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PROTEIN CONFORMATIONS OF OCTOPUS RHODOPSIN AND ITS DEPROTONATED PHOTOCYCLE INTERMEDIATE MONITORED BY ABSORPTION AND PROTEIN FLUORESCENCE

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Abstract — Picosecond time-resolved and static protein fluorescence spectra and absorption spectra of octopus rhodopsin, a photorecepting protein, are measured and compared with those of bacteriorhodopsin, a photon-induced proton pumping protein, to understand the protein conformations and functions of octopus rhodopsin and its deprotonated photocycle intermediate. The bluer and weaker absorption of retinal indicates that octopus rhodopsin is better in thermal noise suppression but less efficient in light harvesting than bacteriorhodopsin. The protein fluorescence of octopus rhodopsin shows the characteristic of Trp only and the quantum efficiency and lifetime variations may result primarily from variations in the coupling strength with the retinal. The stronger intensity by four times and larger red shift by 12 nm of fluorescence suggest that octopus rhodopsin has more open and looser structure compared with bacteriorhodopsin. Fluorescence decay profiles reveal two decay components of 300 ps (60%) and 2 ns (40%). The deprotonation of protonated Schiff's base increases the shorter decay time to 500 ps and enhances the fluorescence intensity by 20%. The fluorescence and its decay time from Trp residues near retinal are influenced more by the deprotonation. The increase of fluorescence intimates that protein structure becomes loosened and relaxed further by the deprotonation of protonated Schiff's base. The driving force of sequential changes initiated by absorption of a photon is too exhausted after the deprotonation to return the intermediate to the ground state of the begun rhodopsin form.

INTRODUCTION

Rhodopsin is a photorecepting protein of visual cells and bacteriorhodopsin (bR)[‡] is a phototrans-ducing protein of purple membrane. In spite of their significantly different biological roles, natural selection has converged on very similar designs for both proteins. Both rhodopsin and bR share the same basic chromophore system of a retinal, 11-*cis* in rhodopsin and all-*trans* in bR, bound to opsin *via* protonated Schiff's base (pSb) linkage.[1-8] Light absorption induces a sequential cyclic spectroscopic transformations, reflecting changes in the structures of both retinal and opsin, which induce the specific biological activities. These similarities have prompted comparative experimental and theoretical studies.[1,9-12]

Rhodopsin is a photoreceptor protein of visual cells in invertebrate retina as well as in vertebrate one. Upon absorption of light, Rhodopsin undergoes a photocycle that allows it to activate G-protein and thus to initiate a transduction cascade, leading to an electrical signal in photoreceptor cells.[1] Although the above photoresponse can be found in any animal, many aspects in the physiology and structure of the photoreceptor are quite different between invertebrates and vertebrates.[13] One of the differences is that the chromophore eventually detaches from apoprotein in vertebrate pigments but does not in invertebrate ones. Among the invertebrate pigments, octopus rhodopsin (oR) is employed as an excellent system to study structural changes during the photocycle,[14] because the final photoproduct, metarhodopsin intermediate of oR (M_{oR}) is stable at physiological conditions and Rhodopsin intermediate of oR (Rh_{oR}) can be almost completely photoregenerated from M_{oR} at neutral pH as well as from any other intermediates.[15] The Rh_{oR} and M_{oR} can be reversibly interconverted under illumination of

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‡ *Abbreviations*: bR, bacteriorhodopsin; M_{oR}, metarhodopsin intermediate of octopus rhodopsin; oR, octopus rhodopsin; pSb, protonated Schiff's base; Rh_{oR}, rhodopsin intermediate of oR.

appropriate wavelength light. Blue-violet light (wavelength 400-440 nm) induces the phototransformation from Rh_{oR} to M_{oR} *via* the photocycle intermediates of bathorhodopsin, lumirhodopsin and mesorhodopsin, while orange-yellow light (wavelength > 540 nm) does the reverse phototransformation. oR is a 455 amino acid polypeptide chain with 11 Trp and 22 Tyr and a 11-*cis* retinal as chromophore, which is linked *via* protonated Schiff's base to the ϵ-NH_2 group of Lys-306.[13]

bR, one of the protein pigments found in the purple membrane of *Halobacterium halobium*, is a polypeptide chain of 248 amino acid residues with 8Trp and 11 Tyr as well as a single retinylidene chromophore.[16] Upon absorption of a photon, bR undergoes a photocycle[17] consisting of intermediate states labeled K_{630}, L_{550}, M_{412}, N_{550} and O_{640}[18] with lifetimes ranging from picoseconds to milliseconds.[19] The pSb is deprotonated and reprotonated with the formation and decay of M_{412} intermediate, respectively, leading to a proton-pumping process that increases the proton concentration on the outside surface of the membrane. The created proton gradient across the membrane is then used to transform ADP into ATP.[20]

Trp fluorescence has been used as a sensitive probe for conformational perturbations in proteins and polypeptides.[21-25] The variations of the fluorescence lifetime, emission maximum and intensity are often affected by the environments of the emitting Trp molecules. Various environments of different Trp molecules in multitryptophan protein then give informations on protein structure and its changes with perturbations, photocycle, denaturation and interaction with a chromophore molecule. Protein fluorescence of bR is known to have the characteristic of Trp fluorescence only and the excitation of other amino acid residues may indirectly contribute to the Trp emission by energy transfer.[26] Most of the Trp residues in bR interact with the retinal.[22,27] The variations in the quantum efficiency and observed lifetimes of Trp fluorescence have been suggested[22] to result from the variations of the energy transfer efficiency between excited Trp molecules and the retinal in bR. Energy transfer from Tyr and Trp to the retinal in bR has a quantum yield of 0.7-0.8 and leads to a photocycle identical with that triggered by the excitation of the visible absorption bands of retinal.[28] Trp fluorescence during the photocycle of bR is reported[23] to have two additional photocycle-dependent quenching processes, a rapid one on the time scale of L_{550} formation and a slow one on the time scale of M_{412} formation.

In this paper we report picosecond time-resolved and static protein fluorescence and absorption spectra of oR and compare the observations of oR with those of bR to understand the protein conformations and functions of oR and its deprotonated photocycle intermediate.

MATERIALS AND METHORDS

Materials. The method to isolate the microvillar membrane of oR is similar to that described previously.[15,29] All the procedures of sample preparation and handling were done under a dim red light. The final oR samples were solubilized in aqueous solution of 2 m*M* 4-morpholinepropanesulfonic acid (pH 7.4) and 2% detergent (L-1690). The purple membrane was purified from the ET1-101 strain of *H. halobium* according to the methods of Oesterhelt and Stoeckenius[30] and Becher and Cassim.[31] The purified purple membrane was suspended in doubly deionized water. The sample concentrations of bR and oR were measured and normalized by the protein absorption.

Methods. Absorption spectra were measured with a Beckman DU-68 diode-array spectrophotometer and steady-state fluorescence spectra were obtained using an Spex Fluolog2 Spectrofluorometer. All the fluorescence spectra reported here were not corrected for the variation of detector sensitivity as a function of wavelength. Fluorescence lifetimes were measured by using a previously described[32] time-correlated single photon counting system of a 70-ps-fwhm response time equipped with a Coherent Antares YAG-pumped, hybrid mode-locked and cavity-dumped dye laser. Samples had to be excited only by frequency-doubled R6G dye laser pulses because of limited laser availability. Fluorescence was collected from the front surface of sample excitation. Decay time constants were deconvoluted from fluorescence kinetic profiles using a relative nonlinear least-square method.

RESULTS AND DISCUSSION

The absorption spectra of oR and bR in Figure 1 are significantly different in two respects. The absorption spectrum of oR with the same retinal chromophore system bound to opsin *via* a protonated Schiff's base is weaker and shifted to the shorter wavelength compared with that of bR. Absorption maximum shift of retinal in protein reflects the influence of the protein structure to retinal, so that the shift can be used as a probe to deduce the environment of binding site. The opsin shift of retinal absorption is defined as the magnitude of the red shift of absorption maximum relative to the absorption maximum of the chloride salt of the protonated Schiff's base in methanol solution.[33] It depends on the geometry of retinal, the flatness of conjugated double bonds in retinal and the location of counter ion of retinylidene Schiff's base.[34] The energy gap between excited and

ground states of retinal is minimized by the delocalization of positive charge on the Schiff's base in the photoexcited state. The possibility of *cis-trans* isomerization with thermal energy is also related to the double bond character of retinal, thus can be measured by opsin shift. Thermal noises such as thermal deprotonation are reduced when the pigment has blue-shifted absorption.[35,36] The blue-shifted absorption maximum of oR at 476 nm relative to that of bR at 558 nm implies that oR has a less delocalized positive charge in the pSb and an appropriate protein conformation for thermal noise suppression. The integrated retinal absorption band of oR is about 4 times smaller for the same protein absorbance than that of bR, admitting that there might be more retinal-free opsins in oR. The difference of absorbance between the two pigments is ascribable to the different oscillator strengths of the chromophore. Considering the biological role, proton translocation with light energy, of bR, the stronger absorption implies that bR has appropriate structures of protein and chromophore for light-harvesting. Absorption at 280 nm is mostly due to Trp residues in Trp containing proteins.[37] The 280 nm absorption ratio of two pigment proteins in Figure 1 is approximately similar to the ratio of Trp numbers 11 and 8 of two proteins.

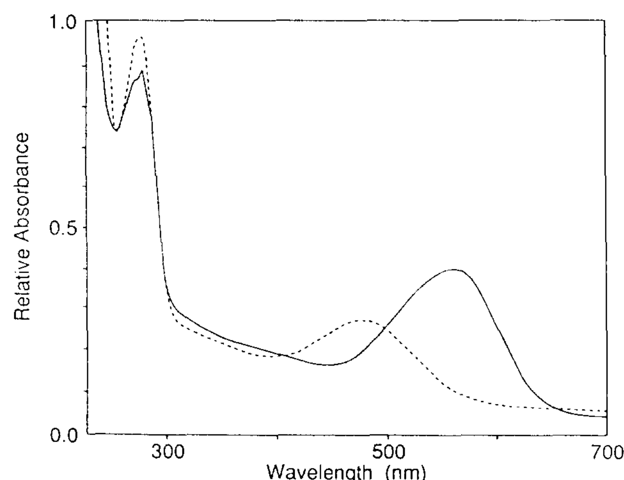

Figure 1. Absorption spectra with the same concentrations of Rh_{oR} form (dotted curve) and dark-adapted bR (solid curve). The retinal absorption maxima of oR and bR are at 476 and 558 nm, respectively.

The protein fluorescence of oR is about four times stronger for the same protein absorbance and red-shifted by 12 nm, compared with the fluorescence of bR, as shown in Figure 2. Much stronger intensity and more significant red shift of protein fluorescence in oR indicate that protein structure is not tight as that of bR. It has been reported[22,26] that protein fluorescence of bR is characteristic of Trp only and that excitation of Tyr or Phe indirectly contributes to Trp emission by energy transfer. The same argument can be asserted to oR from the greater red shift of protein fluorescence. The (π,π^*) transition of Trp[38] shifts to the red in a hydrophilic environment.[39] The larger red shift by 12 nm of the fluorescence peak wavelength in oR suggests that emitting Trp residues are more exposed to water environment and that protein has a looser structure. This argument is supported from the great increase of fluorescence intensity by four times, since the quantum efficiency of protein fluorescence in bR is reported[22] to be determined by the energy transfer from excited Trp and the retinal. The large increase of the fluorescence intensity is due to the decrease of energy transfer that results from the increase of distance between Trp residues and the retinal. The looser protein structure may increase the interaction distance although the distance increase could be mainly owing to the increase of the total amino acid residue number from 248 in bR to 455 in oR.

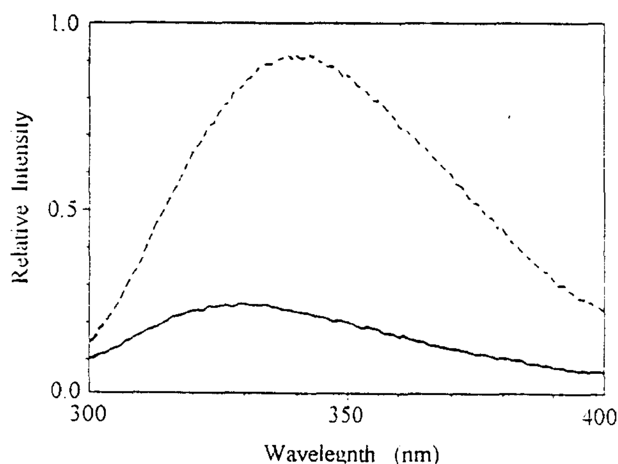

Figure 2. Protein fluorescence spectra, excited at 280 nm, of Rh_{oR} (dotted curve) and bR (solid curve) for the same protein absorbance. The emission peak wavelengths of oR and bR are 339 and 327 nm respectively and the emission intensity of bR is about 25% of that of oR for the same protein concentration and conditions.

Pigment proteins may undergo conformational changes during their photocycle, which modify Trp environments and vary the intensity, lifetime and wavelength of Trp fluorescence. We can obtain a lot of information about conformational changes during the photocycle by monitoring Trp fluorescence. Figure 3 shows that the protein fluorescence increases by 16% as the major fraction of Rh_{oR} transforms into M_{oR}. The pSb of retinal is deprotonated in M_{oR} intermediate while protonated in Rh_{oR}. The observation of fluorescence increase is quite interesting since the deprotonation does not alter Trp structure itself directly. Trp fluorescence in

bR is reported[23] to decrease by the deprotonation of pSb that modifies the protein conformation and enhance the energy transfer to retinal from excited Trp residues. However, fluorescence increases by the deprotonation of the pSb in oR. This indicates that the deprotonation reduces the quenching efficiency of Trp emission by retinal. This reduced quenching could suggest that the average distance between Trp residues and retinal increases by the deprotonation, although the quenching also could be affected by changes in relative orientation between Trp and retinal, protonation states of aromatic acids, effects of proximal charges and/or in polarity environments of Trp residues. oR becomes more relaxed and looser by the deprotonation. The difference spectrum is spectrally shifted to a shorter wavelength by several nanometers from the spectrum of either Rh_{oR} or M_{oR}, admitting the poor spectral resolution and low signal-to-noise ratio of the difference spectrum. More change in the fluorescence intensity at the shorter wavelength region tells us that Trp residues at relatively more hydrophobic environment are affected more significantly by the deprotonation of pSb.

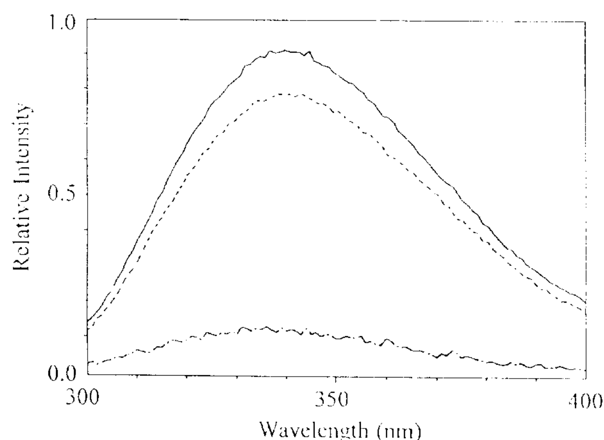

Figure 3. Protein fluorescence spectra, excited at 280 nm, of Rh_{oR} (dotted curve) and M_{oR} forms (solid curve). The two spectra were taken from the identical sample except that the Rh_{oR} spectrum was measured with red light illumination while the M_{oR} spectrum with blue light illumination. The dot-and-dashed curve is the difference spectrum from the above two curves. The photoconversion to M_{oR} from Rh_{oR} increases the protein emission intensity about 16% and the enhanced fluorescence of the difference spectrum is spectrally shifted to the blue by several nanometers from the fluorescence of Rh_{oR}.

The decay kinetic profiles of M_{oR} protein fluorescence reveal two decay components with the employed temperal resolution as shown in the time window of Figure 4. The fraction of the shorter decay component increases as the monitored emission wavelength decreases (Table 1). This points out that the faster decaying fluorescence is emitted from Trp at relatively more hydrophobic environment, compared with the slower decaying one. The decay kinetic profile of Rh_{oR} at 340 nm, shown in Figure 5, exhibits the decay components of 290 ps (60%) and 2.1 ns (40%). The two time constants at the same wavelength grow into 490 ps (55%) and 2.3 ns (45%) by the deprotonation of pSb. Trp residues affected more notably by the deprotonation are located at relatively more hydrophobic environment and near the retinal since the shorter fluorescence decay is ascribable to more efficient quenching by the retinal. This observation meets our expectation, for the pSb is an edge of the retinal. The calculated fluorescence enhancement, based on the changes in the time constants and fractions of decay components, predicts about 30% increase by the deprotonation, however, the static fluorescence in Figure 3 is observed to grow 16% by the deprotonation. This discrepancy is probably resulting from incomplete trapping of Rh_{oR} and M_{oR} in the static fluorescence measurement.

Table 1. Protein Fluorescence Decay Time Constants of oR Intermediates

Intermediate	Collected wavelength* (nm)	Fluorescence lifetime: fast component (ps)	Fluorescence lifetime: slow component (ns)
	320	400 (56%)†	1.9 (44%)
M_{oR} form	340	490 (55%)	2.3 (45%)
	360	570 (49%)	2.7 (51%)
Rh_{oR} form	340	290 (60%)	2.1 (40%)

* Samples were excited at 285 nm.
† The percentages in parentheses indicate relative amplitudes for the respective components.

Longer lifetime and greater intensity of fluorescence, compared with those of bR, indicate a reduced interaction between Trp and retinal in oR. This fact with the larger red shift of fluorescence suggests that the protein structure of oR is more open, more hydrophilic and looser than that of bR. As the pSb is deprotonated, fluorescence decay times become even longer but also the fraction of the slower component increases. The Trp at relatively more hydrophobic environment near retinal are affected more significantly and they become exposed to more hydrophilic environment by the deprotonation. Upon the deprotonation of the retinylidene Schiff' s base, bR tightens its retinal pocket to receive

a proton and proceeds the photocycle,[22,23] however, oR loosens its retinal pocket to relax itself and cannot complete the photocycle probably because the driving force obtained by absorption of a photon is not left enough to render the photocycle to proceed. The difference can be explained based on their biological functions and roles. The biological role of bR is to transport proton across the membrane, thus it is benefit for the efficiency of proton pumping to shorten its photocycle for a fast proton transport. However, the biological role of oR is to produce optical impulse, thus it needs time to initiate biochemical reaction followed by the deprotonation. So the protein conformation changes of both oR and bR are appropriate for their respective biological roles and functions. In vertebrate rhodopsin, moreover, retinal completely separates from opsin.

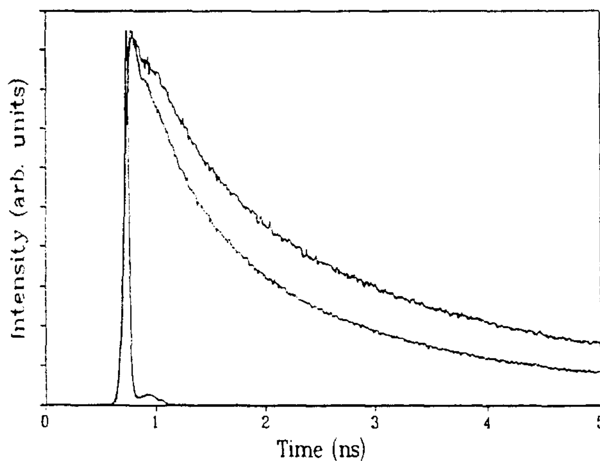

Figure 4. Fluorescence decay profiles of M_{oR} at 360 nm (solid curve) and 320 nm (dotted curve). The deconvoluted time constants are 570 ps (49%) and 2.7 ns (51%) at 360 nm and 400 ps (56%) and 1.9 ns (44%) at 320 nm.

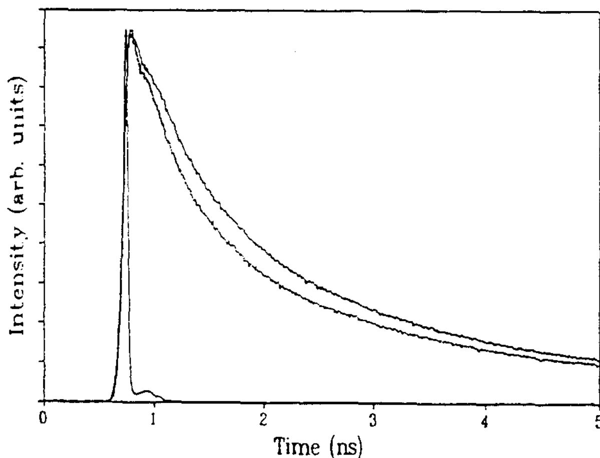

Figure 5. Fluorescence decay profiles of Rh_{oR} (dotted curve) and M_{oR} (solid curve) at 340 nm. The deconvoluted time constants in Rh_{oR} are 290 ps (60%) and 2.1 ns (40%) and those in M_{oR} are 490 ps (55%) and 2.3 ns (45%).

Thus, vertebrate rhodopsin is expected to relax its protein structure to an extreme extent for the detachment of retinal.

CONCLUSION

Protein conformation of oR and its change by the deprotonation of pSb are studied by measuring picosecond fluorescence kinetic profiles and absorption and emission spectra and by comparing the observations with those of bR. Relatively better thermal noise suppression and less efficient light absorption of oR are explained well by their respective roles of the two light-transducing membrane proteins that oR converts light into an optic nerve impulse while bR harvests light to pump protons. Protein fluorescence is characteristic of Trp only, fluorescence quantum efficiency and lifetimes are determined mainly by the interacting strength with the retinal and the fluorescence from Trp near the retinal is affected more significantly by the deprotonation of pSb as reported in bR. However, the extremely enhanced intensity, larger red shift and larger lifetime of fluorescence hint that oR has more open and looser protein structure compared with bR. Fluorescence kinetic profiles show two decay components of 300 ps (60%) and 2 ns (40%). The deprotonation of pSb increases the shorter decay time to 500 ps and enhances fluorescence intensity by 20%. The enlargements in both fluorescence intensity and decay time by the deprotonation of pSb imply that the deprotonation relaxes the protein further, having the result that the pSb cannot be reprotonated unless a additional energy is supplied by red light illumination or biochemical process.

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