Ultraviolet resonance Raman evidence for the absence of tyrosinate in octopus rhodopsin and the participation of Trp residues in the transition to acid metarhodopsin

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Abstract The ultraviolet (244 nm) resonance Raman spectra of octopus rhodopsin and its photoproduct, acid metarhodopsin, do not give any evidence for a tyrosinate. This finding excludes the possibility that Tyr-112 serves as the counter anion to the protonated Schiff base as does Glu-113 in bovine rhodopsin. Upon photoconversion from rhodopsin to acid metarhodopsin, Trp and Tyr Raman bands decrease in intensity and concomitantly a Trp band shifts in frequency. The changes of Trp Raman bands are ascribed to changes in hydrophobic interactions and conformation, suggesting a possible role of Trp in the photoconversion process of octopus rhodopsin.

Key words: Octopus rhodopsin; Ultraviolet resonance Raman spectroscopy; Protonated Schiff base; Tyrosine; Tryptophan

1. Introduction

The visual pigment rhodopsin consists of the apoprotein opsin and an 11-*cis* retinal chromophore, which is covalently attached to the opsin via a protonated Schiff base (PSB) linkage [1,2]. Absorption of a photon by the pigment triggers isomerization of the retinal from 11-*cis* to all-*trans*, followed by formation of a series of intermediates including the biologically active photoproduct that initiates a biochemical reaction cascade leading to an electrical signal [3]. The intermediates of rhodopsin are characterized by individual visible absorption maxima (λ_{max}) reflecting the protonation state of Schiff base, conformation of retinal, and retinal-opsin interactions [4].

In the retinal binding pocket of rhodopsin, the positive charge on the nitrogen atom of PSB is believed to be balanced by a negatively charged amino acid residue. Site-directed mutagenesis of bovine rhodopsin has shown that the counterion to the PSB is the COO^- group of Glu-113, which is conserved in all vertebrate visual pigments [5–7]. In invertebrate rhodopsins, on the other hand, no Glu residues are found near position 113 and instead a tyrosine residue is found at the corresponding position: Tyr-112 in octopus rhodopsin [8] and Tyr-111 in squid rhodopsin [9]. Accordingly, one may speculate that Tyr-112 of octopus rhodopsin is ionized to a tyrosinate (Tyr^-) and serves as the counterion to the PSB [5]. Alternatively, since the λ_{max} of octopus rhodopsin shows a red shift from 476 to 514 nm upon formation of acid metarhodopsin, perhaps caused by altered electrostatic interactions [10], the red shift might be explained by a change in the ionization state of Tyr-112. Thus, it is important to determine the ionization state of Tyr-112 in both rhodopsin and acid metarhodopsin.

Ultraviolet resonance Raman (UVRR) spectroscopy can be used to sensitively detect the ionization state of Tyr residues as demonstrated for bacteriorhodopsin [11–13]. Tyr^- exhibits UV absorption significantly differently from neutral Tyr and UVRR scattering from Tyr^- can be enhanced by tuning the excitation wavelength to an absorption band of Tyr^-. In this study, we have examined UVRR spectra of octopus rhodopsin and acid metarhodopsin to determine the ionization state of Tyr-112 and to investigate the molecular mechanisms of retinal-opsin interactions. The UVRR spectra show that Tyr^- is not contained in rhodopsin or acid metarhodopsin, and so cannot be the counterion to the PSB. Additionally, upon photoactivation of the pigment to acid metarhodopsin, we have detected changes in Trp environment and conformation, which are attributed, at least partly, to a change in hydrophobic interaction between the side chain of Trp-275 and the chromophore in the retinal binding pocket.

2. Materials and methods

Sodium sulfate, 2-morpholinoethanesulfonic acid (MES), sucrose monolaurate, L-tyrosine, and L-tryptophan were purchased from Nacalai Tesque and used without further purification. Octopus (Mizudako, *Paroctopus defleini*) microvillar membranes were prepared as described previously [14] and solubilized with 1% sucrose monolaurate. Since the microvillar membranes contain large amounts of unsaturated lipids [15], very strong UVRR bands from the lipids obscured rhodopsin UVRR bands. To reduce the interference from the lipid Raman bands, rhodopsin was purified on DEAE-cellulose and Con-A Sepharose (Pharmacia Biotech) columns. The rhodopsin-containing fraction eluted from the DEAE-cellulose column [16] was applied to the Con-A Sepharose column, which had previously been equilibrated with a solution containing 10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1 mM $MnCl_2$, and 0.1% sucrose monolaurate. Rhodopsin was eluted from the Con-A Sepharose column with 250 mM α-methyl-D-mannopyranoside in the equilibrating buffer and then dialyzed against 10 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate.

The sample solution for UVRR measurements was prepared as follows. The purified octopus rhodopsin suspension was diluted in 20 mM MES buffer (pH 5.6) containing 100 mM sodium sulfate as an internal intensity standard for Raman scattering. The concentration of rhodopsin was determined using $\varepsilon = 30\,000\ cm^{-1}\ M^{-1}$ at 476 nm [17,18]. The sample solution was divided into two parts of equal volume and one of them was irradiated with 457.9 nm light from an

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Abbreviations: MES, 2-morpholinoethanesulfonic acid; PSB, protonated Schiff base; Tyr^-, tyrosinate; UVRR, ultraviolet resonance Raman; λ_{max}, absorption maximum wavelength

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Ar^+ laser (NEC, GLG 3300) for 3 min. The blue laser light converted rhodopsin to acid metarhodopsin as confirmed by its absorption spectrum. The other half of the sample was kept in the dark.

Raman spectra were excited with the 244 nm line (2 mW) from a continuous wave UV laser (Coherent, Innova 300 FReD) and were recorded on a fore-prism UVRR spectrometer (JASCO, TR-600UV) equipped with a liquid nitrogen-cooled charge-coupled device detector (Princeton Instrument, LN/CCD1152) [19]. Raman intensity saturation, which is sometimes encountered with pulsed laser excitation, was avoided by use of the continuous wave UV laser. About 2 ml of protein solution was recirculated through a quartz capillary (2 mm i.d.) using a peristaltic pump. Since irradiation with the UV (244 nm) probe beam caused a partial (~10%) conversion from rhodopsin to acid metarhodopsin, the rhodopsin solution was irradiated, downstream from the probe beam, with orange light transmitted through a cutoff filter (>582 nm, Toshiba O58) to convert acid metarhodopsin generated by the probe beam back into rhodopsin. Similarly, the acid metarhodopsin solution was irradiated with blue light (355–455 nm) using a Toshiba V40 band-pass filter to convert any rhodopsin generated back into metarhodopsin. Each spectral acquisition required 5 min and the final spectrum was obtained by averaging six recordings. After this 30 min exposure to the UV laser, the visible absorbance decreased by about 20% compared to the initial solution. Irrespective of the bleaching, there was no difference between the first and last acquisitions of the UVRR spectrum except for a decrease in intensity of the lipid C=C stretch band. Wavenumber calibration was performed using the Raman bands of cyclohexanone-acetonitrile (1:1, v/v). Peak wavenumbers of sharp Raman bands were accurate to within ± 1 cm^{-1}.

3. Results

3.1. Structural information provided by the UVRR spectrum of octopus rhodopsin

Fig. 1A shows the UVRR spectrum excited at 244 nm of octopus rhodopsin. The strong band at 1647 cm^{-1} and a broad band around 1270 cm^{-1} are assigned to the C=C stretch and =C-H in-plane bend of lipid acyl chains, respectively [20]. These lipid Raman bands are due to unsaturated lipids originating from the microvillar membranes [15]. (The intensities of lipid Raman bands were greatly reduced by rhodopsin enrichment, though they are still very strong.) A broad Raman band at 1302 cm^{-1} decreases in intensity to some extent but does not disappear completely in D_2O buffer (spectrum not shown). Accordingly, the band is ascribed to an overlap of Raman bands due to the amide III (N-H bend) mode of α-helical structure and lipid vibrational modes (CH_2 twist and CH_2 rock) [20].

A shoulder peak at 1443 cm^{-1} is assigned to the imide II vibration of the Pro C(O)-N bond. The frequency of the imide II band has been shown to be a sensitive marker of the strength of hydrogen bonding of the imide C=O group [21]. The 1443 cm^{-1} imide II band of octopus rhodopsin indicates that the hydrogen bonds at the imide C=O sites of some Pro residues are very weak and the C=O groups are shielded from the solvent water. Octopus rhodopsin contains 43 Pro residues and 25 of them are localized at the end of a long C-terminal domain that extends to the cytoplasmic side of the membrane [8]. Possibly, the Pro-rich end of the peptide chain is folded back and inserted into the membrane. It is known that the Pro-rich region contributes to the mobility of octopus rhodopsin in the membrane [22].

The Raman bands of Trp and Tyr vibrations are labeled W and Y, respectively, with their mode designations proposed previously [23]. Octopus rhodopsin contains 22 Tyr and 11 Trp residues. Fig. 1E shows a UVRR spectrum computed for an amino acid mixture of 22 Tyr+11 Trp by adding the

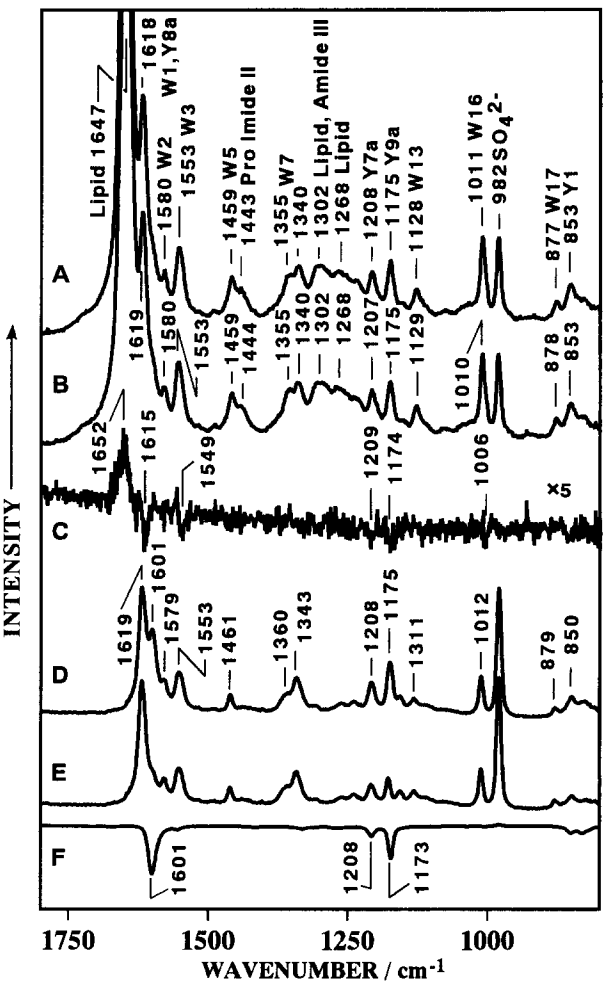

Fig. 1. Resonance Raman spectra excited at 244 nm of octopus rhodopsin and aqueous amino acids. A: octopus rhodopsin; B: acid metarhodopsin; C: difference spectrum (spectrum B minus spectrum A); D: sum of the spectra of aqueous Tyr^-, Tyr, and Trp in a 1:21:11 ratio; E: sum spectrum for 22 Tyr and 11 Trp; F: difference spectrum (spectrum E minus spectrum D). The spectra of rhodopsin were measured at room temperature and pH 5.6 in 20 mM MES buffer. The 982 cm^{-1} band is due to SO_4^{2-} added as an internal standard of Raman scattering intensity.

244-nm Raman spectra of aqueous Tyr and Trp in a 22:11 ratio. The intensity of the amino acid spectrum is scaled using the 982 cm^{-1} band of SO_4^{2-} such that a direct comparison of intensities can be made with the spectrum of rhodopsin. It is known that the relative intensity of the doublet at 1355 and 1340 cm^{-1} (W7 doublet) and the absolute intensity of Trp Raman bands serve as a measure of hydrophobicity of the Trp environment [11,24]. The I_{1355}/I_{1340} intensity ratio of rhodopsin (0.9) is nearly twice that of aqueous Trp (0.5), and the intensities of the 1553 (W3) and 1011 cm^{-1} (W16) bands of rhodopsin are much larger than those of aqueous Trp. These observations indicate that some of the Trp residues of rhodopsin are buried in the protein and their environments are hydrophobic. According to a topological model for octopus rhodopsin [8], 5 Trp residues are within the transmembrane helices. Site-directed mutagenesis and photochemical cross-linking studies on bovine rhodopsin have shown that Trp-265 (Trp-275 in octopus rhodopsin) is close to the β-ionone ring of retinal [25–27]. The side chain of Trp-275 probably

constitutes a hydrophobic pocket for retinal binding in octopus rhodopsin. The other Trp residues within the transmembrane helices may also be in hydrophobic environments.

The intensities of Tyr UVRR bands in octopus rhodopsin at 1205, 1175, and 853 cm^{-1} (Fig. 1A) are almost doubled compared to those in aqueous amino acid solution (Fig. 1E). The Tyr UVRR intensity has been shown to increase when the phenolic side chain is hydrogen bonded as a proton donor in hydrophobic environments [28]. The intense Tyr Raman bands of octopus rhodopsin imply that some of the Tyr residues are buried in the protein with the side chain hydrogen bonded.

Fig. 1D shows a UVRR spectrum computed for another amino acid mixture of 1 Tyr^-+21 Tyr+11 Trp using the spectra of the component amino acids in aqueous solution. The prominent 1601 cm^{-1} band in spectrum D is characteristic of Tyr^- and the 1208 and 1175 cm^{-1} bands are due to overlapping Tyr and Tyr^- bands. Tyr^- has a λ_{max} at 240 nm and the Raman scattering from Tyr^- is particularly enhanced when excited around 240 nm. Thus, the 244-nm excitation employed here is suitable for sensitive detection of Tyr^-. However, even the strongest Raman band of Tyr^- at 1601 cm^{-1} cannot be detected for octopus rhodopsin (Fig. 1A). Therefore, it is concluded that octopus rhodopsin does not contain Tyr^-. A weak shoulder around 1600 cm^{-1} on the 1618 cm^{-1} band envelope in Fig. 1A is assigned to the Y8b mode of Tyr, but not to Tyr^-, because the corresponding weak shoulder is seen in the spectrum for the aqueous amino acid mixture 22 Tyr+11 Trp (Fig. 1E). To confirm this assignment, we subtracted the interfering C=C stretch band at 1647 cm^{-1} from the spectrum in Fig. 1A using oleic acid as a model compound for the unsaturated acyl chains in the lipid. The intensity of the 1600 cm^{-1} shoulder compared to the other bands in the resultant difference spectrum (not shown) was nearly identical to that in Fig. 1E (22 Tyr+11 Trp) and much lower than that in Fig. 1D (1 Tyr^-+21 Tyr+11 Trp), lending support to the assignment that the 1600 cm^{-1} shoulder arises from Tyr. Since the UVRR intensity of Tyr^- is expected to be higher in protein environments than in aqueous solution [29], the intensity at 1600 cm^{-1} would be even higher than that of the 1601 cm^{-1} band in Fig. 1D if octopus rhodopsin contained Tyr^-.

3.2. Structural changes associated with the transition to acid metarhodopsin

Fig. 1C shows the difference UVRR spectrum, acid metarhodopsin (B) minus rhodopsin (A). The intensity was expanded by a factor of 5. A positive peak around 1650 cm^{-1} is due to a slight difference between the two spectra in the degree of UV-induced bleaching of the lipid C=C stretching band. Fig. 1F shows a spectrum obtained by subtracting spectrum D (1 Tyr^-+21 Tyr+11 Trp) from spectrum E (22 Tyr+11 Trp). The difference spectrum demonstrates that Tyr^- can be readily detected by UVRR spectroscopy even if only one of the 22 Tyr residues is ionized. The strongest negative peak at 1601 cm^{-1} in the amino acid difference spectrum (Fig. 1F) has no counterpart in the acid metarhodopsin−rhodopsin difference spectrum (Fig. 1C). This gives evidence that every Tyr residue of octopus rhodopsin remains protonated upon conversion to acid metarhodopsin. The weak features at 1209 and 1174 cm^{-1} and part of the 1615 cm^{-1} negative peak in Fig. 1C are assigned to Tyr. Since the UVRR intensity of Tyr is affected by its environmental hydrophobicity and state of hydrogen bonding [11,28,30], the intensity decreases may reflect a decrease in environmental hydrophobicity and/or weakening of hydrogen bonding for some Tyr residues.

Negative peaks at 1549 and 1006 cm^{-1} in Fig. 1C are ascribed to intensity decreases of the W3 and W16 bands of Trp side chains. The 1615 cm^{-1} negative peak is also ascribed partly to an intensity decrease of the Trp W1 mode. These intensity decreases indicate that the environments of some Trp side chains become less hydrophobic in acid metarhodopsin [11]. The frequency of the W3 band in the difference spectrum is 4 cm^{-1} lower than that of rhodopsin (compare spectrum A and C). This frequency difference indicates conformational changes of one or more Trp residues because the W3 frequency is sensitive to the dihedral angle, $\chi^{2,1}$, about the bond connecting the indole ring of Trp to the peptide main chain [31]. The photoisomerization of retinal induces, directly or indirectly, conformational changes of Trp residues. Trp-275 in the retinal binding pocket may be one of such Trp residues and its conformational change would result in weaker hydrophobic interactions with the β-ionone ring of retinal. Conformational changes of Trp in the process of photoactivation have also been detected by linear dichroism for frog and cattle rhodopsins [32], both of which contain a Trp residue (Trp-265) corresponding to Trp-275 in octopus rhodopsin.

4. Discussion

The present UVRR study has shown that octopus rhodopsin does not contain Tyr^- and therefore Tyr-112 cannot be a counterion to the PSB. Another candidate for the PSB counterion in octopus rhodopsin is Asp-81, which is also located near the PSB and ionizable [8]. However, an FTIR study has shown that Asp-81 is protonated both in rhodopsin and acid metarhodopsin [33]. Since octopus rhodopsin does not have any other Asp or Glu in the α-helix bundle accommodating the PSB [8], the positive charge of the PSB may not be stabilized by negative charges of amino acid residues. The pK_a of the Schiff base of bovine rhodopsin is above 16 [34] and is greatly reduced to 6.0 in the E113Q mutant, in which the counterion Glu-113 is replaced with neutral Gln [6]. The Schiff base pK_a in octopus rhodopsin is estimated to be 10.6 [35], which is in between the values for the wild-type and E113Q mutant of bovine rhodopsin. The pK_a is also suggestive of electrically neutral environments for the PSB in octopus rhodopsin. The protonated state of Schiff base in octopus rhodopsin is probably stabilized by mechanisms other than direct charge neutralization. Polarization of and hydrogen bonding with nearby amino acid residues (including Tyr-112 and Asp-81) or water molecules are candidates for the stabilization mechanisms [36,37].

The Schiff base of octopus rhodopsin remains protonated in acid metarhodopsin [2] and no protonation of Tyr (this work) or Asp [33] takes place during the rhodopsin→acid metarhodopsin transition as described above. For bovine rhodopsin, on the other hand, the PSB deprotonates and concomitantly Glu-113 is protonated on going to the biologically active photoproduct metarhodopsin II [38]. Studies on mutants of bovine rhodopsin have shown that the proton translocation from the PSB to Glu-113 is not strictly required for photoconversion to the active state [6,39]. The difference in the

stabilization mechanism of the PSB between vertebrate (bovine) and invertebrate (octopus) rhodopsins may not be important for the activation of rhodopsin.

A site-directed mutagenesis study on bovine rhodopsin has shown that replacement of Trp-265 in the retinal binding pocket by Tyr, Phe, or Ala greatly reduces the ability to activate transducin, the next protein in the cascade of signal transduction [26]. Illumination of bovine rhodopsin regenerated with a retinal analog lacking the β-ionone ring fails to produce the active state metarhodopsin II [40]. These observations suggest that the steric interaction between the β-ionone ring and Trp-265 may be important for generating the biologically active state of bovine rhodopsin. The present UVRR study on octopus rhodopsin has provided evidence for environmental and conformational changes of Trp residues concomitant with the retinal isomerization in the transition from rhodopsin to acid metarhodopsin. Interactions between the β-ionone ring of retinal and Trp-275 (corresponding to Trp-265 in bovine rhodopsin) may play a role in the photoactivation process of octopus rhodopsin.

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