS	[95]
Cct6p	G416D	Normal	[95]
Cct6p	G416E	CS	[95]

CS: Cold sensitive; HS: heat sensitive; TBZ^S: Thiabendazole sensitive; TBZ^{SS}: Thiabendazole-hyper sensitive; $NaCl^S$: Sodium chloride sensitive.

3.4. Mutations Affecting ATP Hydrolysis. The high-resolution crystal structure of GroEL in ATP-bound and -unbound formed and that of GroES has identified an ATP binding domain encompassing N- and C-terminus of GroEL subunits [42, 90, 91]. This domain contains highly conserved GDGTT (residues 86–90) ATP binding residues along with loop structural motifs, LGPKG (residues 31–35), ITKDG (residues 49–53), and GGG (residues 414–416) which are found to be conserved among chaperonins. Sequence homology analysis of CCT subunits with that of GroEL identified almost identical ATP binding motif containing residues GDGTT and other loop structural motifs suggesting their conserved role in ATP binding/hydrolysis in prokaryotes as well in eukaryotes. Though the conserved loop elements appear to have certain common functions in ATP binding/hydrolysis, they could possess some functions which are specific to certain loop elements in a subunit. This is inferred from the observations that certain alleles of the same gene, affecting residues from different conserved loop structures have different degrees of cytoskeletal dysfunctions [92, 93]. Besides, there might be functional hierarchy among the paralogous motifs of different subunits. For example, homologous replacement of the fifth conserved glycine residue to glutamic acid in the LGPKG motif causes a lethal phenotype in Cct2p whereas the same mutation in Cct1p makes it heat sensitive in yeast *Saccharomyces cerevisiae*. However, the same replacement in Cct6p does not have much effect (Table 1 and Figure 2) [92, 94]. Furthermore, it has been shown that the conserved ATP binding/hydrolysis motif GDGTT → AAAAA replacement in the Cct6p in *S. cerevisiae* does not have much effect whereas same replacement is lethal for Cct1p [95]. From the above-mentioned experimental data, it can be suggested that different subunits play different role for ATP binding/hydrolysis in the CCT. This might be required for folding different substrates and modulating intraring and interring interactions.

3.5. Substrate Recognition by CCT. One of the most well-studied chaperonins is the GroEL from *E. coli*, and the recognition of substrate by this chaperonin has been studied to a large extent, and so we will briefly discuss substrate recognition mechanism of GroEL before we go into the details of substrate recognition by CCT complex. A number of techniques have been used to unveil the mechanism of substrate binding by GroEL. The localization of substrate binding region appears to be at the entrance of central cavity of GroEL which has been shown by electron microscopy [34, 97]. The X-ray crystal structure revealed this substrate binding region in each subunit and has been termed "apical domain" [42]. A systematic mutational study was used to understand the role of different amino acids in this region, and it revealed that A152, Y199, S201, Y203, F204, L234, L237, L259, and V263 play important roles in binding the substrates as the mutations in these residues in the apical domain affect the substrates binding to GroEL severely [96]. Interestingly, most of these residues have hydrophobic side chains which can generate a hydrophobic surface for the binding of the substrate. On the other hand, mutations of charged amino acids in the apical domain appear to have no effect on the binding of the substrates. This suggests that hydrophobic residues in the apical domain are mainly responsible for creating hydrophobic surfaces in the central cavity for binding of substrates through hydrophobic interactions. However, the single-residue replacements in the intermediate domain (I150E, S151V, A152E, A383E, A405E, and A406E) exert global effect on the functioning of GroEL [96]. All of these mutants showed severe defect in ATPase

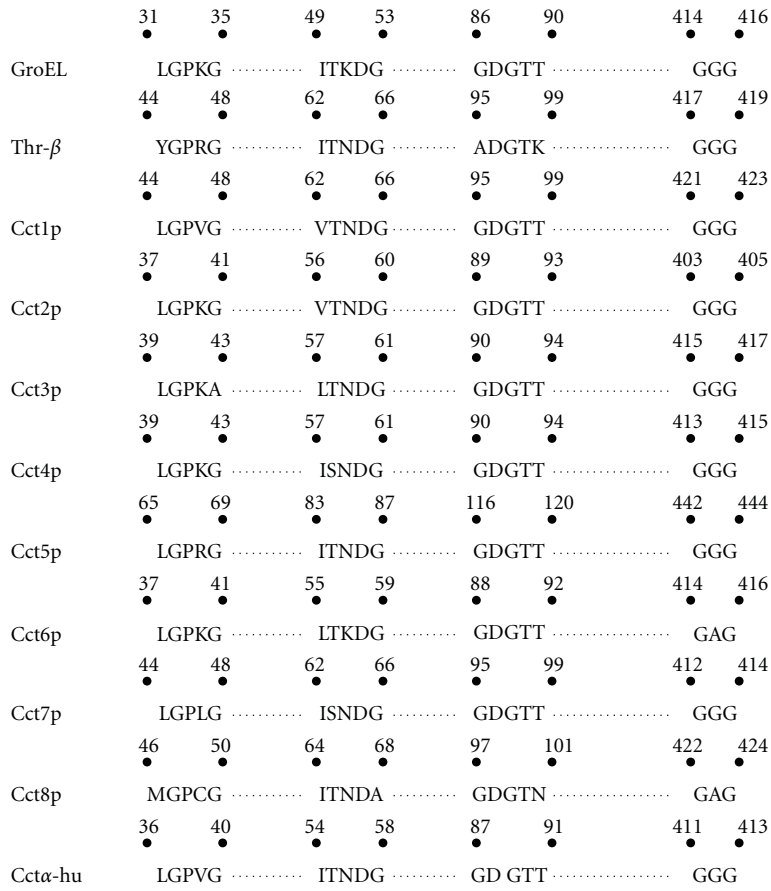

Figure 2: Comparison of highly conserved ATP binding/hydrolysis motifs in equatorial domain. GroEL, chaperonin of *E. coli*; Cct1p-Cct8p subunits of CCT complex of yeast *S. cerevisiae*; Cctα-hu, Cct1α subunit from human and Thr-β, β subunit from *Acidianus tengchongensis*. Starting from *E. coli* (homooligomeric chaperonin) to human (heterooligomeric chaperonin), all the chaperonin subunits have maintained conserved regions and any changes would have severe effects.

activity though they fall outside the ATP-binding domain. Also the mutants at positions, 150, 151, 383, and 405 could bind polypeptide but the release of the polypeptide was severely affected. On the other hand, D87K/D87N mutation in the conserved domain, GDGTT, in the equatorial domain, lost the ATPase activity completely, though it has the ability to bind ATP. It has also reduced the ability to bind polypeptide; however, there was a complete block of polypeptide release [96].

Several techniques have been used to implicate the importance of hydrophobic interactions between GroEL surface and the substrates [98–102]. However, there are some exceptions to this substrate recognition principle and certain other forces such as electrostatic interactions might play an important role as well for binding the substrate to GroEL efficiently [103–105].

Although group I chaperonin (GroEL) and group II chaperonin (CCT) have double ring structure and share sequence similarities, they differ from each other in two major aspects. First, group I chaperonin is composed of identical subunits and has seven subunits per ring whereas group II chaperonin is composed of 2–8 paralogous subunits with 30–40% homology to one another and each ring has 8–9 subunits [16, 17, 26, 106, 107]. For example, CCT is composed of eight paralogous subunits [52, 70]. However, the functional relevance of this subunit diversity is not well understood. As the sequence divergence in the apical domain is more among the paralogous subunits, it has been hypothesized that different subunit in CCT has different substrate specificity [107, 108]. Second, group II chaperonins do not have GroES-like cofactor; however, it possesses a helical protrusion that acts as "built-in lid." These two major differences might be related to the evolution of these group II chaperonins for assisting the folding of different archaeal and eukaryotic proteins. The structural and mechanistic differences between two groups might have profound functional impact on the substrate specificity [13, 109]. For example, several eukaryotic protein including actin and tubulin can be folded by CCT only whereas the bacterial proteins which require the assistance of GroEL for their folding, are not able to fold in eukaryotic cytosol [13, 58, 109, 110].

The specificity of GroEL and CCT towards the substrates is thought to be due to chemical nature of their interactions with substrates. It has been well established that GroEL recognizes the exposed hydrophobic surfaces of unfolded substrates [111–113]. On the other hand, CCT subunits

possess specific binding sites for unique polar motifs of certain cellular proteins [82, 114–116]. However, using a biochemical approach, it has been shown that for the binding of actin, von Hippel-Lindau tumor suppressor and Gβ WD-40 protein to CCT, hydrophobic interactions are involved [117–119]. In the absence of well-defined structural surfaces or motifs present in the substrates of CCT, three model proteins, actin, tubulin, and von Hippel-Lindau tumor suppressor have been studied thoroughly to find the recognition sites present in these substrates as well as in the interacting subunits of CCT.

3.5.1. Recognition Sites in Actin and Tubulin. Several studies have pointed out that the nature of actin and tubulin conformations bound to CCT are not of any nonspecific structures as in the case of GroEL substrates rather they must have some kind of defined, quasinative conformations before they are recognized by CCT for the final steps of their folding [80, 120–122]. The quasinative conformation may be achieved themselves or they may be guided by prefoldin kind of cochaperonin to reach that conformation. The atomic structure of actin has shown the presence of two domains, small and large [123]. The three-dimensional reconstruction analysis of alpha actin with CCT using electron microscopy has shown that CCT interacts with these two domains of actin by two specific and distinct interactions. The small domain of actin interacts with Cct4p subunit whereas large domain interacts with either Cct2p or Cct5p (both are 1,4 position with respect to Cct4p). This observation led to the suggestion that CCT interacts with actin in subunit-specific and geometry dependent-manner [115]. The three-dimensional reconstruction analysis combined with immunomicroscopy and screening study using actin peptide arrays have identified residues in four regions of actin molecule [114–117, 124]. Two of these regions (R37-D51 and R62-T66) are located at the tip of small domain and interacts with Cct4p subunit of CCT. The other two regions (E195-R206 and T229-I250) are present at the tip of large domain and interact with Cct2p or Cct5p subunit. Mutational analysis coupled with electron microscopy and biochemical assay have shown that major determinants of actin binding to CCT are present at the tip of the large domain [116, 125]. A mutation (G150P) in the conserved putative hinge region between small and large domains resulted in the accumulation of actin on the chaperonin CCT. Furthermore, electroncmicroscopic studies have shown that actin interacts with Cct2p or Cct5p subunits rather than Cct4p subunit. It was thought that Cct2p and Cct5p might have highest substrate affinity, and this possibility has been strengthened by immunoprecipitation experiments of actin-CCT complexes [114].

On the other hand, the interaction of tubulin with CCT seems to be much more complex and it does not confine to a few regions of tubulin rather it is spread along its entire sequence and interacts with several domains at a time [82, 117, 122, 124, 126, 127]. Several studies have shown that CCT binding sites in tubulin are present in loops exposed to the surface of native protein [67, 82, 128, 129]. Of the eight binding sites present in tubulin, three are located at N-terminal domain and five are placed in C-terminal domain. The N-terminal binding sites are T33-A57, S126-Q133, and E160-R164. Immunomicroscopic experiments have shown that residues T33-A57 interact with Cct1p or Cct4p whereas the residues S126-Q133 and E160-R164 interact with Cct7p or Cct8p [82]. The interaction of C-terminal domain of tubulin with CCT is much more complex than that of N-terminal domain. Five putative segments present in the C-terminal domain responsible for interaction with CCT are T239-K254, P261-H266, S277-V288, V355-P359, and W407-E417, and they interact with multiple CCT subunits at a time. The segments T239-254 and P261-H266 interact with Cct6p or Cct3p subunits, the segments S277-V288 and V355-P359 interact with Cct5p or Cct2p whereas the segment W407-E417 interacts with Cct2p or Cct8p subunits [82].

Though it was suggested that tubulin binding sites could have weak interactions with CCT, the residues S277-V288 are thought to be hot spot for the binding of tubulin to CCT and it might have higher affinity to CCT compared to other sites [117, 122, 126, 127, 130]. Recently, Jayasinghe et al. used a computational approach to pinpoint the interactions between gamma subunit of CCT and its stringent substrate beta-tubulin. It has been shown that the substrate binding sites in CCT are composed of helical region (HL) and helical protrusion region (HP). Interaction of substrate at helical region involves both hydrophobic and electrostatic contacts while binding to helical protrusion is stabilized by salt bridge network [131].

3.5.2. Recognition Sites in von Hippel-Lindau (VHL). The tumor suppressor protein von Hippel-Lindau (VHL) has been extensively studied from substrate point of view of CCT and has been shown to be an obligate substrate of CCT [14, 132]. VHL is a subunit of a ubiquitin ligase complex that targets cellular proteins, like HIF-1α, for proteolysis [133, 134]. Loss of function mutations in VHL is responsible for the tumor formation in kidney, adrenal glands, and central nervous system [135, 136]. VHL is composed of 213 amino acid residues of which 55-amino acid domain (100–155 residues) is necessary and sufficient for binding to CCT [132, 137]. Interestingly, most of the mutations in this domain are responsible for VHL diseases [138–140]. Using alanine-scanning mutagenesis procedure, the 55-amino acid segment has been completely analyzed to identify minimal regions responsible for CCT binding. This has revealed that two small regions of VHL, amino acid 116–119 (Box1) and 148–155 (Box2; Figure 3) are absolutely required for stable binding with CCT [118].

Furthermore, contribution of individual amino acid in these two boxes has been evaluated using single alanine substitution mutants. This analysis has shown that alanine replacement of W117 and L118 within Box1 or F148, I151, L153, or V155 within Box2 (Figure 3) substantially reduce the binding of VHL to CCT [118]. Though these two boxes are distant in the primary structure, they are located in the adjacent strands within the β sheet domain of folded VHL and the side chains of two boxes are projected in the same

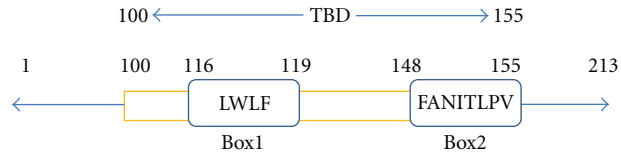

Figure 3: von Hippel-Lindau (VHL) protein containing two boxes, Box1 and Box2, required for binding to CCT. VHL is 213 amino acids long protein. Amino acid residues 100–155 constitute TRiC binding domain (TBD; this diagram was made on the basis of [118]).

direction to generate a hydrophobic surfaces required for its interaction with CCT.

3.6. Allosteric Regulations in CCT. Allosteric regulation plays an important role for transitions between different functional states among the molecular machines in response to changes in environmental conditions. As it was strongly believed that allosteric regulation of chaperonins is crucial for assisting the protein folding, the chaperonins GroEL and CCT complex were studied from allosteric point of view to understand their functioning [47, 141]. The allosteric transitions of GroEL can be described by a nested allosteric model in which each of its rings is in equilibrium between a *T* state and an *R* state. The *T* state has low affinity for ATP and high affinity for unfolded substrate proteins whereas *R* state possesses high affinity for ATP and low affinity for unfolded substrates [142–144]. It has been shown that *T* and *R* states interconvert in a concerted manner in accordance with Monod-Wyman-Changeux (MWC) model of cooperativity [145]. However, in the presence of high concentration of ATP, GroEL ring switches from *TT* state to *RR* state via *TR* state in a sequential manner in accordance with Koshland-Némethy-Filmer (KNF) model of cooperativity [146]. Though the overall structure of both GroEL and CCT is similar, CCT is different from GroEL with respect to its subunit composition and so, it was important to unveil whether intra-ring transitions are concerted or sequential for CCT. Both biochemical and genetic approaches have been adopted to understand this mechanism. Kinetic studies have shown that CCT undergoes two ATP-dependent transitions and they most likely correspond to each of its two rings. ATP-induced conformational changes have been detected by monitoring changes in fluorescence and visualized using cryo-EM and single-particle reconstructions [66, 147–149]. It has been found that the binding of ATP to CCT generates an asymmetric particle in which one ring will have slight conformational changes whereas the other ring undergoes a substantial movement in the apical and equatorial domains [66]. Using the powerful yeast genetics, it has been suggested that intra-ring conformational changes in CCT are not concerted rather it occurs in a sequential manner around the ring. This was inferred from the suppression analysis of different mutant alleles of Cct1p, Cct2p, Cct3p, and Cct6p [70]. Moreover, EM analysis has shown two important differences between GroEL and CCT. First, a lot of conformational heterogeneity has been observed in the apo state of CCT but not in GroEL. Second, ATP-induced conformational changes take place in a sequential manner in CCT whereas concerted mechanism is observed for GroEL [150]. Biochemical as well as genetic analysis data suggested that ATP-induced conformational changes in CCT take place in the order Cct1p → Cct3p → Cct2p → Cct6p [70, 150].

3.7. Cochaperones of CCT. The role of CCT has been well established for the folding of a large number of proteins. However, it was not clear in the beginning whether CCT alone is sufficient for the folding of nascent chains to their maturity or other components are also required. Later, it was found in a genetic screen during the identification of synthetic lethals for gamma-tubulin that GimC, also known as prefoldin (PFD), participates in the maturation of cytoskeletal proteins [151]. Using biochemical approach, it has been shown that prefoldin plays an important role for the formation of functional actin and tubulin by transferring unfolded protein substrates to CCT [152]. The role of prefoldin (GimC) has been established in the folding of actin using chaperone trap and suggested that prefoldin acts along with CCT for the maturation of the substrate protein [153]. Using *in vitro* transcription/translation of actin, it has been shown that unfolded actin polypeptide chain remains bound to prefoldin until it is transferred to CCT. Similar observations were made for the maturation of α- and β-tubulin as well [154]. Prefoldin is a heterohexameric protein complex that exits both in archaea and eukaryotes. However, eukaryotic prefoldin is composed of six different subunits whereas archaeal prefoldin has only two different kinds of subunits: α and β subunits. It is possible that like CCT, eukaryotic prefoldin has been developed to more complex structure from simpler archaeal form to heterohexameric structure to participate in the more complex protein folding processes. Three-dimensional reconstruction of CCT with prefoldin based on electron microscopy analysis has shown that prefoldin interacts with each of CCT rings in a unique conformation with two specific subunits that are placed in a 1,4 arrangement. Therefore, it is highly desirable that PFD : actin complex will interact with CCT through Cct4 and Cct2 subunits or Cct4 and Cct5 subunits [74]. A large body of evidence show that heterohexameric complex of prefoldin uses its jellyfish or octopus-like structure to grip nonnative protein substrates and transfer it to CCT for proper folding [74, 155, 156].

Another set of proteins implicated in the regulation of CCT function are phosducin-like proteins (PhLPs) which were originally identified as modulators of heterotrimeric G protein signaling [157]. Subsequently, they were found to play an important role in the regulation of CCT function [158–162]. PhLPs are subdivided into three families like PhLP1, PhLP2, and PhLP3 and they share structural similarities at N-terminal helical domain, a central thioredoxin-like fold, and a C-terminal extension [163]. PhLP1 has been shown to have inhibitory effect on CCT and this may be required for regulating the protein folding capacity of CCT [158]. Electron microscopy reconstruction of mammalian CCT : PhLP1 has demonstrated that PhLP1 binds to apical

domains of several chaperonin subunits [159]. Furthermore, the interaction of PhLP2 with CCT was suggested in proteome-wide studies. To substantiate this observation, *in vitro* study was done using human PhLP2A and has been shown that it does inhibit the folding of actin and forms ternary complex with CCT and actin in mammalian system [164]. However, recent study has suggested stimulatory role of PLP2 in yeast *S. cerevisiae* [165]. It has been shown that PLP2-CCT-ACT1 complexes produce 30-fold more actin than CCT-ACT1 complexes in a single ATP driven cycle. PLP2 itself can bind to actin through its C-terminal of thioredoxin fold and CCT-binding subdomain 4 of actin [165]. The inhibitory effect of human PDCL3, an orthologue of PLP2, can be relieved by exchanging the acidic C-terminus extension of that of PLP2 of yeast [165]. Therefore, it seems that higher eukaryotes have developed another level of regulatory control of CCT by phosducin-like proteins. The third member of this family, PhLP3, has been shown to bind CCT as well. The PhLP3 forms ternary complex with CCT and actin or tubulin, and does inhibit the folding process. It has been suggested that this negative impact is not due to direct competition for substrates rather by diminishing the ATPase activity of CCT by PhLP3 [162]. Moreover, *in vivo* experiments have shown that yeast PhLP3 might coordinate the proper biogenesis of actin and tubulin with prefoldin [162]. So, it is clearly established that phosducin-like proteins are responsible for regulating the function of CCT along with normal function of G protein signaling [161, 162, 164, 166]. Therefore, both prefoldin and phosducin-like proteins are working as co-chaperones to modulate the function of CCT.

In another study, it has been shown that caveolin-1 can interact with CCT and modulates its protein folding activity [167]. The caveolin-1-TCP interaction involves the first 32 amino acids of the N-terminal segment of caveolin. Phosphorylation at tyrosine residue 14 of caveolin-1 induces the detachment of caveolin-1 from CCT and activates actin folding [167]. Recently, Bag3 protein has been identified as another co-chaperone of CCT. Bag3 protein is known as co-chaperone of Hsp70/Hsc70 and involved in the regulation of various cell processes, such as apoptosis, autophagy, and cell motility. Using RNAi, it has been shown that strains lacking Bag3 activity slowed down-cell migration and also influenced the availability of correctly folded monomeric actin [168]. Altogether, it shows that CCT is highly regulated by cochaperones for its folding activity of actin and other proteins and the interaction of different co-chaperones with CCT decides the fate of the final folding process.

3.8. Phosphorylation of CCT. Recently, it has been demonstrated that p90 ribosomal S6 kinase (RSK) and p70 ribosomal S6 kinase (S6K) can phosphorylate CCT in response to tumor promoters or growth factors that activate the Ras-mitogen activated protein kinase (MAPK) pathway [169]. RSK and S6K phosphorylate Ser-260 of Cct2p (Figure 4). Furthermore, it has been shown that Cct2p plays an important role in regulating cell proliferation and especially the phosphorylation of Cct2p at Ser-260 contributes substantially to this [169]. Though Cct2p has been implicated in this process, how the phosphorylation of Cct2p modulates the function of CCT is not clear. However, there could be two implications of this phosphorylation. First, phosphorylated Cct2p subunit itself might be interacting with certain factors of Ras-MAPK and PI3K-Mtor-pathways and regulate the cell proliferation in response to multiple agonists in diverse mammalian cells. Second, the phosphorylation of Cct2p subunit of CCT might change the folding rate and reduce the stress-related unfolded proteins in the cell. On the other hand, it has been shown that a fraction of GroEL is phosphorylated at least one phosphate at each of its subunits in *E. coli* [170, 171]. This phosphorylation of GroEL subunits enhances 50–100 -fold capacity of this chaperonin to bind to denatured proteins. Possibly, the phosphorylated form of GroEL might be responsible for refolding or degradation of certain damaged polypeptides [171]. In another study, it has been shown that GroEL of *Thiobacillus ferrooxidans* is phosphorylated in response to phosphate starvation suggesting its role in sensing and regulating stress responses in bacteria [172]. It is plausible that CCT carrying phosphorylated form of Cct2p might be playing similar roles in eukaryotes.

3.9. Cooperation of CCT with Upstream Chaperones. Many newly translated proteins may interact with several different chaperones before they reach their biologically functional three-dimensional structures. One of the most abundant molecular chaperones is Hsp70 which was found to associate with CCT *in vivo* suggesting their cooperation in protein folding [59, 132]. *In vitro* experiments were performed to elucidate the cooperative nature of Hsp70 and CCT using mammalian cell-free lysates. It has been shown that short chains of actin and firefly luciferase can interact with Hsp70 whereas longer ones interact with CCT [173, 174]. Besides, the cooperation between prefoldin (GimC) and CCT were found for folding of actin and tubulin [152–154]. Using three-dimensional reconstruction of CCT : PFD based on cryoelectron microscopy, it was shown that prefoldin binds to CCT through two specific subunits [74]. Moreover, several studies have also suggested the interaction between phosducin-like proteins and CCT indicating their cooperative nature for protein folding [158, 159, 165]. The interaction of caveolin-1 with CCT also modulates the protein folding function of CCT [167]. Furthermore, it has been shown that sequential cooperation between Hsp70 and Hsp90 plays an important role for the folding of steroid hormone receptors and kinases [175, 176].

From the above-mentioned experimental evidence, it appears that cooperation between different chaperones is a central principle to the protein folding process. However, cooperation may not be required for each newly synthesized polypeptide chain. For example, it has been shown that many chaperones are recruited to the protein synthesis machinery, and as soon as the polypeptide chains are coming out, they are protected by these chaperones including CCT. It has been demonstrated that chaperones can bind to ribosome-bound polypeptide chains in both prokaryotes and eukaryotes [173, 174, 177–183]. It seems that the cellular proteins follow different chaperone-dependent and chaperone-independent

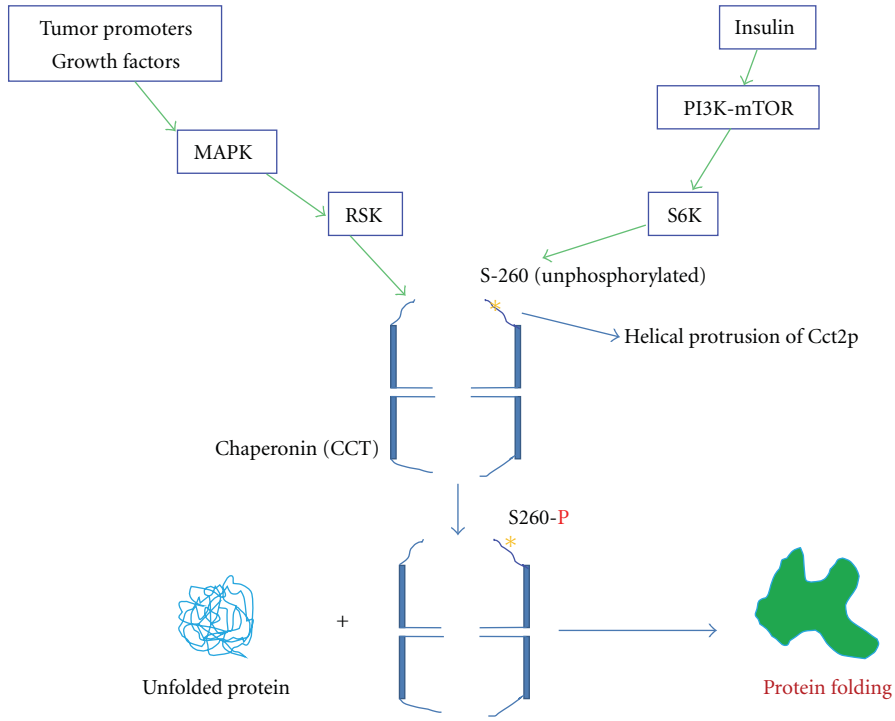

Figure 4: Schematic of Cct2p phosphorylation by p90 ribosomal S6 kinase (RSK) and p70 ribosomal S6 kinase (S6K) and the function of CCT containing phosphorylated Cct2p. *Indicates the S-260 position in the helical protrusion of Cct2p (this schematic is made based on the [169]).

pathways for reaching their biologically functional three-dimensional structures. The proteins in the chaperone-independent pathway will probably be small proteins and they can fold without any help from any chaperones. The chaperone-dependent folding process might follow three different strategies depending upon the nature of the proteins. First, newly synthesized proteins will be bound by Hsp70, Hsp90, or other small chaperones transiently at the exposed hydrophobic regions present on the surface of nonnative proteins and will be protected from aggregation and will fold. Second, some proteins will be bound by Hsp70 and Hsp90 sequentially and will proceed for folding. Third, a small fraction of newly synthesized polypeptide chains will bind sequentially to Hsp70, Hsp90, and prefoldin/phosducin-like proteins and then transferred to CCT for final folding.

3.10. Protein Misfolding Diseases and CCT. The concerted and cooperative action of a large number of molecular chaperones leads to the production of three-dimensional and biologically functional protein molecules. The failure in this process may result in the aggregation and misfolding of many essential proteins and may cause a severe effect on the overall functions in a cell. Now it has been well recognized that protein aggregation and misfolding are the root causes for many diseases known as "protein misfolding" or "protein conformational" diseases [184]. The toxic effect of the aggregated or milfolded protein could be because of gain-of-function which will have titrating effect on interacting proteins or due to the loss of function of misfolded proteins [185, 186]. Aggregation process is a multistep process having stable or metastable intermediates that lead to the formation of homotypic fibrillar aggregates that may interact with other proteins and result in the formation of inclusion bodies or plaques that deposit outside or inside the cells [187–189]. A large body of evidence shows that the intermediates of the aggregate formation process are responsible for disease pathogenesis rather than the final products which may be inactive or protective [190, 191]. For example, increased level of diffuse polyglutamine- (polyQ-) expanded huntingtin is thought to be cause of cell death in Huntington's disease and inclusion bodies could enhance the survival [192]. Similarly, proteinaceous deposits or inclusion bodies are found to be not associated with toxicity in Parkinson's and Alzheimer's diseases [192–194].

Though chaperonins are generally responsible for maintaining the cellular protein homeostasis, they are now implicated in the pathogenesis of misfolding human diseases as well. Both the group I and group II chaperonins are found to be participating as modulators of misfolding diseases. For example, inactivation of mitochondrial Hsp60 is responsible for hereditary spastic paraplegia, a late-onset neurodegenerative disease [195, 196]. Recent studies have clearly shown that polyQ-expanded huntingtin is a potential substrate of CCT [197–199]. Moreover, an RNA interference screen in *C. elgans* has identified six out of eight subunits of CCT as suppressors of polyQ aggregation, suggesting that CCT can bind polyQ and inhibit the formation of toxic aggregates [200]. It has been shown that

overexpression of Cct1p of CCT was effective at inhibiting huntingtin aggregation and subsequently increased the viability [198]. On the other hand, the deletion of Cct6p of CCT increases the huntingtin aggregation and toxicity [52, 199]. Generally, the CCT substrates are large, hydrophobic proteins containing the regions with β-strand propensity, and they are highly prone to the formation of toxic aggregates [63, 117, 118, 201]. Aggregation of these proteins may begin with conformational transition from native monomer to mature amyloid fibrils [197, 202, 203]. Therefore, it is quite possible that CCT binds directly to β-sheets and protects the protein from being aggregated which can be otherwise toxic for the cell. It has been shown that overexpression of certain subunits of CCT can protect from misfolding diseases. Therefore, drug-mediated induction of molecular chaperones can be considered as one of the methods for treating these diseases [50]. Otherwise, certain CCT subunits can be injected di-rectly on regular basis like insulin in case of diabetic patients and misfolding diseases can be handled.

4. Conclusion

Molecular chaperones are crucial for the production of biologically functional three-dimensional protein structures. A large number of molecular chaperones are present in all the three kingdoms of life implying their importance in biological system. Chaperonins are cylindrical structures having central cavity for encapsulating unfolded protein substrates and assist in protein folding in an ATP-dependent manner. The chaperonin CCT is composed of eight different but related subunits of differential functional hierarchy in which catalytic cooperativity of ATP binding/hydrolysis takes place in a sequential manner rather than concerted cooperativity as found in GroEL. Moreover, substrate recognition in CCT takes place through diverse mechanisms involving hydrophobic and electrostatic interactions. For the fine-tuning of protein process, many cochaperones like prefoldin, phosducin-like proteins act as upstream factors and transfer the substrate to CCT. These upstream molecular chaperones and chaperonins might be responsible for generating a protective chaperone cage for the newly synthesized polypeptide chains to minimize the chance of aggregation and misfolding. Recently, it has been shown that certain CCT subunits are phosphorylated in response to tumor promoters or growth factors suggesting the possible roles of different kinases and possible certain phosphatases in regulating the activity of CCT. Any abnormal function posed by any chaperone at any stage of protein folding might have severe consequences. Many mutations in the molecular chaperones are now linked to Parkinson's and Alzheimer's diseases. Better understanding of chaperonin CCT and other molecular chaperones will be helpful to develop drugs for the treatment of misfolding or conformational diseases.

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