Applications of vibrational spectroscopy in the study of flavin-based photoactive proteins

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Abstract. Flavin cofactor is known to perform diverse biological functions. Recently, its role as a photoreceptor has been identified. So far, three classes of photoactive flavoproteins have been recognized: phototropin with LOV (Light, Oxygen and Voltage) domain, blue light sensory protein with BLUF (Blue Light sensing Using Flavin adenine dinucleotide) domain and photolyase/cryptochrome protein with PHR (Photolyase Homology Region) domain. Photochemistry of flavin is the key to unravel the reaction mechanisms of photoactive flavoproteins in their biological functions such as DNA repair or signal transduction. Vibrational (Infrared and Raman) spectroscopy is a useful and sensitive tool to investigate the photochemistry of flavin in protein environments and has significantly contributed to elucidate the reaction mechanisms of these photoactive proteins. This study will survey recent advances in vibrational spectroscopic studies on this topic and remaining questions to be answered.

Keywords: Flavin, flavoprotein, photochemistry, vibrational spectroscopy, infrared, Raman, photosensor, DNA repair

1. Introduction

Flavin, which constitutes of a group of compounds having the heteroaromatic ring system of 7,8-dimethylisoalloxazine, is contained in protein as a cofactor and plays critical roles in various important processes in cells [36,47]. The traditional biological function of flavin is of catalysis in redox reactions. It was found recently, however, that this cofactor also works as a photoreceptor to initiate the light-induced DNA repairs and signal transductions. As an isoalloxazine skeleton could not be synthesized in human cells, it is obtained from the diet through riboflavin (vitamin B₂) to maintain lives. The structure of flavin and its atomic numbering are illustrated in Fig. 1(a), where riboflavin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) reside. Flavin is an extremely versatile molecule with three redox states. Figure 1(b) shows the oxidized (quinoid), one electron reduced (semiquinoid), and two electron reduced (hydroquinoid) states of flavin and their physiological ionic states.

Among the photoactive flavoproteins, FAD was first identified as a catalytical cofactor of photolyase in 1987 – a repair enzyme of photo-damaged DNA [20]. Successively in 1995, FAD was discovered in cryptochrome (CRY), which has a sequence homologous to photolyase [29]. CRY serves as a blue-light

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Fig. 1. (a) Structure of flavin derivatives (riboflavin, FMN, FAD) and the atomic numbering for isoalloxazine ring. (b) Redox and ionic states of flavin under physiological conditions.

receptor that regulates the light-induced responses of plants and also controls the circadian rhythm of animals. Recently, it was noted that CRY works as an important player in rapid light perception like rhodopsin [8]. In both photolyase and CRY, FAD is buried in a PHR domain. Secondly, phototropin, which regulates the phototropism responses in plants, came into the scene in 1997 [11]. Phototropin binds two FMN within a pair of LOV domains. At almost the same time, the third type of photoactive flavoproteins was found in blue light sensory proteins with BLUF domain in 1997 [13], and to date, six BLUF proteins have been characterized. So far, the DNA repair mechanism of FAD in PHR domain and the signal transduction mechanisms of FMN in LOV domain and of FAD in BLUF domain have been well explained. However, the photochemistry of FAD in the PHR domain of CRY is still elusive.

In the last decade, photochemistry of flavin in these photoactive proteins has been studied extensively with vibrational spectroscopy including Infrared (IR) and Raman spectra. The peak frequencies in vibrational spectra are discussed on the basis of the normal modes that are related to the strength of chemical bonds and effective masses of vibrating groups. Thus, the vibrational spectroscopy is a powerful tool to investigate structures of molecules. Here, we summarize the information provided to date on these photoactive flavoproteins by vibrational spectroscopy and discuss the remaining questions to be answered.

2. FMN in LOV domain

Phototropin contains two ~ 12 kDa FMN-binding domains, LOV1 and LOV2, at their N-terminus, and a serine/threonine kinase domain at the C-terminus [6]. As shown in Fig. 2(a), the oxidized FMN-bound LOV domain in the dark absorbs maximally around 450 nm (LOV 447) [6]. Blue light illumination triggers a photocycle involving the formation of the electronically excited triplet state of FMN, a reversible formation of a blue-shifted signaling state (LOV 370, absorbing maximally around 370 nm), and a slowly restoration to the original LOV 447 state in the dark. The signaling state was demonstrated to involve a light-induced formation of a covalent bond between C(4a) of flavin and a nearby cysteine (Fig. 2(a) right) [42]. Soon after this report, Swartz et al. presented the evidence for the formation of flavin C(4a)-thiol adduct using the Fourier Transform Infrared (FTIR) spectroscopy. The observed

Fig. 2. Photochemistry of flavin. (a) In LOV domain, signaling state: LOV 370. (b) In BLUF domain, signaling state: BLUE RED.

spectra are consistent with the frequencies calculated for a flavin attached with a sulfur at the C(4a) position [51]. The presence of S–H groups in the dark state and its disappearance upon photoreaction was confirmed by IR spectroscopy [16]. More recently, a more comprehensive vibrational assignment of the flavin-thiol adduct was provided by Kikuchi et al. [21].

A formation mechanism of flavin-thiol adduct is one of the basic questions of LOV domain signaling. Phenomenologically, first a protonated cysteine (Cys57) near FMN was identified in the dark state LOV domain [4]. Second, a protonated cysteine and unprotonated flavin were detected for the excited triplet state of FMN by steady-state and time-resolved IR spectroscopy [1,40,43]. These results ruled out the ionic mechanism for the formation of flavin-thiol adduct suggested by other studies. Third, Alexandre et al. [3] found that the proximity of the cysteine to FMN not only enables the formation of a covalent adduct between flavin and cysteine, but also facilitates the rapid formation of the reactive flavin triplet state from their investigation of electronic structures and molecular vibrations of flavin. Interestingly, the formation of flavin-thiol adduct was engineered by introducing a cysteine residue into the BLUF domain, in which the occurrence of a LOV-like photoreaction of flavin was identified by FTIR and UV-vis spectroscopy, suggesting that the cysteine works as the key factor in determining the reaction of flavin in LOV domain [50]. Another basic question of LOV domain signaling is the signal transduction pathway. The conformational changes of protein induced by the C(4a)–S(Cys) bond formation were studied by IR spectroscopy, mostly by Kandori's group, who determined the key residues and protein fragment [2, 15,18,23,38,41,56,57]. In addition, FTIR studies also revealed the C=O stretching band of flavin [14] and the conformational heterogeneity of the LOV domain [2,44]. Although the photochemistry of flavin in the LOV domain has been well characterized, some questions remain unanswered. First, the mechanism by which the covalently bound flavin-thiol adduct relaxes to the initial dark state is still unknown. Second, the dynamics of the formation of flavin-thiol adduct has not been directly clarified.

3. FAD in BLUF domain

BLUF domain exhibits typical features of an oxidized FAD in the dark. Upon blue light illumination, the FAD forms an intermediate signaling state with a ~ 10 nm red-shifted absorbance (BLUF RED) without apparent variations in the redox state of flavin. The red-shifted state of FAD returns to the original form in the dark. As shown in Fig. 2(b), it has been identified that the BLUF RED signaling state is formed when the hydrogen-bond between flavin and glutamine is switched from N(5) to C(4)=O of flavin. Indeed, vibrational spectroscopic methods played an important role in clarifying this mechanism.

Laan et al. [25] characterized the primary photochemistry of FAD in the BLUF domain using FTIR. The difference spectrum between dark and signaling states was dominated by a band shift from 1710 to 1698 cm^{-1} [25]. Such a large downshift as $\sim 15 \text{ cm}^{-1}$ was observed by many successive IR and Raman studies and was assigned to the C(4)=O stretching of the isoalloxazine ring (Fig. 1(a)) [9,30,39,55]. By examining the BLUF domain containing isotope-labeled FAD, Masuda et al. [33] revealed that the C(4)=O stretching of isoalloxazine ring was the only band assignable exclusively to FAD, and that all the other bands came from protein moiety. The downshift indicates the formation of a strong H-bond at C(4)=O when FAD is brought into the signaling state, which yielded the red-shifted absorption [10]. Unno et al. carried out Raman spectroscopic studies on wild-type and mutated BLUF domains, and clarified that the key mechanism is the H-bonding switch of Gln63-NH₂ from N(5) to C(4)=O of flavin as shown in Fig. 2(b) [54]. Iwata et al. [17] demonstrated that the switch is accompanied by the donation of H-bond from Tyr21-OH to Gln-NH₂. The formation of the signaling state was further investigated by ultrafast IR spectroscopy [49]. On the other hand, Bonetti et al. observed the light-induced electron transfer from tyrosine to flavin through the formation of anionic semiquinoid flavin and a bleach of a vibrational mode of tyrosine, which suggests that the H-bond between glutamine and flavin is switched through a radical pair mechanism [5]. Other residues in signal transduction pathway also have been investigated [24,31,32,53]. Although the primary photochemistry of FAD in the BLUF domain has been well examined, the dynamics of structural changes around N(5) and C(4)=O of flavin and more details of signal transduction pathway after light-excitation need to be clarified.

4. FAD in PHR domain

Photolyase is a blue-light activated enzyme that repairs ultraviolet (UV)-damaged DNA. There are two major UV-damaged DNA, cyclobutane pyrimidine dimer (CPD) and (6-4) photoproduct (64PP), which are repaired by CPD photolyase and (6-4) photolyase, respectively. The photo-excited FAD in photolyase adopts the anionic two-electron-reduced state (Fig. 1(b)) and splits the UV-damaged DNA dimer through a cyclic electron transfer between FAD and DNA. Schelvis and colleagues examined the CPD photolyase combined with DNA substrate by resonance Raman spectroscopy and discussed the vibrational modes of neutral one-electron-reduced FAD and their perturbations by substrate binding [37, 45]. On the other hand, FTIR spectroscopy was used by Schleicher et al. to monitor the vibronic bands of one- and two-electron-reduced FAD in this enzyme with and without substrate. Also, the characteristic bands of both FAD and DNA were assigned [46]. These studies provided the basis for understanding the structure and environment of FAD when placed in a PHR domain. For example, frequencies of bands between 1615 and 1150 cm^{-1} observed for neutral one-electron-reduced FAD are all lower in photolyase compared with those in neutral semiquinoid flavin in other flavoproteins, which suggests a stronger H-bonding environment in photolyase. In fact, the crystal structure of CPD photolyase has showed a very special U-shaped conformation of FAD, in which an adenine ring approaches the N(10)–C(10a) of the isoalloxazine ring (Fig. 3(a)) and an intramolecular H-bond is formed between the 2'-OH of the ribityl chain and the N(1) of isoalloxazine ring [35]. Such a bent FAD is unique in this protein family, but whether the adenine bridges the electron transfer between isoalloxazine and DNA substrate is still under debate.

Little was known about (6-4) photolayse when we first investigated this enzyme by resonance Raman spectroscopy [27,28]. We found that the H–D exchange at N(5) of isoalloxazine in a semiquinoid form was much more difficult compared with that at N(3). Complete H–D exchange was confirmed only when

Fig. 3. Active sites of photolyase (a) and cryptochrome (b). (a) Structure of FAD and DNA substrates of 64PP and CPD (light-grey). (b) Structure of FAD and (1) the ATP in plant CRY that may accept an electron from light-excited flavin; (2) the Trp that donates an electron to flavin in the photoreduction; (3) residues (Asp in plant CRY, Cys in insect CRY) that determines the ionic state of semiquinoid flavin.

flavin was oxidized once and photoreduced to a semiquinone form again. Such a difficult H–D exchange at N(5) of flavin was recognized also for CPD photolyase (unpublished data). Therefore, the assignments of vibrational bands involving the N(5) of flavin in this protein family by H–D exchange [22,45] have to be made with caution. With the help of the selectively deuterated isoalloxazines at N(3) or N(5), we assigned the observed Raman bands with the normal mode calculations for neutral semiquinoid flavin for the first time [27]. Based on this assignment and the Raman spectra of both (6-4) and CPD photolyases with and without DNA substrate, we suggested that the DNA disturbed the bent conformation of FAD in both enzymes, but only the ring I of isoalloxazine in CPD photolyase. 64PP was predicted to interact with adenine ring of FAD through H-bonding (like CPD) but not to interact with isoalloxazine ring (unlike CPD) as shown in Fig. 3(a), and two years later this was, in fact, observed by X-ray crystallography [34]. Recently Li et al. observed that the electron transfer rate from FAD to substrate in (6-4) photolyase is similar to that in CPD photolyase [26]. All these results strongly suggest that the electron transfer from isoalloxazine to DNA substrate is bridged by adenine in both photolyase enzymes. In addition, a recent IR study on (6-4) photolyase noted that the substrate binding changed only the protein backbone and a protonated carboxylic acid side chain, whereas photoactivation yielded changes of FAD and surrounding protein [58]. It has been known that (6-4) photolyase is much flexible than CPD photolyase [7], and is highly homologous to the photoreceptor of CRY [52]. Thus, further investigations on the light-induced conformational changes of (6-4) photolyase may shed light on the molecular mechanism of CRY.

CRY is defined as photolyase homology without a DNA repair function and is classified into two groups: plant CRY and animal CRY. DASH-CRY was identified as a photolyase that repairs CPD in a single strand DNA. Resonance Raman spectroscopy has revealed that DASH-CRY has significant spectroscopic similarities to CPD photolyase [48]. The reaction mechanisms of plant and animal CRYs are better characterized in plant *A. thaliana* CRY (AtCRY) and insect *D. melanogaster* CRY (DmCRY), respectively. Upon irradiation with blue light, the conserved C-terminal extension domain undergoes a light-induced conformational change in plant and insect CRYs, which results in the modulations of

downstream signaling partner proteins. Although the FAD cofactor in this protein must initiate the signal transduction, its catalytically active redox state in plant CRY is under debate. The semiquinoid anionic FAD was suggested as the signaling state in animal (insect) CRY, but the light-induced response of C-terminal extension has not been observed directly. In both CRYs, it is left to be solved what kind of photochemistry occurs to the flavin for initiating the conformational changes of protein. Figure 3(b) shows several possible mechanisms that may disturb the protein structure of CRY: (1) photolyase-like electron transfer from flavin to an ATP bound in AtCRY1; (2) electron transfer from nearby conserved Trp to flavin in photoreduction reactions; (3) the changes of H-bonding or electrostatic interaction between N(5) of flavin and its nearby residues. Kottke et al. [22] observed deprotonation of an aspartic or glutamic acid upon illumination to AtCRY, which is probably the Asp near flavin (Fig. 3(b)). IR spectroscopy has revealed the changes in secondary structure of protein in response to the photoreduction of FAD [12,22] and noted a low frequency of the carbonyl stretch in plant CRY [12]. Interestingly, the conserved Cys near the N(5) of flavin in insect CRY stabilizes an anionic semiquinoid form of FAD, which is believed to be the signaling state. Such a stable anionic semiquinoid FAD was also observed in the Asp to Cys mutant of DASH CRY [19]. However, it is not clear why the Cys near flavin does not form the flavin-thiol adduct like the LOV domain and mutated BLUF domain. So far, the vibrational studies on animal CRY are lacking. Similar to the progresses in understanding LOV and BLUF domains, low temperature difference IR spectra before and after light illumination and site-directed mutagenesis as well as time-resolved techniques at room temperature are expected to clarify the photochemistry of FAD in CRY and to determine key residues in the signal transduction pathway.

5. Concluding remarks

Unlike the light-induced E/Z isomerization of photoactive cofactors in other biological photoreceptors such as retinoid proteins, phytochrome and photoactive yellow protein, flavin does not initiate the light-induced signal transduction by a large structural change of itself. Vibrational spectroscopy is a powerful tool to detect the delicate conformational change of flavin and surrounding proteins and has indeed significantly contributed to the understanding of photochemistry in flavin-based photoactive proteins with LOV, BLUF and PHR domains. We believe that the recently-developed ultrafast time-resolved vibrational spectroscopy will unravel more details about the structural dynamics involved in initial signaling process, especially for the unknown photochemistry of FAD in PHR domain of CRY. Femtosecond IR spectroscopy has been applied to the studies on flavin-based photoactive proteins [1,5,49], but the spectral window was limited by the available probe frequencies and solvent absorption. Recently, femtosecond stimulated Raman spectroscopy (FSRR) has been successfully applied to the study of the photochemistry of bacteriorhodopsin, phytochrome, and green fluorescent protein. In contrast to femtosecond IR spectroscopy, FSRR can detect a wide UV-visible/near IR spectral region. In addition, resonance conditions can be chosen to selectively detect the specific ionic redox state of flavin without the influence of protein matrix. Therefore, FSRR may be a promising vibrational spectroscopic technique to investigate the mechanisms of flavin-based photoactive proteins in the future.

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