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Spectral Tuning and Protein Structures of Photoactive Yellow Protein by Resonance Raman Spectroscopy

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論　文　内　容　要　旨

The photoactive yellow protein (PYP) from phototrophic bacterium *Halorhodospira halophila* is a small water-soluble photoreceptor protein, and it has been an attractive model for studying protein structures and dynamics. Recently PYP gained further attention as the structural prototype for the PAS and LOV domains of a large class of receptor proteins. This protein has the 4-hydroxycinnamyl chromophore, which is covalently linked to Cys69 through a thiolester bond. In a dark state (PYP_{dark}), the chromophore is stabilized in the *trans* configuration as a phenolate anion. The phenolate oxygen of the chromophore forms hydrogen bonds with the hydroxyl group of Tyr42 and the protonated carboxyl group of Glu46 (Figure 1A). In addition, the carbonyl oxygen of the chromophore forms a hydrogen bond with the amide group of Cys69. Photoexcitation of PYP triggers a photocycle that involves at least two intermediate states denoted as PYP_L and PYP_M (Figure 2). A long-lived blue-shifted PYP_M intermediate is the putative signaling state of this photoreceptor protein (Figure 1B). Upon lowering the pH below 3, PYP_{dark} is reversibly converted into a stable bleached state, $PYP_{M,dark}$ (Figure 2). *H. halophila* displays negative phototaxis to blue light and PYP has been proposed to function as a photosensor in this response.

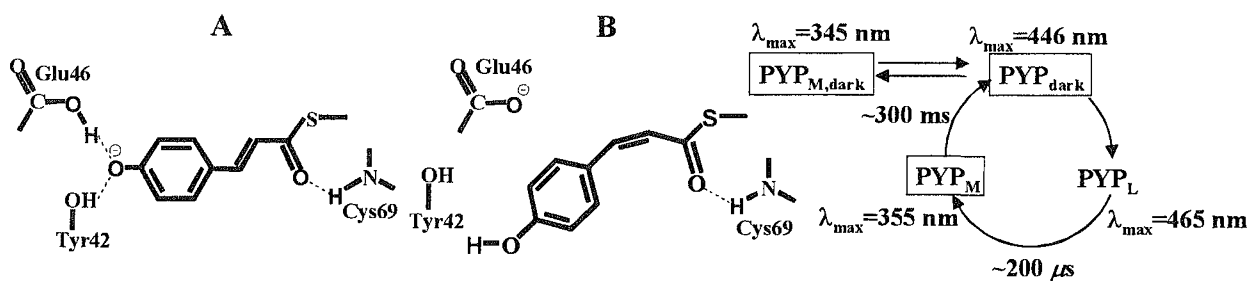

Fig. 1. Active site structures of (A) PYP_{dark} and (B) PYP_{M}.

Fig. 2. Photo- and pH-induced changes in PYP.

Resonance Raman (RR) spectroscopy is a powerful method to study the proteins functions at an atomic level because it detects the vibrational frequencies and the Raman intensities of the reactive protein groups with high sensitivity in a real time. RR spectroscopy requires the excitation within an electronic absorption band and results in large increase of scattering. The intensity enhancement associated with the resonance phenomenon introduces the possibility of selectively observing the vibrational spectrum of a single chromophore in a system containing many components (e.g., a biological system).

The main purpose of my study is to understand at the atomic level how a protein and the chromophore interact to sense light, undergo defined conformational changes, and send a biological signal. In the present studies, I have used resonance Raman spectroscopy in combination with site-directed mutagenesis to understand the following:

(1) Spectral Tuning of Photoactive Yellow Protein

Previous studies have shown that either removal of a hydrogen bond with Tyr42 or addition of chaotropes such as thiocyanate produces a blue shifted species called an intermediate wavelength form, in which the absorption maximum ranges from 355 to 393 nm. In order to determine the origin, the protonation state of the chromophore is key information. Resonance Raman spectroscopy is ideally suited for this purpose, because several Raman bands are sensitive to the protonation state of the chromophore.

PYP samples were excited with either the 406.7 nm line from a krypton ion laser or the 325.0 nm line from a helium-cadmium laser at a 90° angle relative to the axis of the collection optics to record the RR spectra. I have performed resonance Raman investigations of WT PYP and Tyr42 → Ala, Tyr42 → Phe, Glu46 → Gln, and Thr50 → Val mutants in the presence and absence of potassium thiocyanate to elucidate the structure of the intermediate wavelength form. The RR spectra of WT and PYP mutants have shown that the chromophore of the intermediate wavelength form is protonated. This finding indicates that the protonation of the chromophore is responsible for a blue-shift in the absorption maximum from 446 to 355-393 nm. I have also shown that the WT PYP contains a minor component whose chromophore is protonated even at neutral pH.

The DFT calculations support the idea that the chromophore-protein hydrogen bond is important for controlling the protonation state of the chromophore, where removal of the hydrogen bond between the phenolic oxygen and Tyr42 increases ΔpK_a by a 3-6 pH unit. Another noteworthy result of the DFT calculations is that the hydrogen bond at the carbonyl oxygen contributes little to the stabilization of the deprotonated chromophore in PYP. Furthermore, the DFT calculations demonstrate that the surrounding dielectric constant significantly affects the protonation state of the chromophore. I have also examined the effect of Y42A mutation on the structure of the chromophore in the

PYP_M, where the weak interactions between Tyr42 and the chromophore in PYP_M are suggested.

(2) Protein Structures of Photoactive Yellow Protein

The three-dimensional structure of PYP_M has been determined at (very) high resolution, providing important information on the structural change in the PYP protein to study photosensory signaling at the atomic level. The crystallographic structure of PYP_M revealed that the structural changes are limited to a small region in the vicinity of the chromophore. For example, the phenolate oxygen of the chromophore becomes protonated, while Glu46 becomes deprotonated. In addition, the hydrogen bonding network among the chromophore, Tyr42, and Glu46 is broken in PYP_M (Figure 1B). In contrast, various spectroscopic studies in solution showed global structural changes. For instance, the structural analysis of PYP_M by multinuclear, multidimensional NMR indicated a link between formation of the signaling state and protein unfolding in PYP.

In the present study I have used ultraviolet resonance Raman (UVRR) spectroscopy to obtain site specific information about the structural changes occurred upon the formation of PYP_M for the first time. The UVRR spectroscopy is used to probe the protein structure by monitoring both the Trp and Tyr residues.

PYP samples were excited with 220-240 nm lines from OPO laser at a 90° angle relative to the axis of the collection optics to record the RR spectra. I have performed all the measurement with a low laser power to avoid the saturation and/or photochemical transformation. In addition, I performed most of the UVRR experiments in the presence of sodium perchlorate as an internal standard, which showed no significant effect on the spectra.

The UVRR spectra provide information on the conformation, the hydrogen bonding, and the environmental hydrophobicity of Trp (W) and Tyr (Y) residues. For example, the Trp W17 and W7 vibrations are marker bands for the hydrogen bonding interaction and the hydrophobicity of Trp, respectively. In the PYP_{dark}, these vibration modes have indicated that the indoly nitrogen of the Trp119 is moderately hydrogen bonded to an acceptor in a hydrophobic environment. In addition, the Y8b and Y9a can be used as marker bands for the Tyr hydrogen bonding interaction. These bands have shown that some Tyr residues form a donor hydrogen bond to an acceptor. Moreover, the Raman excitation profiles of the Trp W16 and the Tyr Y8a are red shifted for WT PYP_{dark} from those of the Trp and Tyr in aqueous solution, respectively. This red shift is consistent with the hydrogen bonding interactions and the environmental hydrophobicity of PYP_{dark}. Furthermore, I have compared the REP of the Trp W16 and Tyr Y8a vibration modes in PYP_{dark} and PYP_M. Except for a slight difference in the intensities, the effects are similar for the PYP_{dark} and PYP_M states of the protein.

Upon the formation of PYP_M, the UVRR spectra have shown large structural changes. For example, the reduction of the Trp W18, W16, W7, and W3 intensities during $PYP_{dark} \rightarrow PYP_M$ process has indicated that the Trp119 becomes solvent exposed (hydrophilic) in PYP_M. In addition, the intensities changes of the Y9a, Y7a, and Y8a have shown that the part of the Tyr residues become solvent exposed (hydrophilic) upon the formation of PYP_M. Another evidence for the solvent exposure of the Trp119 and some Tyr residues is obtained by comparison with the UVRR spectra of the $PYP_{M,dark}$ state, in which the protein is unfolded.

The UVRR spectra of WT PYP have shown that the Tyr Y9a and Y8a are upshifted upon the formation of PYP_M. The upshift of those vibration frequencies suggests decreasing hydrogen bond donation or increasing hydrogen bond

acceptance during PYP_{dark} → PYP_M process. The decreasing of the hydrogen bond donation of the Tyr residues in PYP_M is consistent with our resonance Raman result of the PYP $Y42A_M$, where very weak interactions between Tyr42 and the chromophore in PYP_M are suggested.

In order to examine the contribution of the Tyr42 to the observed structural changes during PYP_{dark} →PYP_M process, I have used T50V mutant in which the hydrogen bond between the OH group of Tyr42 and Thr50 side chain is removed. The vibration modes of Tyr42 are perturbed by the mutation and the UVRR spectra of the T50V mutant have indicated the environment of Tyr42 is altered during the PYP_{dark} →PYP_M. In addition, the hydrogen bond between the OH group of Tyr42 and side-chain oxygen of Thr50 is preserved during the PYP_{dark} → PYP_M process.

[Summary]

(1) RR investigations have shown that the chromophore in the intermediate wavelength form is protonated.
(2) Both the low dielectric constant in the vicinity of the chromophore and the hydrogen bonds at the phenolic oxygen are key factors that lower the chromophore pK_a in PYP_{dark}.
(3) Very weak interactions between Tyr42 and the chromophore in PYP_M were suggested.
(4) The UVRR investigations have shown that theTrp119 and part of Tyr residues become hydrophilic (solvent exposed) upon the formation of PYP_M.
(5) By use of the T50V mutant it was indicated that Tyr42 undergoes environmental changes in PYP_M.
(6) The hydrogen bond between Tyr42 and Thr50 was preserved during the PYP_{dark} → PYP_M process.

論文審査の結果の要旨

エルマシュトーリーサミール提出の論文では，近年活発な研究が展開されている光センサータンパク質の一つの光活性イエロープロテイン(PYP)について，共鳴ラマン分光法を用いて，光サイクル中間体の構造，特にタンパク構造の変化が明らかにされた。共鳴ラマン分光法ではラマン散乱の励起波長を選ぶことで特定の化学種の振動スペクトルを選択的に得ることができる。例えば，PYPでは，励起波長として可視光を使うと，可視部に吸収帯を持つ発色団（クマール酸）の振動スペクトルが選択的に得られる。また，励起波長が220～250 nmの紫外共鳴ラマン分光法を使うと，芳香族アミノ酸残基の振動スペクトルが選択的に観測できる。波長によりアミノ酸残基の種類を選択することも可能である。本研究ではこの共鳴ラマン分光法がもつ特徴を最大限に利用して光活性イエロープロテインにおける吸収波長の制御（色の制御）および光によって引き起こされる中間体構造変化のうち主にタンパク構造の変化についての研究を行った。

得られた結果は次のようにまとめられる。

（１）光活性イエロープロテインの吸収極大は，活性部位のアミノ酸残基を他のアミノ酸で置換したり，溶液の塩基性度を変えることで大きく変化することが知られている。そこで吸収波長が変化したいくつかの成分のラマンスペクトルを測定し，発色団周りの構造決定を行った。その結果，発色団の構造変化やプロトン状態の変化が明らかになった。このことから発色団とタンパク間の相互作用，特に発色団のプロトン化状態の変化がPYPの吸収波長制御（色の制御）に重要なことを見いだした。

（２）光による変化は，反応中心の発色団の構造変化だけでなく，タンパク部分の構造変化を伴うと考えられる。本研究ではタンパク構造の変化を追跡するために，紫外共鳴ラマン分光法による測定・解析を行った。その結果，発色団の構造変化に伴なって，種々の部位のアミノ酸構造も変化していることがわかった。特に，信号伝達に関与すると考えられているM中間体において，発色団周りだけでなく，活性部位から10Å離れた部位のタンパク構造にも変化が及んでいることを見いだした。

これらの成果は本人が自立して研究を行うに必要な能力と学識を持つことを示している。よって，エルマシュトーリーサミール提出の論文は理学博士の学位論文として合格と認める。