Research Article

***In Silico* Characterization and Homology Modeling of a Cyanobacterial Phosphoenolpyruvate Carboxykinase Enzyme**

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ATP-dependent phosphoenolpyruvate carboxykinase (PEPCK) is a key catabolic enzyme found in various species of bacteria, plants, and yeast. PEPCK may play a role in carbon fixation in aquatic ecosystems consisting of photosynthetic cyanobacteria. RuBisCO-based CO₂ fixation is prevalent in cyanobacteria through C₃ intermediates; however, a significant amount of carbon flows into C₄ acids during cyanobacterial photosynthesis. This indicates that a C₄ mechanism for inorganic carbon fixation is prevalent in cyanobacteria with PEPCK as an important β -carboxylation enzyme. Newly available genomic information has confirmed the existence of putative *PEPCK* genes in a number of cyanobacterial species. This project represents the first structural and physicochemical study of cyanobacterial PEPCKs. Biocomputational analyses of cyanobacterial PEPCKs were performed and a homology model of *Cyanotheca* sp. PCC 7424 PEPCK was generated. The modeled enzyme consists of an N-terminal and C-terminal domains with a mixed α/β topology with the active site located in a deep cleft between the two domains. Active site residues and those involved in metal ion coordination were found to be conserved in the cyanobacterial enzymes. An active site lid which is known to close upon substrate binding was also predicted. Amino acid stretches that are unique to cyanobacterial PEPCKs were also identified.

1. Introduction

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.32) catalyzes the reversible ATP- or GTP-dependent decarboxylation of oxaloacetate (OAA) to yield phosphoenolpyruvate (PEP). This reaction uses the phosphate group from the nucleotide triphosphate and, as a result, produces CO₂ and the corresponding nucleoside diphosphate. PEPCK has a strict requirement for divalent cations with Mn²⁺ as its best activator [1]. Two classes of PEPCKs exist in nature, and they are classified in the basis of the nucleotide substrate: ATP-utilizing enzymes are found in bacteria, plants, and yeast, while GTP-dependent PEPCKs are found mostly in higher eukaryotes [2]. GTP-dependent PEPCKs also occur in some bacteria such as *Corynebacterium glutamicum* [3]. While there is no significant sequence identity between the two classes, a number of residues are completely conserved across all PEPCKs in the regions of the enzyme that are necessary for nucleotide binding and metal ion coordination [1]. The

crystal structures of PEPCKs from representative species of plants, bacteria, and mammals have been published, and conservation in metal and substrate binding were confirmed [4].

In mammals, two forms of PEPCKs exist: PEPCK-M, which is expressed in mitochondria, and PEPCK-C, the cytosolic form of the enzyme. Mammalian PEPCK plays a major role in gluconeogenesis and glyceroneogenesis. The enzyme is also a contributor to other downstream processes [7]. The significance of the PEPCK reaction has been studied in *Streptococcus bovis*, *Selenomonas ruminantium*, and other bacteria where PEPCK was found to be involved in growth initiation and amino acid synthesis [8]. *E. coli* overexpressing PEPCK exhibited slower growth rate and increased ATP production [9]. PEPCK has also been investigated as the sole anaplerotic enzyme in the yeast *Saccharomyces cerevisiae* [10]. In plants, where PEPCK isoforms are expressed in various tissues, regulation of the enzyme by phosphorylation is dependent upon location and illumination [11]. The same is true for CAM plants such as *Ananas comosus* [12].

Cya8801	16	LKIAPHLSQGLDYPKVHPSSVTTSDREDGIKF	PTYGLE	TLGIHN	SVVYRNL	PV	AEL	VEH
Cyaccy0110	16	QPNLSLSSQ----	PSYSGNTTLEKDSEEAIR	PTYGLE	DLGLYN	IGRVYRNL	PIAQL	VEH
Cya7424	16	TLPGQSKTNHLAGCSEAITPPNYLVENDTFHQ	PTYGLE	SLGMKN	LGRVFHNL	SV	PQL	VEQ
Thermus	1	-----	MQRLEAL	GTHPKRVF	WNTVSPV	L	VEH	
A. succi	1	-----	MSLS----	ESLAKYGITGATN	IVHNPS	SHEEL	F	AA
E. coli	1	-----	MRVNGLTPQEL	EAYGISD	VHD	IVY	NPSYD	LLYQE
A. thal1	106	KVVRGDPAEK--	KT	DGSTTPAYAHGQHSIFSPATGAVSD	SSLKF	THV	LYNL	SPAEL
A. thal2	113	KLIRGDP-----	TSAAKVAHVPTPTSLPAADVSD	SLKFT	THLHNL	SPAEL	YEQ	
Z. mays	100	KVVKGDPAAKGEAAAQ	GAPSTPRAHQQRHPA	APAI	AVSD	SSLKF	THV	LYNL
T. cruzi	1	-----	MPPTIHR	N	L	SPEL	V	LQW
H. sapiens	43	IHICDGSEENGRL	LGMEEGILRLK--	KYDNCW	LALTD	PRD	V	ARIESKTV
Rattus	1	-----	KYDNCW	LALTD	PRD	V	ARIESKTV	ITQEQR
Gallus	1	-----	GKELLRGLQDD	GVHLPLP--	KYDNCW	LARTD	PRD	V
C. glut	36	VVFVDGSQAEWDRMAED	LVEAGTLIKLNEEKRPNSY	LARSNPSD	V	ARVESRTF	I	CSEKEE

Cya8801	121	HHNVAISERQFEQ	LYQRVL	SV	QGR-ELYIF	DG--	YVGAD	RNYRHGVRVINE	L	ASQNL	FV
Cyaccy0110	117	QHNVPISEAKFNQ	YQKVIA	QGR-DIYIF	DG--	YIGAD	TNYRCGVRI	NE	L	ASQNL	FV
Cya7424	121	KDNVPISEAKFEQ	YRKVIT	VQGR-DLYIF	DG--	YVGAD	PKYRF	GVRI	NE	L	AYQNL
Thermus	73	EVNQPFAPAE	FEALYQRVVQ	YLSER-DLYVQDL	--	YAGAD	RRYRLAVRV	VT	SP	UHAL	F
A. succi	85	NDNKPVTEA	QAQLKALAGKEL	SNK-PLYVVDL	--	FCGAN	ENTRLKIR	FVME	VAUQ	AHFV	
E. coli	92	NDNKPLSPET	QAHLKGLVTRQ	LSGK-RLFVVD	A--	FCGAN	PDTRL	SVR	FITE	VAUQ	AHFV
A. thal1	212	SPNIEMDEHT	FVNRRERAVD	YLN	SLDKVFVNDQ--	YLNWD	PENRIK	VRI	SARAY	HSLFM	
A. thal2	211	SPNIEMDEKTF	LVNRRERAVD	YLN	SLDKVFVNDQ--	YLNWD	PENRIK	VRI	SARAY	HSLFM	
Z. mays	208	SPNIEMDEKTF	LINRRERAVD	YLN	SLDKVFVNDQ--	YLNWD	PENRIK	VRI	SARAY	HSLFM	
T. cruzi	65	-VNMKLSEES	FARVRKIAKEFL	D	TREH	LFVVD	C--	FAGH	D	ERYRLKVRV	FTTRPYHALFM
H. sapiens	155	KIGIELTDS	SPYVVASMRIMTR	M	GTPVLEA	LDG	-EFVK	CLHSVGCPL	PLQKPL	VNNWPCN	
Rattus	85	KIGIELTDS	SPYVVASMRIMTR	M	GTSVLEA	LDG	-EFIK	CLHSVGCPL	PLKPL	VNNWACN	
Gallus	104	KLGVQVTD	SPYVLSMRIMTR	V	GPAVLQR	DD--	DFVR	CLHSVGRPL	PLTEP	VSSWPCD	
C. glut	142	KLGVQVTD	SEYVVM	SMRIMTR	M	GIEALDK	T	GANGS	FVR	CLHSVGA	LEPGQDVA-WPCN

Cya8801	178	HQLFLRP----	SPEEL	ANH-QADFT	VIAVPG	HGDPEID	-GLR	SEAFIV	HL	TKRL	VIIIG
Cyaccy0110	174	HQLFIRP----	SPEEL	NNH-QADFT	VIAIPG	HGDPEND	-DIR	SEAFIV	HL	TKRL	VIIIG
Cya7424	178	HQLFLRP----	SPMELISH	-KADFT	VICVPG	HGDPIDD	-GIN	SEAFIV	HL	TKRL	VIIIG
Thermus	130	RNMFILPRRG	NDDEVEAF	-VPGFT	VVHAPYFQ	AVPERD	-GTR	SEVFV	GT	RRLL	VIIIV
A. succi	142	TNMFIRP----	TEEL	KGF-EPDF	VVLNASKAK	VNFKEL	GLN	SETAV	VFN	AEKMQ	IIL
E. coli	149	KNMFIRP----	SDEEL	AGF-KPDF	IVMNGAKCTNP	QWKEQ	GLN	SENFV	AFNL	TERMQ	LIG
A. thal1	270	HNMCI	RP----	TQEEL	ESFGTPDFTI	YNAGQFPCNRYTH	-YMTS	STSVD	N	LARRE	MVIL
A. thal2	269	HNMCI	RP----	TQEEL	ENFGTPDFTI	YNAGQFPCNRYTH	-YMTS	STSVD	N	LARRE	MVIL
Z. mays	265	HNMCI	RP----	TQEEL	EDFGTPDFTI	YNAGQFPCNRYTH	-YMTS	STSVD	N	LARRE	MVIL
T. cruzi	122	RDMLIVP----	TPEEL	ATFGE	PDYVIYNAGECK	ADPSIP	-GLT	STTCVA	N	FKTR	EQVIL
H. sapiens	214	PELT	LIAHLP--DRRE	ISF----	GSGYG	GNSLLGKKCFAL	RNASRLAKEEGW	LAEHML	LIL		
Rattus	144	PELT	LIAHLP--DRRE	ISF----	GSGYG	GNSLLGKKCFAL	RNASRLAKEEGW	LAEHML	LIL		
Gallus	162	RSRVLVAHIP	-SERRIVS	F----	GSGYG	GNSLLGKKCFAL	ATASRLAKEEGW	LAEHML	LIL		
C. glut	201	-DTKYITQFP	-ETKEI	WSY----	GSGYG	GNA	LAKKCYALR	ASVMAREEGW	LAEHML	LIL	

Cya8801	232	GSRYSGEMKKS	SV	SM	N	VIA	E	V	P	CA	---	IDKRA	-HTTL	GLSGTGKT	A
Cyaccy0110	228	GSRYSGEIKKS	SV	LMMNY	F	MTKRN	VLP	PMHGAAN	---	IDKRG	-HTNL	GLSGTGKT	A		
Cya7424	232	GSRYSGEIKKS	SV	FMMNY	L	MTKQ	N	VLP	PMHGAAN	---	IDKKS	-HTALFF	GLSGTGKT	TL	SA
Thermus	188	GTQYAGEIKKS	I	FTVM	N	YLM	PKRG	VFP	PMHASAN	---	VGKEG	-D	VAVFFGLSGTGKT	TL	ST
A. succi	197	NTWYGGEMKK	G	MFS	M	NFY	L	PLQ	GIAAMHCSAN	---	TDLEG	KNTA	IFFGLSGTGKT	TL	ST
E. coli	204	GTWYGGEMKK	G	MFS	M	NYL	L	PLK	GIAAMHCSAN	---	VGKEG	-D	VAVFFGLSGTGKT	TL	ST
A. thal1	325	GTQYAGEMKK	G	LFS	V	MHY	L	MPK	RILSLHSGCN	---	MGKDG	-D	VALFFGLSGTGKT	TL	ST
A. thal2	324	GTQYAGEMKK	G	LFG	V	MHY	L	MPK	RILSLHSGCN	---	MGKDG	-D	VALFFGLSGTGKT	TL	ST
Z. mays	320	GTQYAGEMKK	G	LFG	V	MHY	L	MPK	RILSLHSGCN	---	MGKDG	-D	VALFFGLSGTGKT	TL	ST
T. cruzi	177	GTEYAGEMKK	G	ILT	V	MFEL	MPQ	M	NHLCMHASAN	---	VGKQG	-D	VTVFFGLSGTGKT	TL	SA
H. sapiens	269	GITNPEGE	KKYL	AAAF	PSACGK	SNL	AM	MNP	LPGWK	VECV	GDD	I	AWMKFDAQGHL	RAINP	
Rattus	199	GITNPEGE	KKYL	AAAF	PSACGK	SNL	AM	MNP	LPGWK	VECV	GDD	I	AWMKFDAQGHL	RAINP	
Gallus	217	GVTSPSGE	KRYM	AAAF	PSACGK	TNL	AM	MTP	LPGWRI	HCV	GDD	I	AWMKFDDRGR	LRAINP	
C. glut	255	KLINPEGK	AYHI	AAAF	PSACGK	TNL	AM	ITP	IPGWT	AQVV	GDD	I	AWLKLRE	DG-LYAVNP	

FIGURE 1: Continued.

FIGURE 1: Multiple sequence alignment of selected regions of ATP- and GTP-dependent PEPCKs. The yellow block highlights are amino acid stretches that are conserved and exclusive to cyanobacterial PEPCKs; the amino acids in the blue blocks make up the pyruvate binding site; the kinase-1a and kinase 2 motifs are highlighted by the red and pink boxes, respectively; the active site lid amino acids are shaded in green; the adenine binding site is highlighted in olive green. The alignment was generated with Clustal W [5].

PEPCK is also prevalent in marine ecosystems where a significant amount of CO_2 fixation takes place. The enzyme is involved in light-dependent carbon fixation, and its activity varies with changes in water temperature and nitrogen concentration [13]. Furthermore, PEPCK contributes to photosynthetic carbon fixation as an addition to the C_3 pathway catalyzed by RuBisCO [14]. The same may be said of marine cyanobacteria where β -carboxylation reactions involving

phosphoenolpyruvate carboxylase (PEPC) and PEPCK are prevalent [15].

Cyanobacteria are unable to derive metabolic energy from the Krebs cycle since they lack α -ketoglutarate dehydrogenase and NADH oxidase. Furthermore, C_4 acid concentration fluctuates in cyanobacteria in response to light-dark cycles. This indicates that C_4 mechanisms for inorganic carbon fixation involving PEPC and PEPCK are widespread

TABLE 1: Phosphoenolpyruvate carboxylase sequences retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/>).

Organism	Accession number	Nucleotide specificity
<i>Cyanothece</i> sp. PCC 8802	ACV02875.1	ATP
<i>Cyanothece</i> sp. PCC 7424	ACK71186.1	ATP
<i>Cyanothece</i> sp. PCC 8801	ACK67979.1	ATP
<i>Cyanothece</i> sp. ATCC 51142	ACB49859.1	ATP
<i>Microcystis aeruginosa</i> NIES-843	BAG00438.1	ATP
<i>Cyanothece</i> sp. CCY0110	EAZ91985.1	ATP
<i>Cyanothece</i> sp. PCC 7822	ADN17293.1	ATP
<i>Microcoleus chthonoplastes</i> PCC 7420	EDX72609.1	ATP
<i>Microcystis aeruginosa</i> PCC 7806	CAO87080	ATP
<i>Arthrospira platensis</i> str. Paraca	ZP-06380836	ATP
<i>Trypanosoma cruzi</i>	AAA50780.1	ATP
<i>E. coli</i> K12	BAE77888.1	ATP
<i>A. succiniciproducens</i>	AAC45394.1	ATP
<i>C. glutamicum</i>	ABB53264.1	GTP
<i>Homo sapiens</i>	AAH23978.1	GTP
<i>Rattus norvegicus</i>	AAH81900.1	GTP
<i>Gallus gallus</i>	NP-990802	GTP
<i>Thermus thermophilus</i> HB8	YP-143544.1	ATP

in those organisms [16]. In marine ecosystems, PEPCK may be involved in light-independent carbon fixation (LICF) in diatoms [17].

Despite their potential importance in net CO₂ fixation, PEPC and PEPCK from cyanobacteria have not been extensively studied. A model of PEPC from a marine cyanobacterium is available, but no such model exists for cyanobacterial PEPCK [18]. In this work, a homology model for a cyanobacterial PEPCK is presented for the first time. Furthermore, the enzymes' physicochemical properties were characterized *in silico*.

2. Materials and Methodology

2.1. PEPCK Protein sequences. Phosphoenolpyruvate carboxykinase (PEPCK) protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) [<http://www.ncbi.nlm.nih.gov/>]. The search of the protein database yielded 21 cyanobacterial sequences from which 10 cyanobacterial enzymes were selected after redundant sequences were excluded. Bacterial, mammalian, and plant sequences were also obtained from the NCBI protein database (Table 1). The sequences were converted to FASTA format using the ReadSeq sequence conversion server [19].

2.2. Sequence Alignments. Multiple sequence alignments were performed with Clustal W [5]. The Clustal W alignment file was imported into the BoxShade sequence alignment editor. Identical and similar amino acids were shaded or

colored. Phylogenetic analysis of protein sequences was generated using the alignment obtained with Clustal W.

2.3. Structural Analysis. The amino acid composition of the cyanobacterial PEPCK sequences was computed using the PEPSTATS analysis tool [24]. The physicochemical parameters such as the molecular weight, isoelectric point (pI), extinction coefficient, half-life, aliphatic index, amino acid property, instability index, and grand average hydropathy (GRAVY) were calculated using the ProtParam tool of the ExPASy proteomics server. Secondary structure elements prediction was performed using the Network Protein Sequence Analysis (NPS@) server and the Secondary Structural Content Prediction (SSCP) server [6, 25]. The consensus secondary structure content and predicted disulfide patterns of each cyanobacterial PEPCK are tabulated in Table 3. The presence of disulfide bridges was analyzed using the CYS-REC tool which predicts the most probable bonding patterns between available cysteine residues (<http://linux1.softberry.com/berry.phtml>). The 3D models of cyanobacterial PEPCKs were constructed using the protein structure homology model building program SWISS-MODEL with energy minimization parameters [21]. The modeled tertiary structures were built on the basis of sequence identity with the high-resolution crystal structures of the enzyme from *Thermus thermophilus*. The Swiss PDB viewer [26] was used to visualize and refine the models, and PyMOL (Schrödinger Inc.) was used to generate publishable images of the PEPCK models. The modeled 3D structures were evaluated and validated with the WHAT IF and RAMPAGE programs [22, 27].

2.4. Molecular Docking. The molecular operating environment (MOE) program was used to calculate the docking energies between oxaloacetate (OAA) and the active site of *Cyanothece* sp. PCC 7424 PEPCK. OAA was drawn using the MOE software [23]. The PDB file of the modeled *Cyanothece* sp. PCC 7424 PEPCK was imported into MOE, and a binding pocket in the area between the C-terminal and N-terminal domains was selected as the target area in a 3D docking box. OAA was imported to the protein's 3D box, and the docking program was initiated to search for favourable binding configurations between OAA and *Cyanothece* sp. PCC 7424 PEPCK. MOE's docking program flexes the substrate and attempts to fit it into the active site using an energy minimization algorithm. Docking studies were carried out at the Laboratory of Molecular Computations and Bioinformatics at Howard University, Washington, DC.

3. Results and Discussion

The primary structure analysis of cyanobacterial phosphoenolpyruvate carboxykinase enzymes showed that the most abundant amino acid is leucine which accounts for 9% of the enzyme's primary structure. The least common amino acids were tryptophan and cysteine. Cysteine residues are much more prevalent in GTP-dependent PEPCKs as such a greater number of disulfide bridges were predicted for

TABLE 2: Parameters of cyanobacterial PEPCKs calculated using the ProtParam program: molecular weight (MW) (g/mol); isoelectric point (pI); extinction coefficient (EC) ($M^{-1} \text{ cm}^{-1}$); instability index (Ii); aliphatic index (Ai); grand average hydropathy (GRAVY); number of negative residues (−R); number of positive residues (+R) [6].

Organism	Seq length	MW	pI	EC	Ii	Ai	GRAVY	−R	+R
<i>Cyanothece</i> sp. PCC 8802	571	63940.3	6.07	72770	33.35	84.36	−0.309	63	50
<i>Cyanothece</i> sp. PCC 7424	572	63646.3	6.06	58790	34.49	85.59	−0.228	64	53
<i>Cyanothece</i> sp. PCC 8801	571	63966.3	6.07	72770	33.83	85.04	−0.299	63	50
<i>Cyanothece</i> sp. ATCC 51142	569	63388.7	5.96	60280	28.65	84.01	−0.262	62	50
<i>Microcystis aeruginosa</i> NIES-843	540	60169.2	5.60	65780	29.75	85.07	−0.226	63	47
<i>Cyanothece</i> sp. CCY0110	568	63649.1	6.09	60780	33.59	84.98	−0.283	62	51
<i>Cyanothece</i> sp. PCC 7822	612	67961.3	6.42	58790	37.70	84.30	−0.260	65	58
<i>Microcoleus chthonoplastes</i> PCC 7420	555	61947.4	5.82	61770	32.67	83.44	−0.268	63	51
<i>Microcystis aeruginosa</i> PCC 7806	540	60123.2	5.53	65780	30.22	85.80	−0.223	64	47
<i>Arthrospira platensis</i> str. Paraca	573	63546.8	5.45	67270	33.70	87.09	−0.240	73	56
<i>Trypanosoma cruzi</i>	472	52585.2	8.20	55350	41.65	77.50	−0.254	56	59
<i>E. coli</i> K12	540	59643.4	5.46	74830	21.19	78.24	−0.315	68	57
<i>A succiniciproducens</i>	532	58642.8	5.77	68870	21.81	79.96	−0.233	67	61
<i>C. glutamicum</i>	610	66874.3	4.64	128340	36.86	75.74	−0.295	89	51
<i>Homo sapiens</i>	622	69177.4	5.80	104390	44.95	80.48	−0.282	77	66
<i>Rattus norvegicus</i>	622	69415.7	6.09	108860	44.43	79.53	−0.308	78	71
<i>Gallus gallus</i>	640	70970.1	8.16	122380	53.61	72.09	−0.329	71	74
<i>Thermus thermophilus</i> HB8	529	59314.4	6.27	78270	39.59	79.96	−0.282	65	60

TABLE 3: Predicted consensus secondary structure content and predicted disulfide patterns of cyanobacterial PEPCKs. The data was generated from the Protein Sequence Analysis server and CYS REC (<http://linux1.softberry.com/berry.phtml>).

Organism	α -helix	β -sheet	Coil	Disulfide bridge prediction
<i>Cyanothece</i> sp. PCC 8802	23.64	16.99	59.37	291–440
<i>Cyanothece</i> sp. PCC 7424	26.05	16.26	57.69	None
<i>Cyanothece</i> sp. PCC 8801	23.64	17.51	58.84	291–440
<i>Cyanothece</i> sp. ATCC 51142	23.55	20.21	56.24	288–437, 310–370
<i>Microcystis aeruginosa</i> NIES-843	26.30	16.85	56.85	None
<i>Cyanothece</i> sp. CCY0110	23.77	18.66	57.57	287–369, 370–432
<i>Cyanothece</i> sp. PCC 7822	25.82	15.20	58.99	None
<i>Microcoleus chthonoplastes</i> PCC 7420	24.68	17.30	58.02	None
<i>Microcystis aeruginosa</i> PCC 7806	25.74	15.56	58.70	None
<i>Arthrospira platensis</i> str. Paraca	23.56	17.98	58.46	None
<i>Trypanosoma cruzi</i>	22.67	22.25	55.08	8–9
<i>E. coli</i> K12	25.19	18.70	56.11	None
<i>A succiniciproducens</i>	25.56	18.23	56.20	None
<i>C. glutamicum</i>	31.31	15.57	53.11	90–199
<i>Homo sapiens</i>	26.21	16.24	57.56	192–245, 212–413, and 399–407
<i>Rattus norvegicus</i>	27.17	18.01	54.82	192–399, 245–307, and 407–413
<i>Gallus gallus</i>	27.50	17.81	54.69	11–93, 325–425, and 417–431
<i>Thermus thermophilus</i> HB8	25.90	19.47	54.63	None

GTP-dependent enzymes (Table 3). The computed isoelectric point (pI) of cyanobacterial PEPCKs was 6.03 on average; this indicates that the enzyme is likely to precipitate in acidic buffers (Table 2). The extinction coefficient (EC) of cyanobacterial and prokaryotic PEPCKs was lower than that of GTP-dependent enzymes. The instability indices (Ii) of bacterial PEPCKs were below 40, indicating that they would be stable in solution. The ProtParam tool calculated

instability indices above 40 for selected eukaryotic PEPCKs [6]. All PEPCKs have negative GRAVY scores attesting to their solubility in hydrophilic solvents. The aliphatic index (Ai) which evaluates the relative volume occupied by the side chains of hydrophobic amino acids was generally higher in bacterial PEPCKs. A high aliphatic index indicates that a protein may remain stable over a wide range of temperatures.

FIGURE 2: Cladogram of selected PEPCKs based on amino acid sequences of ATP- and GTP-dependent enzymes.

A multiple sequence alignment of selected ATP- and GTP-dependent PEPCKs was performed (Figure 1). While sequence identity was insignificant between the two types, domains involved in catalysis and metal ion coordination were conserved between ATP- and GTP-dependent PEPCKs. A phylogenetic tree was constructed from the alignment (Figure 2). The cladogram illustrated the high degree of divergence between ATP- and GTP-utilizing enzymes. The multiple sequence alignment also revealed amino acid sequences that are only found in cyanobacterial enzymes and do not occur in PEPCKs from eukaryotes and other prokaryotic organisms (Figure 1).

The predicted secondary structure composition of *Cyanosethece* sp. PCC 7424 PEPCK was determined using the NPS@ server which generates a consensus report from twelve secondary structure prediction methods [6]. All PEPCKs shared similar alpha helical and β -sheet content (Table 3). A more detailed analysis of the secondary structural elements of *Cyanosethece* sp. PCC 7424 PEPCK was performed using the PDBsum tool [20]. The secondary structure prediction server revealed that 26.05% of amino acids resided in α -helices, while 16.26% of residues were in β -sheets. The rest of the amino acids were found in other conformations such as β -hairpins and β -turns. The topology of *Cyanosethece* sp. PCC 7424 PEPCK is illustrated in Figure 3(b). The overall fold of the enzyme is similar to other PEPCKs for which crystal structures are available. The protein's main chain consists of a C-terminal domain and an N-terminal domain that are both folded into a mixed α/β topology.

The predicted secondary structures generated by the PDBsum tool were generally in agreement with the

three-dimensional structure of *Cyanosethece* sp. PCC 7424 PEPCK (Figure 3(a)). However, while the enzyme's computed schematic diagram illustrated the existence of α -helices and β -sheets in the N-terminal between residues 50 and 88, no such structures exist in the corresponding region of the 3D model. This may be due to the fact that PEPCKs across species align poorly in this particular area of the N-terminal domain. According to the schematic diagram, the active site lid ⁴¹⁷SKLAGTERGITAP⁴²⁸ is located between two β -strands. This was accurately represented in the 3D structure. The same can be said for the locations of the kinase-1a and kinase 2 motifs as well as the ATP-binding site.

The homology model of *Cyanosethece* sp. PCC 7424 PEPCK was generated with the SWISS-MODEL software using *Thermus thermophilus* PEPCK as a template (Figure 4). The overall fold of the *Cyanosethece* sp. PCC 7424 enzyme is similar to that of *Thermus thermophilus* PEPCK as expected from a sequence identity score of 51% between the two proteins. According to the Ramachandran plot generated with the RAMPAGE server, 96.2% of residues are found in the most favoured region, while 2.7% of amino acids reside in the generously allowed region (Figure 5). Furthermore, the model's calculated QMEAN4 score of 0.771 long with a Z score 0.3 confirmed its reliability [28]. The protein is organized into a C-terminal domain and an N-terminal domain with mixed α/β content. The active site is located in a deep cleft between the two domains.

Based on what is known of ATP-dependent PEPCKs the purported active site residues of *Cyanosethece* sp. PCC 7424 PEPCK are R100, K240, K241, H260, K282, D297, K316, and R362. In the well-characterized *E. coli* enzyme, the lysine

FIGURE 3: Schematic and topology diagrams showing the secondary structural elements in the *Cyanothece* sp. PCC 7424 PEPCK. (a) α -helices are labeled with the letter “H”, and β -strands are lettered in uppercase. β , γ , and hairpin turns are also labeled. (b) Helices are represented as cylinders and β -strands as arrows. The secondary motif map and topology diagram were calculated using the PDBsum tool [20].

FIGURE 4: Predicted 3D structure of the *Cyanothecae* sp. PCC 7424 PEPCK. The models were generated with SWISS-MODEL using PDB template 1xkvB. PyMOL was used to visualize the model. The active side lid is shown as a purple loop in the C-terminal domain [21].

FIGURE 5: The Ramachandran plot for the modeled *Cyanothecae* sp. PCC 7424 PEPCK. 96.2% of residues are found in the most favoured regions, 2.7% of residues are in the most allowed regions, and 1.1% of residues are found in the outlier regions. The plot was generated with the RAMPAGE program [22].

residues homologous to K240 and K241 are involved in OAA and PEP binding [28]. Furthermore, R100 along with R362 are thought to be involved in pyruvate binding; additionally, R100 and Y235 form H-bonds with CO₂ [29]. The residues form a positively charged active site that is well suited for OAA and PEP binding (Figure 6). Metal ion coordination is also

FIGURE 6: The electrostatic surface representation of the active site of *Cyanothecae* sp. PCC 7424 PEPCK generated by MOE [23]. The surface residues are color coded based on their nature: the blue coloration indicates that the enzyme's substrate-binding pocket is positively charged (MOE; Chemical Computing Group, Inc.).

an important aspect of the catalytic mechanism of PEPCK. In *Cyanothecae* sp. PCC 7424 PEPCK, active site residues H260, D297, and K316 are most likely involved in Mn²⁺ or Mg²⁺ binding. Furthermore the cyanobacterial protein features the sequence ³⁰⁶IFNFEGCYAK³¹⁵ which forms the Ca²⁺-binding domain in ATP-dependent PEPCKs [30].

Cyanobacterial PEPCKs also feature the nucleotide-binding motif kinase-1a which consensus sequence is GXXGXGKT [31]. This motif, which forms the phosphate-binding loop, is common in ATPase enzymes. Another motif found in cyanobacterial PEPCKs is kinase 2 with the consensus sequence XXXXD, where X is a hydrophobic amino acid. This motif is often present in nucleotide-binding proteins. In the C-terminal domain of *Cyanothecae* sp. PCC 7424 PEPCK, the kinase-1a motif is ²⁷⁶GLSGTGKT²⁸³, and the kinase 2 motif is ²⁹³LIGDD²⁹⁷. In *E. coli* PEPCK, D296 and D297 are involved in Mn²⁺ binding. The N-terminal domain of *Cyanothecae* sp. PCC 7424 PEPCK includes two additional motifs that appear to be exclusive to cyanobacterial enzymes: ⁴⁸PTYGLE⁵³ and ²⁰³GLHGDPE/I²⁰⁹. The adenine-binding motif, ⁴⁸²RIPIKHT⁴⁸⁸, is found in the C-terminal of *Cyanothecae* sp. PCC 7424 PEPCK. H487 is found in all cyanobacterial sequences, while the equivalent amino acid is found as either a valine or aspartate residue in other ATP-dependent PEPCKs (Figure 1).

The sequence ⁴¹⁷SKLAGTERGITAP⁴²⁸ makes up the active site lid which is prevalent in ATP-utilizing PEPCKs [32]. This loop stands above the active site cleft and prevents water molecules from interfering with the substrate (Figure 4). The Ω-loop is also present in GTP-dependent enzymes, but it has a different location and sequence. The 3D model of *Cyanothecae* sp. PCC 7424 PEPCK generated with the SWISS-MODEL software was that of the unliganded, lid open conformation of the enzyme. Additional models of the protein were built with the Phyre2 and ESyPred3D automated homology modeling programs using default parameters [33, 34]. The generated 3D structures depicted the enzyme in the

FIGURE 7: (a) Predicted 3D structure of liganded *Cyanothece* sp. PCC 7424 PEPCK. The binding pocket is shown in red with the bound OAA substrate in green. (b) Active site of *Cyanothece* sp. PCC 7424 PEPCK showing active site residues as sticks and OAA as spheres. The model was generated with SWISS-MODEL, and docking studies were performed with MOE (Chemical Computing Group, Inc.); [21, 23].

lid closed conformation as both programs used the ATP-Mg²⁺-oxalate ternary complex of *E. coli* PEPCK [35].

The MOE software correctly predicted the binding site of *Cyanothece* sp. PCC 7424 PEPCK (Figure 7). While the program flexes the substrate, the conformation of the modeled protein is left unchanged when energy minimization calculations are being performed. The substrate is able to bind to the active site when the lid is in the open conformation, but the lid must close in order for all active site residues to interact with the substrate and to initiate catalysis [36]. Furthermore, the binding of ATP to the nucleotide binding site causes a conformational change which correlates to lid closure and catalytic activity [37].

4. Conclusion

This study provided the 3D structure of *Cyanothece* sp. PCC 7424 PEPCK through a homology modeling approach. This particular enzyme was modeled because of this group's particular interest in investigating the role of PEPCK in overall carbon assimilation by *Cyanothece* sp. PCC 7424 an obligate autotroph with unique metabolic properties. Docking studies of the interaction of OAA with the enzyme's active site showed that the spatial arrangement of catalytic amino acids in the cyanobacterial enzyme was similar to that of other modeled PEPCKs; this suggests a catalytic mechanism that is similar to other forms of the enzyme. In general, the enzyme's primary structure varies considerably among species suggesting significant evolutionary divergence. The precise role of PEPCK in cyanobacteria has yet to be investigated, and whether PEPCK is present in marine cyanobacteria remains to be seen. More work will be needed to determine the potential role of PEPCK in cyanobacterial carbon fixation.

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