Eu^{3+} binding to europium-regenerated bacteriorhodopsin upon delipidation and monomerization

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Abstract **We have studied the effect of monomerization of the purple membrane lattice, as well as removal of 75% of the lipids, on the binding properties of Eu^{3+} ions. We found that delipidation and monomerization do not cause the cations to lose their binding ability to the protein. This suggests that the three most strongly bound Eu^{3+} cations do not bind to the lipids, but directly bind to the protein. Furthermore, we found that delipidation actually causes a *slight* increase in the binding affinity. This is likely a result of reduced aggregation of europium-regenerated bacteriorhodopsin (bR) upon lipid removal causing more exposure of the binding sites to the Eu^{3+} cations. These results, taken with those from our previous publication [Heyes and El-Sayed, Biophys. J. 85 (2003) 426–434], might suggest that the cations remain bound upon delipidation of bR, but have no effect on the function. This is discussed with respect to the role of cations in the function of native bR.**

Key words: Cation binding; Membrane protein; Proton pumping; Europium fluorescence

1. Introduction

Bacteriorhodopsin (bR) is the only membrane protein in the purple membrane of *Halobacterium salinarum*. It consists of seven α-helical transmembrane segments, which span the membrane approximately perpendicular to its surface, acting as a channel for the unidirectional transport of protons from the cytoplasmic side to the extracellular side of the membrane [1,2]. This proton transport is initiated by the absorption of a photon by the retinal chromophore ($\lambda_{max} \approx 570$ nm), which is bound to the lysine-216 residue, causing *trans–cis* isomerization of the 13–14 C=C bond. The isomerization causes a sequence of protonation–deprotonation reactions throughout the protein, with intermediates characterized by the temporal shift in the visible absorption spectrum (for reviews see [3–7]). The resulting pH gradient creates a proton-motive force in the cell membrane and is used by the organism for ATP synthesis [8]. This photosynthesis is much simpler than chlorophyll-based systems, thus bR has become the model of study of bacterial photosynthetic membrane proteins and ion pumps. Additionally, it has become a promising biomaterial in a wide-ranging number of optical and electronic applications [8,9].

Even though bR has been the focus of intense study from researchers in a wide range of fields from biologists, chemists and physicists through spectroscopists and crystallographers to biomolecular and electrical engineers, there are still a number of unanswered questions in this system. A number of questions that have been examined in our lab include: How and why is the protein so stable over a wide variety of conditions? What are the locations and the exact role of bound cations in the structure and function? What are the effects of the cations and lipids in controlling the structure and function? [10].

It was discovered that well-washed native purple membrane contains ~1 mol Ca^{2+} and ~4 mol Mg^{2+} per mol of bR, and that deionization of the native purple membrane forms a red-shifted absorption spectrum centered at 603 nm – called the blue membrane [11–13]. This blue bR can be formed by passing through a deionizing column [12], removal of cations by EDTA chelation [13] or by acidifying the solution [11]. All spectroscopic and biochemical tests suggest that the blue membrane formed by each method is the same species [14,15]. Furthermore, the proton pumping function of bR is halted upon deionization but can be recovered by addition of a number of different cations to the blue bR [13–16]. This illustrates the importance of cations in the function. We have recently also shown the importance of cations on the structure of the protein measured by Fourier transform infrared, and the different effects of different cations on the thermal stability [17,18]. However, it has become an extremely difficult task to determine where in the bR the cations reside and their exact role in the structure and function. Surprisingly, this task was not facilitated by the huge effort and success in crystallizing bR into three-dimensional crystals [19] and the subsequent elucidation of the structure by X-ray diffraction to 1.55 Å [20] since these structures found no cations at all. Possible reasons that may explain the lack of observed cations is that the cations are removed (partially or completely) upon crystal preparation or that the cations are not in the same location throughout the crystal (i.e. multiple heterogeneous sites throughout the crystal). In an effort to discover which of these explanations apply, and to possibly determine the exact role of lipids and lattice structure in cation binding, we have used the emission properties of a Eu^{3+} chelating agent to determine the amount of free, and therefore bound, Eu^{3+} in Eu-regenerated bR upon changing the con-

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ditions of lipidic surface charge and lattice structure. We also showed recently that if the lipids are removed from either native or deionized bR, the proton pumping efficiency is similar [21]. This suggests that the cations become less important to the function as the lipids are removed. It is not known if the cations lose binding to bR upon delipidation or if they remain bound, but have no noticeable effects on the function.

2. Materials and methods

bR was grown and isolated according to standard procedures as previously described [22]. Deionization was accomplished by passing the native bR in double-distilled water through a deionizing column (Bio-Rad AG-50W-X8) to form the blue bR according to Kimura et al. [12] and Chang et al. [23]. The stock solution of blue bR was split into three samples at 2 μM blue bR. Regeneration of the blue bR with Eu^{3+} was achieved by addition of 6 μM $EuCl_3$ stock solution to equal amounts of the blue bR. This produced three samples (2 ml volume of each) at 3:1 Eu:bR. The solutions were left to equilibrate overnight. 2 ml of 40 mM CHAPS was added to one sample, and 5% Triton X-100 was added to the second to produce the 75% delipidated and the monomer Eu-bR samples respectively. Double-distilled water was added to the third as a control. After equilibration, the samples were centrifuged and the supernatant analyzed.

The concentration of Eu^{3+} bound to the bR is found using the methods adapted previously [24,25]. Briefly, a chelating agent used to detect the free Eu^{3+} fluorescence in the supernatants of the Eu^{3+} regenerated bR solutions. The chelating agent is a thenoyltrifluoroacetone (TTA)–trioctyl phosphine oxide (TOPO) mixture, which specifically binds trivalent lanthanide and actinide elements [26]. The chelating agent was made up to a concentration of 1×10^{-4} M TTA and 1×10^{-3} M TOPO in 0.1 M acetate buffer at pH 3.6. Free Eu^{3+} in equilibrium with bound Eu^{3+} is measured by separating the bR from the solution by centrifugation and/or filtration, adding the chelating agent and measuring the fluorescence spectrum upon excitation at 346 nm. Standards of Eu^{3+} (from $EuCl_3$) with the chelating agent are prepared and a calibration curve measured beforehand. Control measurements were also performed with Eu^{3+} in the presence of the detergents to ensure that they do not quench the fluorescence from the chelated complex. All experiments are repeated several times to ensure reproducibility.

3. Results and discussion

Following the conclusions of photocycle recovery of deionized bR upon delipidation [21], we were interested to discover if this is due to cation removal, or due to the cation remaining bound, but the binding sites becoming unimportant for the function upon lipid removal. The answer to this question will have important consequences for discovering the role of the cation in native bR. We first attempted this on the native bR, using ICP-OES to determine Ca^{2+} and Mg^{2+} ions after adding the detergents. However, we found these results to be irreproducible. The source of this irreproducibility is unknown, but may be that the detergent matrix causes technical problems in the ICP analysis. We then decided to use the strong emitting nature of Eu^{3+} chelates to investigate the effects of cation binding to bR upon delipidation. This sensitive fluorescence method has been used previously [24,25] to assay specific detection of free Eu^{3+} in equilibrium with membrane-bound Eu^{3+} in order to calculate their binding constants to the deionized blue membrane. Aqueous Eu^{3+} in the presence of the chelating agent TTA in TOPO has an excitation and emission spectrum as shown in Fig. 1. The strong emission at 620 nm is due to $^5D_0 \rightarrow {}^7F_2$ transition. The free ions in solution show weak fluorescence, and two bands (at 620 nm and 590 nm) of approximately equal intensity, due to $^5D_0 \rightarrow {}^7F_2$ and $^5D_0 \rightarrow {}^7F_1$ respectively, are observed. When Eu^{3+} is complexed to TTA–TOPO, the $^5D_0 \rightarrow {}^7F_2$ transition becomes much more intense, and highly favored over $^5D_0 \rightarrow {}^7F_1$ [26]. This effect can be taken advantage of in very sensitive cation binding assays to proteins. Fig. 2B shows that the Eu^{3+} standards in the chelating solution obey Beer's law in the range 0–2 μM, which is the range used for the experiment. These results are in agreement with previously published work on Eu^{3+} assays [24,25].

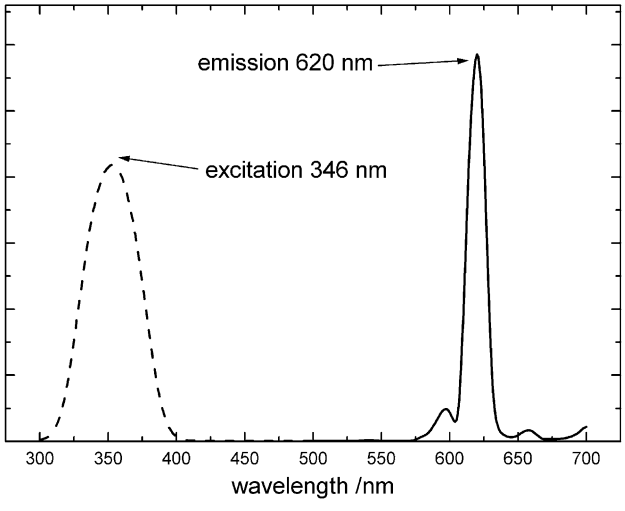

Fig. 1. Excitation and emission spectra of the Eu–TTA chelate.

Table 1
Bound Eu^{3+} per bR for untreated, 75% delipidated (CHAPS) and monomerized (Triton) Eu^{3+}-regenerated bR

	Bound Eu^{3+}:bR at 3:1 Eu:bR regeneration ratio
Non-treated Eu-bR	2.71 ± 0.10
75% Delipidated	2.93 ± 0.12
Monomer	3.00 ± 0.14

Standard deviations are calculated from multiple experiments (>3) and errors in linear fit to calibration curve.

The specific effects of Eu^{3+} binding to bR, which has been regenerated at 3:1, are calculated from the fluorescence spectra shown in Fig. 2B using the calibration curves and correcting for dilution. This provides the free Eu^{3+} in equilibrium with the bound Eu^{3+}. Since we know the total Eu^{3+}, the bound Eu^{3+}:bR ratio is calculated. The results are shown in Table 1. This table shows the effects of the bound Eu^{3+} upon 75% delipidation with CHAPS and upon monomerization into Triton X-100 micelles. At a 3:1 (Eu:bR) regeneration ratio, there are 2.71 mol of Eu^{3+} per mol of unperturbed blue bR. Upon treatment with CHAPS, it was found that the number of bound Eu^{3+} per bR was 2.93, and upon monomerization all 3.00 Eu^{3+} were bound. This suggests that delipidation causes slightly stronger Eu^{3+} binding, not less. Thus, reducing the negative charges on the surface does not eliminate Eu^{3+} binding. This can only be the case if there is specific binding to the protein side chains, and not limited to the negative membrane surface. To ensure that these results are not due to quenching of Eu–TTA fluorescence, control measurements of standards of Eu^{3+} with TTA in the detergent were performed. No quenching was seen in these controls,

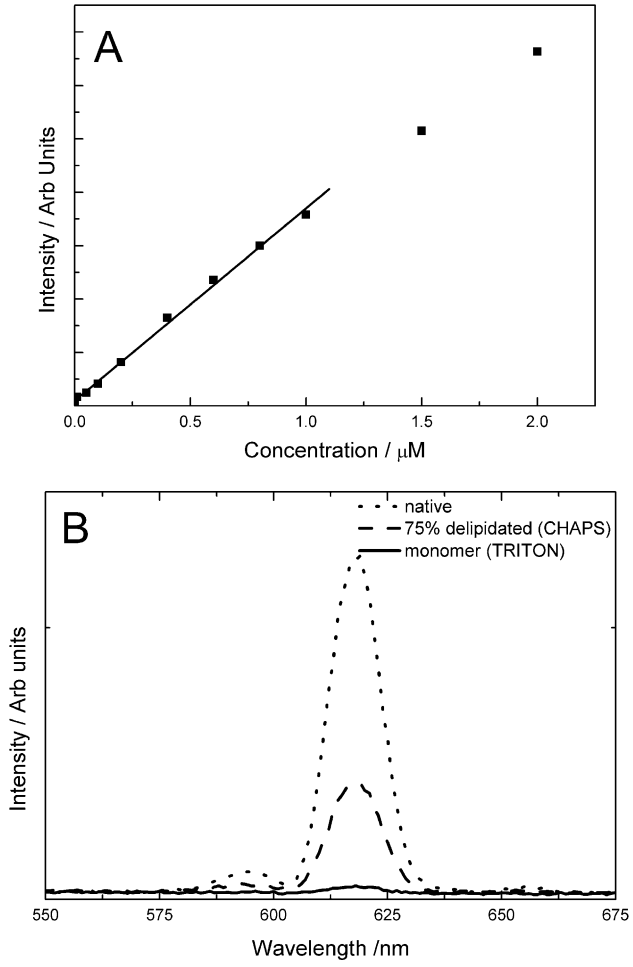

Fig. 2. A: Calibration curve of the emission of Eu^{3+} standards at 620 nm to show that Beer's law is obeyed in the concentration range below 1 μM. B: Fluorescence of the TTA–Eu complexing to free Eu^{3+} for the three Eu-bR samples.

indicating that the results are due to a decreased amount of free Eu^{3+}.

The possibility that the Eu^{3+} remained bound to the detergent-solubilized lipids, rather than the chelating agent, arose since lanthanides are trivalent and have an affinity for the sulfate and phosphate headgroups of the lipids. Thus, we added 300 mM $CaCl_2$ to shield the dissolved lipidic head groups so that the Eu^{3+} is free in solution to be chelated to the TTA/TOPO chelate. It was verified that Ca^{2+} does not bind to TTA/TOPO by comparing the emission intensity with and without Ca added to the standards, and no difference is observed (results not shown). This agrees with the specificity of TTA/TOPO for Eu^{3+} reported [26]. The solubilized lipidic charges produce an electrostatic effect, unaffected by geometry, and should show no such specificity. Since Ca^{2+} is present in much larger concentrations than Eu^{3+} (10^5 times more), it is expected that the lipids would bind Ca^{2+} instead of Eu^{3+}, thus verifying that the Eu^{3+} is still bound to the protein upon delipidation.

The fact that there are more Eu^{3+} per bR upon delipidation is unexpected. However, it is known that Eu^{3+} (and other lanthanides) cause aggregation of bR. We have cut down this aggregation by using low concentrations (1 μM bR), and only 3:1 regeneration ratios, but it is still present. The aggregation is greatly reduced if the sample is treated with the CHAPS or Triton detergents and this may be the reason for the apparent increase of Eu^{3+} binding upon lipid removal. The decrease in aggregation may expose more cation binding sites to the free Eu^{3+}, and thus bind more efficiently. However, this increase is small, and only slightly outside experimental error, and thus the increase is a very minor consequence. In any case, all these results suggest that the three high affinity Eu^{3+} sites are primarily bound to the protein, not to the lipidic surface.

The results presented here suggest that the recovery of the photocycle associated with lipid removal from deionized bR is not connected with the cation being bound [21]. We had found that the photocycle is the same whether delipidation is performed from native or deionized bR. This led to the question of whether delipidation actually removes the bound cations, or whether they remain bound, but do not affect the function. Whilst the question of whether or not the bound native cations (Ca^{2+} and Mg^{2+}) are affected upon delipidation is not answered, it seems that the bound Eu^{3+} ions do remain bound. Thus it is probable that the native cations also remain bound upon delipidation, but become less important for the proton pumping function.

The observation that monomeric bR has three high affinity binding sites for Eu^{3+} is very interesting in terms of the role of cations in the native bR. It has been postulated that the role of cations in the native bR is only to regulate the surface pH by shielding the negative lipidic charges, thereby indirectly stabilizing the deprotonated Asp85 [27,28]. Since 75% delipidation and monomerization still retains the ability of three Eu^{3+} cations to remain bound to the protein, this suggests binding directly to the protein side groups, as previously discussed [13,15,16]. While these experiments cannot determine exactly where in the protein the cations are bound (or even if Eu^{3+} occupies the same sites as the native Ca^{2+} and Mg^{2+}), these results lend support to a direct protein binding model in which their binding must be controlled by the pK_a of the side group binding sites. The ability to function (defined in this case whether the proton transfer intermediate, M, is formed or not) is determined by the initial protonation of Asp85 (the proton acceptor). In native bR, the cation binding sites and Asp85 deprotonation have been proposed to be coupled [29–31], possibly by binding to the extracellular exposed Glu residues [32]. When the lipid bilayer is present, and the surface is highly negatively charged, Asp85 deprotonation is highly unfavored unless a stabilizing positive charge is present. The extra four Glu residues on the extracellular surface would at first sight seem to further hinder Asp85 deprotonation, but may have been specifically designed by nature with two functions related to cation binding. The first is to geometrically chelate cations with very high affinity to shield the negatively charged surface. This geometry is primarily octahedral, but is somewhat flexible due to water being some of the ligands to the cations [32]. This also helps to explain the ability of large organic cations to bind to the bR and retain activity [33]. The second function may be to allow the cations to act through a water-mediated hydrogen-bonded channel from the surface to Asp85, withdrawing electron density and stabilizing the deprotonation of Asp85. This polarization may then help to increase the Asp85 pK_a during the photocycle (together with water movements and H-bond changes in the immediate retinal vicinity) in order for the Schiff base to transfer its proton

during the L to M transition. Removing the lipids does not cause the groups to lose binding to the cation, since the geometry of the extracellular Glu residues is not significantly changed, but the deprotonated Asp85 may already be stabilized enough upon lipid removal for the occupation of the binding sites to be no longer important. The cation would only serve to stabilize even more the deprotonated Asp85, which is probably not necessary once at least 75% of the lipids are removed.

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