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Absence of tryptophan fluorescence quenching by metal cations in delipidated bacteriorhodopsin

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The addition of metal cations to deionized bacteriorhodopsin (bR) quenches the steady-state tryptophan (Trp) fluorescence intensity and reduces the decay times of some of its picosecond components. Similar quenching processes of the Trp emission are not observed in deionized 75% delipidated bR (dLbR). The results are discussed in terms of conformational changes taking place in the protein upon delipidation.

Bacteriorhodopsin; Protein conformation; Tryptophan fluorescence quenching; Delipidation; Metal cation

1. INTRODUCTION

The retinylidene protein bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium halobium* [1,2]. It functions as a light-driven proton pump. Light-adapted bR contains an all-*trans* retinal, covalently bound to Lys^{216} via a protonated Schiff base linkage (PSB) [1,3]. Upon the absorption of visible light, bR proceeds through a photochemical cycle [4]:

$$bR_{568} \longrightarrow J_{625} \longrightarrow K_{610} \longrightarrow L_{550} \longrightarrow M_{412} \longrightarrow O_{640} \longrightarrow bR_{568}$$

The PSB is deprotonated during the $L_{550} \longrightarrow M_{412}$ step, leading to a proton pumping process that increases the proton concentration on the outside surface of the membrane [5]. This electrochemical gradient is in turn used for the formation of ATP.

Acidification or removal of the metal cations that are present in bR shifts the absorption maximum from 568 nm to 605 nm [6–9]. The deionized, blue form is not capable of forming the M_{412} intermediate, although the formation of the other intermediates still takes place [10,11]. However, upon removal of 75% of the lipids, which fill the spaces between the bR molecules and are in close contact with the protein, deionization does not convert bR to its blue form [12]. Moreover, deprotonation of the Schiff base still takes place, although at a lower rate than in native bR [13].

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bR contains 8 tryptophan (Trp) and 11 tyrosine (Tyr) residues which absorb at a relatively long wavelength (~280 nm) compared to other amino acid residues [14]. Trp residues are very useful as optical probes of protein structure and of the interactions of the protein with the retinal and its photocycle intermediates [15–19]. The recent work of Jang and El-Sayed [19] clearly showed the sensitivity of the Trp fluorescence quenching occurring during the bR photocycle on the different acid–base equilibria of the various acids in bR. This strongly confirmed the importance of conformational changes in the protein during the cycle [20] and their effect on not only the deprotonation of the PBS but also the Trp–retinal coupling.

It is known that excited Trp molecules transfer their energy very efficiently to the retinal (quantum yield of 0.7–0.8) which leads to an identical photocycle [16]. Removal of the metal cations from bR results in an increase in Trp fluorescence, probably because of conformational changes which reduce the energy transfer to the retinal [18].

In the present work we have carried out both steady-state as well as picosecond time-resolved studies on the influence of delipidation of bR and the addition of Eu^{3+} and Ca^{2+} to deionized bR and deionized dLbR on the Trp emission. The observed results are interpreted in terms of the sensitivity of the protein conformation on lipid charges, metal cation charges and the charges and dipoles of the protein itself.

2. MATERIALS AND METHODS

The preparation, delipidation and deionization of the bR were described previously [12]. The bR concentration of the samples used was 30 μM. During the measurements the samples were contained in 2 mm spectroscopic cuvettes.

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Steady-state fluorescence spectra were obtained using a SPEX Fluorolog spectrometer. None of the steady-state spectra reported here were corrected for the detector sensitivity as a function of wavelength. The signal from the dark current was subtracted. The fluorescence was collected from the front surface for both the steady-state and ps kinetic measurements in order to reduce the effect of fluorescence depolarization.

The details of the ps decay kinetic measurements and the extraction of the lifetimes from the kinetics were described previously [18]. The samples were excited by the fourth harmonic (266 nm) of a Quantel 471 Nd^{3+} YAG laser which produces a ~35 ps pulse. The Trp fluorescence (290–390 nm) was detected by a Hamamatsu C979 streak camera with a 10 ps temporal resolution, fitted with UV optics and coupled to a Princeton Applied Research intensified 1420 Reticon, which was interfaced to a Digital LSI 11/23 computer.

3. RESULTS

3.1. *Effect of delipidation*

Although it has been shown already that in dLbR the visible absorption maximum of the purple membrane is shifted from 568 to 561 nm [12,13], the absorption and emission maxima of Trp at 280 nm and 320 nm respectively show no shift. It is known that the $S_1(\pi,\pi^*) \leftarrow S_0$ absorption of Trp shifts to the blue in a hydrophobic and to the red in a hydrophilic environment [21]. A large structural change upon delipidation might therefore cause a distinct detectable shift. The absence of the latter clearly argues that the environment of most of the molecules is not altered significantly by the delipidation. However, it is found that delipidation leads to a reduction in the steady-state fluorescence intensity. This suggests that delipidation changes the conformation of the protein as to increase the Trp-retinal coupling leading to more quenching of Trp-emission. In order to be able to detect the subtle conformational changes occurring upon the different perturbations of the bR protein, we looked at the Trp emission before and after addition of metal cations to both deionized bR and deionized dLbR.

3.2. *Characteristics of Trp-emission in delipidated, deionized and reconstituted samples*

In the upper part of fig.1 it is shown that while delipidation decreases the steady-state Trp fluorescence, deionization increases its intensity. The addition of metal cations to deionized bR causes the protein fluorescence emission to be quenched. Jang et al. [18] found that ~20% of the Trp emission is quenched when Ca^{2+}, Mg^{2+} or Mn^{2+} ions are added at concentrations of ≥ 4 cations per bR. For Eu^{3+}, ~35% is quenched at the same concentrations. This can be seen in the middle part of fig.1. A decrease of ~30% is obtained after the addition of 4 Eu^{3+}/bR. We obtained a decrease of ~20% when Ca^{2+} was added in a similar ratio, which confirms the results of Jang et al. [18]. Remarkably enough, however, the addition of neither Eu^{3+} nor Ca^{2+} to deionized dLbR seems to affect the shape of the Trp emission. The very slight reduction that can be observed in the lower part of fig.1 upon the

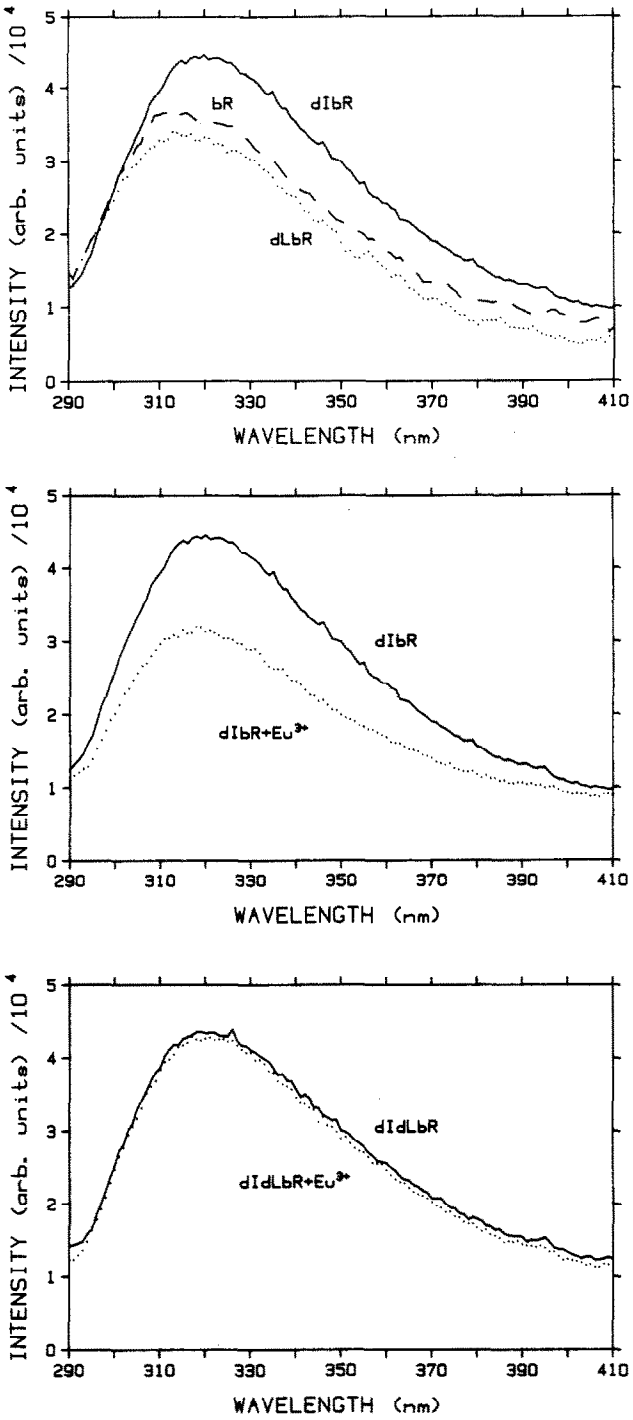

Fig.1. Steady-state Trp fluorescence spectra of bR and dLbR suspensions in water. In the upper part the spectra of dIbR (solid line), dLbR (dotted line) and native bR (dashed line) are compared. Whereas deionization increases the intensity of the Trp fluorescence, delipidation is found to have an opposite effect. In the middle and lower parts, the solid lines show the Trp-emission in the deionized samples and the dotted lines show the Trp emission after adding 4 Eu^{3+}/bR to the respective deionized samples. The samples were excited at 280 ± 3 nm. The addition of Eu^{3+} ions to the deionized bR quenches the Trp emission, but does not seem to affect the deionized dLbR. The slight reduction in the emission intensity after addition of Eu^{3+} to deionized dLbR is due to the dilution effect or to the aggregation of the sample.

addition of Eu^{3+} is probably due to aggregation of the sample, which for the delipidated samples occurs very easily after the addition of metal cations.

The Trp emission decay in native bR has been previously shown to consist of four components with lifetimes varying between 100 ps and 2200 ps [18]. The observed variation in lifetime of the different Trp molecules was attributed [18] to variations in their distances and relative orientation with respect to the retinal, thus giving rise to variations in their coupling. This results in changes in the Trp fluorescence quenching for different Trp molecules. Addition of Ca^{2+} only affects the 1000 ps component, which was shown to decrease to 800 ps [18]. This was proposed to result from changes in the protein conformation by the addition of Ca^{2+}, changing the coupling between emitting Trp molecules in the site(s) with 1000 ps lifetime.

The addition of Eu^{3+} to deionized bR was found to affect only the two middle components, which change from 200 to 150 ps and from 1000 to 700 ps [18]. The more efficient quenching due to the addition of Eu^{3+} over Ca^{2+} was attributed to the presence of electronic energy levels for Eu^{3+} below the emitting Trp energy level leading to direct energy transfer between the Trp excited states and the Eu^{3+} ions [18]. Since the transition moments of the Eu^{3+} ion are relatively small, it is expected that energy transfer occurs over relatively short distances [18].

In fig.2 (upper part) we show the effect of the addition of Eu^{3+} to dIbR on the overall decay of the Trp emission. This clearly shows a faster decay due to more efficient quenching. The bottom part of fig.2 shows that when Eu^{3+} is added to deionized dIbR, no change is observed in the Trp-decay, in agreement with the observations made above for the steady-state spectrum. For comparison all curves were scaled to the same intensity.

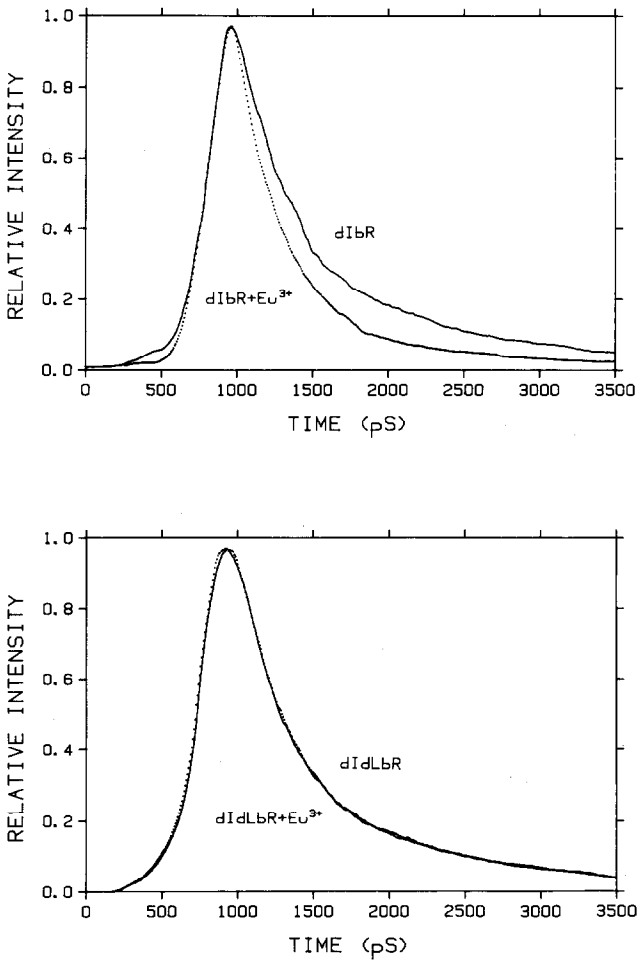

Fig.2. Trp fluorescence decay kinetics of bR (a) and dLbR (b) suspensions in water. The solid lines show the kinetics of the deionized samples and the dotted lines show the kinetics after addition of 4 Eu^{3+}/bR to the respective deionized samples. The samples were excited at 266 nm and the emission was collected at 290–390 nm.

4. DISCUSSION

In 1984, Kimura et al. [8] showed that the addition of Ca^{2+} to deionized bR induces conformational changes in the protein. They mainly based this conclusion on changes in the CD spectrum upon the addition of the metal cations. It seems also fairly obvious that the surrounding lipids control the protein conformation. When dLbR is deionized by passing it through a cation-exchange column, it does not change its color from purple to blue [12]. Moreover, Jang and El-Sayed [13] showed that whereas the removal of metal cations from bR inhibits the deprotonation step $L_{550} \longrightarrow M_{412}$, this step still takes place in deionized dLbR. Apparently, the charges on both cations and lipids are required to induce the proper conformational change within the protein that is needed for the L_{550} intermediate to deprotonate and to form the M_{412} intermediate.

The previous work of Jang and El-Sayed [19] showed that the quenching of the Trp fluorescence during the cycle is strongly dependent on the acid–base equilibria of the various acids present in bR. This confirms that the different protein conformations are sensitive to the charges within the protein as well. In fig.1 (upper part) this point is clearly illustrated: the removal of either the lipids or the metal cations changes the quenching efficiency of the Trp fluorescence, which results from changes in the protein conformation. It is important to point out that while the removal of metal cations decreases the coupling between Trp residues and retinal, the removal of the lipids leads to an increase of this coupling.

The observations presented in this paper can be qualitatively explained in terms of a positive-field model [20,22,23]. The difference between bR and dLbR is the large decrease in negative surface charge density in the latter, since most (80%) of the lipids are acidic. Metal cations, which are found to be mostly

located on the surface [24], might be necessary to balance the lipid surface charges [25]. The total field gradient thus created from the surface charges interacts with the charges and electric dipoles inside the protein and controls the protein conformational changes, which in turn control both the deprotonation process [20,22,23] as well as the Trp-fluorescence quenching process either in bR, during its cycle, or upon its modification.

The fact that the addition of metal cations does not change the intensity of Trp emission, strongly suggests that dLbR regenerated from the addition of metal cations to dIdLbR does not have the same protein conformation as dLbR (formed from the delipidation of bR). If so, then the deprotonation process can be initiated from more than one conformation of the protein in bR.

From the results and discussion presented above, one reaches the conclusion that the protein conformation of bR (and its changes during the cycle) is determined by the positive charges of the metal cations, the negative charges of the phospholipids as well as the charges and electric dipoles within the protein molecule itself. The removal of the lipids undoubtedly changes the protein conformation in such a manner as to change the metal cation binding sites [18]. In such sites the metal cations are ineffective in changing the protein conformation, and thus cannot affect the efficiency of either the Trp fluorescence quenching or the deprotonation process.

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