The study of terbium regenerated bacterirhodopsin

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Abstract   The localization of Terbium (Tb^{3+}) cations binding to deionized bacteriorhodopsin (bR) has been studied by using spectroscopic methods. It was found that adding Tb^{3+} cations to deionized bR affects the fluorescence lifetimes of tryptophan (Trp) in bR, the wavelength of fluorescence peak shifts "blue" and the peak value of fluorescence decreases. It was also found that adding one Tb^{3+} cation to deionized bR can restore the purple state from its blue state obviously. The measurements of absorbance, fluorescence and lifetime of fluorescence also show that when more than three Tb^{3+} cations are added, no further changes can be found. It is suggested that one Tb^{3+} specific binding site for the color-controlling is located on the exterior of the bR trimer structure to negatively charged lipids near Trp-10 and Trp-12. Three Tb^{3+} cations binding per bR is needed for the regenerated bR.

Keywords: cation binding, fluorescence, bacteriorhodopsin, terbium.

Bacteriorhodopsin (bR) is the only retinal-containing protein in the purple membrane of Halobacterium halobium[1]. Upon illumination, the protein undergoes a photocycle and pumps protons across the cell membrane[2,3].

It has been found that well-washed native purple membrane contains ~1 mol Ca^{2+} and ~4 mol Mg^{2+} per mol of bR. The removal of these cations by deionization or acidification causes a color transition from purple (568 nm) to blue (605 nm).

Blue bR does not form the M_{412} intermediate, and thus does not pump protons[4]. The spectroscopic property, photochemical activity and proton pump of the protein can be restored by adding different cations to the deionized blue membrane suspensions[5, 7]. This illustrates the importance of cations in the function of bR.

It is very important to know where the locations of the cations in the bR and their exact role of bound cations in the structure and function of bR are. Electron-crystallographic and X-ray diffraction studies from 0.35 to 0.143 nm do not locate these cations in bR[8, 10]. There are two different models describing the metal binding in bR: specific binding and nonspecific binding[11, 16].

The bR protein contains eight tryptophan (Trp) and eleven tyrosine residues[17]. It has been proposed that the most of Trp residues are buried in the internal region of the membrane, these fluorescences are quenched by retinal chromophore through energy transfer. Surface Trp residues such as Trp-10, Trp-12 were recently specified as the candidates for the unquenched or partly quenched Trp residues[18].

In this paper, we examined the possible localization of Terbium (Tb^{3+}) in regenerated bR using spectroscopic methods. Adding Tb^{3+} cations to deionized bR

regenerates the purple state from its blue state. The wavelength of fluorescence peak shifts "blue" and the peak value of fluorescence decreases. Time-resolved fluorescence measurements show that two lifetime components can be found: a short lifetime component and a long lifetime component. We have found that adding Tb^{3+} cations to deionized bR affects the Trps fluorescence lifetime. We suggest that the one specific binding site for the color-controlling cation involves negatively charged lipids located on exterior of the bR trimer structure near Trp-10 and Trp-12. Three Tb^{3+} cations binding per bR is needed for the regenerated bR.

1   Materials and methods

Purple membrane was prepared from the Halobacterium halobium R_1M_1 as described in ref. [10]. The bR concentration in the pM was determined from absorption at 568 nm, using a molar extinction coefficient e = 63000 (mol L)$^{-1}$ cm^{-1} for its light-adapted state and in the blue membrane at 603 nm, e = 60000 (mol· L)$^{-1}$· cm^{-1}[11].

The deionized membrane (blue membrane) was prepared by passage of purple membrane suspension through a well-washed cation-exchange Dowex AG-50W column. The prepared blue membrane (bM) was always kept in the polystyrene vessels in order to avoid contamination of metal ions. The pH of bM suspension was 5.5±0.1 in the end.

The flash photolysis kinetic spectra were recorded on our homemade instruments[12] and data were analyzed by Govindjee's method[13]. The final curve was obtained by averaging 8 flash pulse curves.

Absorption spectra were measured on a Hitachi U-3200 UV/Visible spectrometer and steady state fluorescence spectra were measured on a Hitachi F4500 spectrometer, where the excitation wavelength was set at 280±5 nm (20±1). All the spectra were recorded at pH 5.5.

The intrinsic fluorescence lifetime of bR was determined from the phase decay at 40 MHz frequency in an Edinburgh 299T nanosecond fluorometer. The samples were excited at 295 nm and the emissions

were monitored at 330 nm. Experiment error is 0.005 ns. The bR concentration was 1.5 ì mol/L. Time-resolved data were analyzed as described by Vos et al.[19].

2   Results and discussion

It was previously proposed that the cations bind to the bR surface and raise the surface pH, or bind to the specific sites in the protein, probably in the retinal vicinity. The removal of the metal cations decreases the surface pH, allowing the protonation of Asp-85. This results in a change in the color to produce blue bR[20]. The Asp-85 residue located in the vicinity of the retinal chromophore plays a key role in the function of bR as a light-driven proton pump and photochemical cycle.

From the absorbance changes at 630 nm with the addition of Tb^{3+} to deionized bR (Fig. 1), it is noticed that the midpoint of this change is about one Tb^{3+} per bR. It suggests that this specific binding site is very important for color transition from blue to purple. When more than three Tb^{3+} cations are added, no further changes at 630 nm can be found. Adding Tb^{3+} cations to deionized bR causes the blue shift of $\ddot{e}_{max}$ of fluorescence and decreases the tryptophan fluorescence intensity of bR (Fig. 2). It means that after the binding of Tb^{3+} to deionized bR, surface Trp residues are located to more hydrophobic environments than in blue membrane, the energy transfer efficiencies are changed due to their relative orientation and distances between tryptophans and retinal, and more fluorescences of Trp-10 and Trp-12 are quenched. Because surface Trp residues such as Trp-10 and Trp-12 are specified as the candidates for the unquenched or partly quenched Trp residues, they are most likely responsible for the steady-state fluorescence emission spectra of bR. Adding two and three Tb^{3+} cations to deionized bR causes the further decrease of fluorescence. It is possible that Tb^{3+} binding induces the conformation of protein and quenches the protein fluorescence. From Fig. 2, it is noticed that steady state Trp fluorescence spectrum of bR of addition four Tb^{3+} is the same to addition three Tb^{3+} to deionized bR.
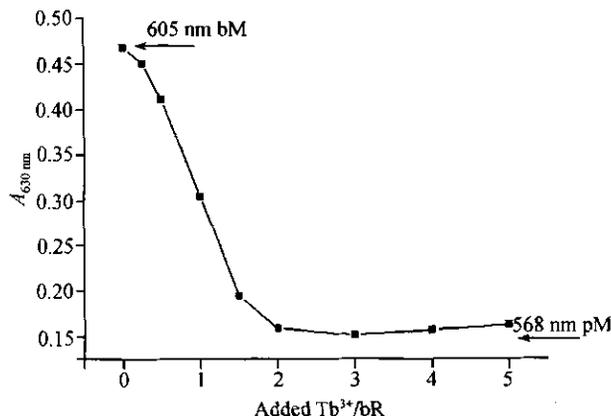
In general, the tryptophans in bR are mainly divided

Fig. 1. Absorbance of the blue membrane at 630 nm, as a function of added Tb³⁺ per bR, showing the blue-to-purple conversion. The bR concentration is 10 ì mol/L; pH 5.5; temperature 15±1 .
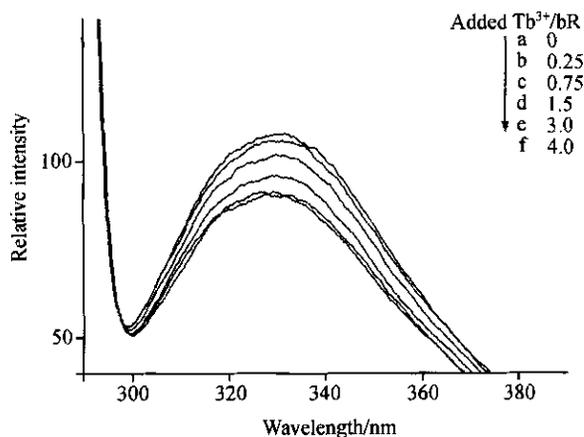

Fig. 2. Steady state Trp fluorescence spectra of bR suspended in water. Successive spectra with decreasing amplitude of bR intrinsic fluorescence correspond to the addition of Tb³⁺.

into three groups: fully quenched, partially quenched and unquenched. The partially quenched tryptophans are associated with the shot-lived component (0.5 0.9 ns). The long-lived component (3.5 5.0 ns) is found to have the same lifetime as that of retinal-free protein and this component is associated with the unquenched tryptophans[18]. The different Trps have different lifetimes according to their microenvironments and conditions. A two-exponential fit is required to describe the intrinsic fluorescence intensity decays of bR in pH under 7[21]. Under our experimental conditions, two fluorescence lifetimes can be characterized for deionized bR. When one Tb³⁺ per bR is added to deionized bR, $\hat{o}_2$ becomes shorter and $\hat{o}_1$ becomes

longer; when more Tb³⁺ are added, both $\hat{o}_1$ and $\hat{o}_2$ become shorter, but the effect is not stronger than that with one Tb³⁺ per bR (table 1). After three Tb³⁺ are added, no further changes can be found.

Table 1 Effect of Tb³⁺ on time-resolved fluorescence lifetimes of tryptophan residues in deionized bR

Tb³⁺/bM	t_1/ns	t_2/ns
Control (0)	0.416	3.44
1	0.428	3.01
2	0.408	2.81
3	0.401	2.68

The excitation and emission wavelengths were 295 nm and 330 nm.

The decay components at 0.416 ns and 3.44 ns can be associated with the residues Trp-10 and Trp-12 mainly. Adding Tb³⁺ cations to deionized bR affects the steady-state fluorescence emission spectra of bR and the fluorescence decay lifetimes of Trps, especially when the first Tb³⁺ is added. This is found to be the case for binding of Tb³⁺ and Ca²⁺ to phosphatidylcholine in which the Tb³⁺ is found to form a bidentate complex with the PO_2^- headgroups, whereas Ca²⁺ did not[22]. These results suggest that the first Tb³⁺ binding site is at the negatively charged lipids located on the exterior of the bR Trimer structure near Trp-10 and Trp-12, as shown in Fig. 3.

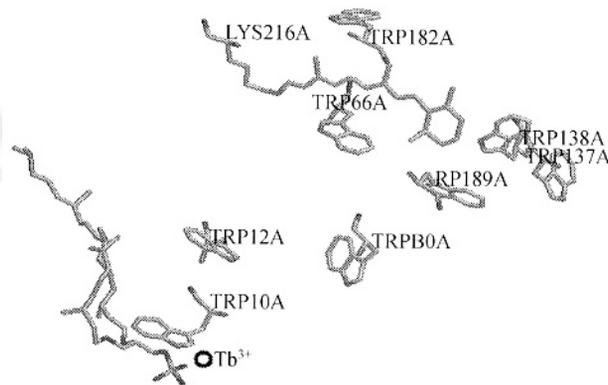Fig. 3. The proposed Tb³⁺ cation binding site in the deionized bR.

Our measurements of the M_{412} yield show the mole ratio of Tb³⁺ to bR is about 1.2 at the midpoint of restoration; after 3 Tb³⁺ are added, M_{412} yield returns to the normal (data not shown). It means that the function of bR is recovered.

The measurements of absorbance, fluorescence and

lifetime of fluorescence also show when more than three Tb^{3+} cations are added, no further changes can be found. These results suggest that three Tb^{3+} cations can bind to deionized bR. The first Tb^{3+} binding site is in the membrane lipid domain near Trp-10 and Trp-12, while others could be in their coo-groups of Asp and Glu residues of bR or PO_2^- groups of other lipids. The first specific binding site is very important for color transition from blue to purple.

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