Detection of a Yb^{3+} binding site in regenerated bacteriorhodopsin that is coordinated with the protein and phospholipid head groups

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ABSTRACT Near infrared Yb^{3+} vibronic sideband spectroscopy was used to characterize specific lanthanide binding sites in bacteriorhodopsin (bR) and retinal free bacteriorhodopsin (bO). The VSB spectra for deionized bO regenerated with a ratio of 1:1 and 2:1 ion to bO are identical. Application of a two-dimensional anti-correlation technique suggests that only a single Yb^{3+} site is observed. The Yb^{3+} binding site in bO is observed to consist of PO_2^- groups and carboxylic acid groups, both of which are bound in a bidentate manner. An additional contribution most likely arising from a phenolic group is also observed. This implies that the ligands for the observed single binding site are the lipid head groups and amino acid residues. The vibronic sidebands of Yb^{3+} in deionized bR regenerated at a ratio of 2:1 ion to bR are essentially identical to those in bO. The other high-affinity binding site is thus either not evident or its fluorescence is quenched. A discussion is given on the difference in binding of Ca^{2+} (or Mg^{2+}) and lanthanides in phospholipid membrane proteins.

Bacteriorhodopsin (bR) is the only protein in the purple membrane of the halophilic archaebacterium *Halobacterium salinarum*. This protein is capable of light-driven transmembrane-proton translocation during a specific photochemical cycle (1–3) involving a retinal chromophore in a protonated Schiff base linkage with the lysine residue Lys-216 (4–6).

The binding of metal cations, specifically Ca^{2+} and Mg^{2+} , is now recognized as essential for the proper functioning of the proton pumping mechanism of bR (7, 8). Removal of the cations by deionization or acidification shifts the absorption maximum of purple membrane to 604 nm (blue bR) (9, 10). The absorption maximum of native bR (560–570 nm) returns upon the addition of different metal cations (9, 10). The initial step in the bR photocycle involves the excitation of the retinal by absorption of a photon (11–15). This step is followed by isomerization of the initially all-trans retinal to form the 13-cis retinal (11–15). Thermal relaxation leads to several intermediates, the net result being translocation of a proton from the cytoplasmic to the extracellular side of the membrane. Eventually, bR returns to its ground state from which it can undergo another photocycle (11–15). While retinal photoisomerization takes place in blue bR, the M_{412} intermediate is not formed and, thus, proton pumping does not occur (9, 10).

The locations of the cation binding sites are still not well known. Stroud and colleagues (16, 17) have observed several Pb^{2+} binding sites in bR via x-ray diffraction. The ligands that bind the cations cannot be identified from such studies due to the present inability to form three-dimensional crystals of bR. To understand the role that these metal cations play in the function of bR, it is necessary to know the location of the

cations in the protein and the chemical nature of the ligands that bind them. For Ca^{2+} binding proteins, such as bR, the latter is difficult to probe because Ca^{2+} possesses no intrinsic spectroscopic properties that could be exploited for study. However, a number of binding studies have been performed with potentiometric titrations using Ca^{2+} specific electrodes and ESR studies of Mn^{2+} binding (18–24). In all of these studies multiple high-affinity binding sites were observed. pH titration studies (21) suggest the presence of a Ca^{2+} high-affinity site involving aspartic residues D85 and D212. Mutation studies of Ca^{2+} binding observed a large change in the binding constant upon replacement of D85 and D212 by neutral amino acid residues (22, 23). The studies on Eu^{3+} emission and its binding results suggest that Eu^{3+} is not far from the retinal to allow efficient quenching of its emission (25). More recently, Fourier transform infrared (FTIR) studies (26) showed that a vibrational band resulting from the interaction of the aspartate group (COO^-) and Ca^{2+} , while present in bR, disappears in the D85N mutant, as well as in deionized bR. This suggests the presence of Ca^{2+} near D85, which is the active site of bR. Most recently, Stuart *et al.* (27) could calculate the two photon spectrum of the retinal in bR only if the Ca^{2+} is placed near D85 and D212 within the active site.

In this paper we use the technique of Yb^{3+} vibronic side band (VSB) spectroscopy (28) to probe cation binding sites in bR. Iben *et al.* (29) first applied Gd^{3+} VSB spectroscopy to study proteins; our method, using Yb^{3+} as the lanthanide probe, extends the VSB technique into the near-infrared (NIR). For this method, the spectroscopically silent native ions are replaced with fluorescent Yb^{3+} cations. When coordinated with ligands, Yb^{3+} luminescence exhibits a sharp 4f–4f electronic feature (zero phonon line; ZPL) corresponding to the $^2\text{F}_{5/2} \rightarrow ^2\text{F}_{7/2}$ electronic transition (≈ 970 nm), as well as weak VSBs shifted to longer wavelengths. These VSBs are the result of short-range dipole–dipole interactions that couple the 4f electronic and ligand vibrational states and reflect the vibrational modes of the ligands (28, 29). Each type of ligand can be shown to exhibit a distinct set of VSBs. Thus, comparison of experimental VSB spectra with those of model ligand complexes allow identification of the ligands bound to the Yb^{3+} in the metal binding protein of interest.

Results are presented for bR and retinal free bacteriorhodopsin (bO) regenerated with Yb^{3+} . The bO sample was investigated because cleavage of the retinal significantly reduces the Raman signal, which could obscure VSBs in bR.

MATERIALS AND METHODS

Purple membrane was prepared from the *H. salinarum* strain ET1001 as described (30). Blue membrane was prepared by

Abbreviations: bR, bacteriorhodopsin; VSB, vibronic side band; bO, retinal free bacteriorhodopsin; ZPL, zero phonon line; PGP, phosphatidylglycerol phosphate; NIR, near-infrared.

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passing the purple membrane over a cation exchange column (Bio-Rad). The final pH of the blue membrane was ≈ 4 with $\lambda_{\max} = 604$ nm. All samples were suspended in nanopure water (resistivity 10–18 M Ω /cm) and kept in plastic tubes to avoid contamination from other cations. Concentration of the protein was determined using the following extinction coefficients: $\epsilon_{570} = 63,000$ cm $^{-1}$ M $^{-1}$ and $\epsilon_{604} = 60,000$ cm $^{-1}$ M $^{-1}$. The purple membrane was regenerated from the deionized blue membrane at ratios of 1:1 and 2:1 cation to bR by addition of YbCl $_3$ solutions. Bleached bR (bO) was prepared by hydroxylamine treatments as described by Wu *et al.* (31). The sample was then washed with nanopure water three times to remove hydroxylamine. Deionized bO was prepared by dialysis against the same cation exchange resin used for native bR. Stoichiometric amounts of YbCl $_3$ were then added to prepare samples with cation to bO ratios of 1:1 and 2:1. The stoichiometry in bR and bO was verified using atomic absorption spectrometry (Perkin–Elmer HGA 300-2380 spectrophotometer). The bR samples were concentrated down to a paste or lyophilized under vacuum from aqueous or D $_2$ O (D = 2 H) solutions, for bO only from aqueous solutions.

Yb $^{3+}$ complexes of phosphatidylglycerol phosphate (PGP) (Avanti Polar Lipids) were prepared by mixing stoichiometric amounts of an aqueous solution of YbCl $_3$ (50 mM in D $_2$ O) with PGP in ethanol (50 mM). The resulting precipitate was concentrated by centrifugation. The Yb $^{3+}$ /P $_2$ O $_7^{2-}$ complex was prepared by mixing 1 ml of a 120 mM Yb $^{3+}$ D $_2$ O solution with 1 ml of 440 mM P $_2$ O $_7^{2-}$ in D $_2$ O solution (pD 7.5). All reagents were analytical grade.

Low temperature (15K) NIR Yb $^{3+}$ VSB spectra were recorded as described (28, 32, 33). Fourier transform Raman spectra of bR using 1064 nm excitation were recorded using the same instrument except that a diode-pumped Nd:YAG laser was used as the excitation source (34).

RESULTS

VSBs of Bacterioopsin. As a control for the bR experiments, we have investigated Yb $^{3+}$ regenerated bO. Fig. 1 shows the Yb $^{3+}$ VSB spectra of bO containing 1:1 and 2:1 ions obtained by excitation at 878 and 965 nm, respectively. Raman bands, which were identified by shifting the wavelength by ≈ 30 cm $^{-1}$ are indicated by R 28 . VSBs that do not shift with excitation wavelength can be clearly identified at 775, 1112, 1192, 1268, 1445, 1510, 1570, 1890, and 1970 cm $^{-1}$.

The intense 775 cm $^{-1}$ (Figs. 1A and 2B) band has not been observed in previous studies of various Yb $^{3+}$ complexes (28). However, none of the previous studies involved integral membrane proteins and, thus, it is possible that this band arises from lipid contributions. To investigate this possibility, VSB spectra were recorded for Yb $^{3+}$ complexes of PGP and P $_2$ O $_7^{2-}$ (Fig. 2C). These spectra show intense 735 and 796 cm $^{-1}$ bands, which can be assigned to the symmetric P–O stretching mode. These observed values correlate well with our VSB frequency of 775 cm $^{-1}$. The assignment of the 775 cm $^{-1}$ band to a phosphate group is supported by previous work of Zheng *et al.* (35). In the mid-frequency region, the Yb $^{3+}$ /P $_2$ O $_7$ complex exhibits VSBs at 1074 and 1190 cm $^{-1}$, whereas the Yb $^{3+}$ /PGP complexes show VSBs at 1115 and 1190 cm $^{-1}$. The lower frequency bands in each complex are attributed to the PO $_2^-$ symmetric stretching mode, whereas the higher frequency bands are assigned to the PO $_2^-$ anti-symmetric stretching mode (36, 37). By analogy, the 1112 and 1192 cm $^{-1}$ VSBs for bO are assigned to the PO $_2^-$ symmetric and anti-symmetric stretches, respectively.

In the bO spectrum, the frequency difference between the PO $_2^-$ symmetric and anti-symmetric bands is $\Delta = 70$ cm $^{-1}$, which indicates (38) that these phosphate groups are coordinated in a bidentate manner to Yb $^{3+}$. Similar conclusions were reached by FTIR for the binding of lanthanides to bR (39).

The intense VSBs in the bO spectrum at 1890 and 1970 cm $^{-1}$ are high in frequency and most likely arise as overtones from combinations of the 775 and 1112 cm $^{-1}$ bands and the 775 and 1192 cm $^{-1}$ bands, respectively. The close agreement in frequency for the combination bands supports the assignment of the 775, 1112, and 1192 cm $^{-1}$ bands as originating from the same chemical group, i.e., the PO $_2^-$ of the phospholipid head groups.

The other observed VSBs (1268, 1445, 1510, and 1570 cm $^{-1}$) cannot be attributed to the phospholipid groups. The 1445 and 1570 cm $^{-1}$ bands can be assigned to the symmetric and anti-symmetric stretching modes, respectively, of carboxylate CO $_2^-$ groups. These modes are expected at approximately 1400 and 1600 cm $^{-1}$, respectively (40). The 125 cm $^{-1}$ difference observed for the 1445 and 1570 cm $^{-1}$ bands in the bO spectra indicates that the ligand(s) binds in a bidentate manner (28, 40).

The 1510 cm $^{-1}$ band is attributable to a phenyl C=C stretching mode (28, 40). In previous studies of various Yb $^{3+}$ complexes, VSBs have been observed at approximately 1500 cm $^{-1}$ in cases where the ligand possessed an aromatic cyclic

FIG. 1. Near infrared Yb $^{3+}$ VSB spectra of bO samples containing one Yb $^{3+}$ (Upper) and two Yb $^{3+}$ (Lower) atoms per bO. (A) Low frequency spectra: 878 nm, 450 mW, 500 scans. (B) High frequency spectra: 965 nm excitation, 450 mW, 10,000 scans.

FIG. 2. Near infrared Yb^{3+} VSB spectra Yb^{3+} regenerated bR and bO. (A) Zero phonon lines for samples of bR containing two Yb^{3+} (Bottom; 870 nm excitation, 20 mW, 100 scans), bO containing one Yb^{3+} (Middle: 878 nm excitation, 650 mW, 1000 scans), and bO containing two Yb^{3+} atoms (Top: 884 nm excitation, 500 mW, 1000 scans). (B) VSB spectra of bR in the 700–2000 cm^{-1} VSB shift region, excited at 965 nm (Lower: 250 mW, 2000 scans) and 962 nm (Upper: 250 mW, 2000 scans). Obvious Raman bands from the retinal chromophore are designated R. (C) VSB bands arising from the phosphate symmetric P–O stretching mode of the PGP (Lower: 886 nm, 180 mW, 200 scans) and $\text{P}_2\text{O}_7^{2-}$ (Upper: 876 nm, 450 mW, 100 scans) Yb^{3+} complexes.

group such as a phenol (e.g., tyrosine) (28). To corroborate the identification of such a phenolic group, it is expected that there would be a VSB at approximately 1280 cm^{-1} corresponding to the C—O stretching mode (28). The 1268 cm^{-1} band seen in Fig. 1A is a good candidate for this mode. Thus, the presence of both the 1510 and the 1268 cm^{-1} bands strongly suggests that tyrosine is a ligand to the Yb^{3+} ion in bO.

Fig. 2A shows the ZPL, which corresponds to the principal Yb^{3+} fluorescence, of 1:1 and 2:1 Yb^{3+} regenerated bO. There is only one ZPL at 10,307 cm^{-1} , which is 25 cm^{-1} broad (FWHM). The ZPL does not change in frequency or width when one or two Yb^{3+} atoms are reconstituted into the protein.

Taken together, the above results indicate that we are observing the emission from one Yb^{3+} population (site) in bO and that this one population of Yb^{3+} ions is coordinated to both the phospholipid head groups and the protein. To verify that only one spectroscopically distinct Yb^{3+} binding site is contributing to the VSB spectra of bO, we have analyzed various VSB spectra using a two-dimensional correlation technique (33). This method consists of recording the VSB spectrum of Yb^{3+} in bO at several different excitation wavelengths and analyzing the spectra for changes in relative band intensities, which arise from variations in excitation efficiencies of Yb^{3+} ions having slightly different spectral properties because of different environments. This technique is extremely sensitive and accurate in resolving two different Yb sites in human serum transferrin, which differed only by 15 cm^{-1} in their respective ZPLs (33). Using this same method for bO samples containing both one and two Yb^{3+} per bO, only one type of binding site could be resolved. If two emitting Yb^{3+} sites are present in bO, then we estimate that they cannot differ by more than 15 cm^{-1} .

VSBs of bR. Fig. 2 shows the VSB spectra of Yb^{3+} reconstituted bR (2 Yb^{3+} per bR) excited at 962 and 965 nm. The spectra contain contributions from Yb^{3+} /bR VSBs as well as Raman bands arising mainly from the strongly scattering retinal chromophore. The VSBs, which do not shift with excitation wavelength, are clearly identified at 775, 1510, 1570, 1890, 1970 cm^{-1} .

To identify less obvious VSBs, which are masked by the Raman bands, we performed two experiments. First, the VSB spectrum was recorded using 1064 nm excitation under comparable experimental conditions. At this wavelength, the transition probability to excite Yb^{3+} fluorescence is low and, thus, only bR Raman bands are observed (Fig. 3). These Raman contributions can be subtracted from the VSB spectrum excited at 965 nm and the difference spectrum is shown in Fig. 3A. Two well-resolved bands at 1190 and 1120 cm^{-1} and a possible weak band at 1268 cm^{-1} are observed in the difference spectrum and can thus be attributed to Yb^{3+} VSBs. Second, a VSB spectrum was recorded using 945 nm excitation. At this wavelength, there is no retinal contribution in the 1400–2000 cm^{-1} VSB region (Fig. 3B). From this spectrum, obvious bands can be seen at 1445, 1510, 1570, 1890, and 1970 cm^{-1} of the VSB spectral region, which do not arise from bR Raman contributions and are also attributable to Yb^{3+} /bR VSBs.

We noted two important points concerning these spectra: (i) The VSBs of bR are the same as those observed for bO and, thus, the interpretation of the Yb^{3+} ligands and their vibrational modes is the same as made earlier for bO. This means that both phospholipid head groups and protein residues are involved in the binding of Yb^{3+} in bR and bO. (ii) The ZPL of bR is the same as that observed for 1:1 and 2:1 bO samples (Fig. 2A). The assignments of the VSBs for bR and bO are summarized in Table 1. As for the bO case, application of the two-dimensional correlation method (33) was unable to resolve two distinct binding sites for bR.

DISCUSSION

The above observations indicate that we are observing only one emitting Yb^{3+} site for bO and that this site is the same observed site for bR. It is proposed that the observed Yb^{3+} is coordinated both by phospholipid head groups and protein residues, specifically carboxylic acids and at least one tyrosine.

As mentioned earlier, the position of the ZPL is sensitive to the nature of the Yb^{3+} ligands. If one Yb^{3+} ion was in an

FIG. 3. (A) Near infrared Yb^{3+} VSB spectrum, excited at 965 nm, of bR containing two Yb^{3+} atoms per bR in the 1100–1300 cm^{-1} VSB shift region (Top: 250 mW, 2000 scans) and the Fourier transform Raman spectrum of the same bR sample excited at 1064 nm (Middle: 280 mW, 2000 scans); 1064 nm excitation results in the observation of essentially only the Raman contributions of the retinal chromophore. Subtracting the retinal Raman contributions (middle spectrum) from the top spectrum results in a difference spectrum (Bottom) revealing VSB bands previously hidden by the Raman contributions. (B) VSB spectrum of the same bR sample excited at 945 nm (Upper: 450 mW, 2000 scans). At this wavelength of excitation, the retinal Raman contributions are not present in the 1400–2000 cm^{-1} VSB shift spectral region. The Fourier transformed Raman spectrum of the same bR sample is shown for comparison (Lower: excitation 1064 nm, 280 mW, 2000 scans).

exclusively membrane domain, whereas the other was in an exclusively protein domain, it is highly unlikely that these two Yb^{3+} ions, which experience different environments, would have the same ZPL. In the event that the two Yb^{3+} atoms are accidentally degenerate, then it is likely that one would observe differences in relative intensities of the VSBs as the laser excitation is changed. We have failed to observe any of these points

A reconstructed VSB spectrum of the specific Yb^{3+} site can be obtained from the two-dimensional correlation method mentioned above (33) and is shown in Fig. 4. The reconstructed spectrum accurately reproduces the 1112, 1192, 1268, 1445, 1510, and 1570 cm^{-1} bands (as well as the 775, 1890, and 1970 cm^{-1} bands, data not shown), which are observed in the raw spectra.

The observation of only one type of binding site for bO is consistent with recent results (41) in which it was observed that the binding characteristics of the two high-affinity sites of bR are altered upon retinal cleavage. Specifically, only one high-affinity site is observed in bO, which exhibits an apparent binding constant intermediate between the first and second high-affinity binding sites in deionized bR. The similarity of

the VSB spectra reported here for bR and bO indicate that one of the two high-affinity binding sites for Yb^{3+} in bR is conserved in bO in terms of the nature of the ligands, although its binding constant is altered. The strong similarity between the VSB spectra of bO and bR suggest that the sites observed for both bO and bR are the same. The other high-affinity site of bR, if it is different, is spectroscopically silent. The failure to observe the second Yb^{3+} in bR is most likely due to a quenching of its fluorescence, probably by a water ligand or other ligand possessing an OH group. It is well known that H_2O ligands strongly quench fluorescence due to strong vibrational coupling (42). Indeed, we have failed to observe the high-frequency VSBs of $\text{Yb}^{3+}/\text{H}_2\text{O}$ complexes under our experi-

FIG. 4. VSB spectrum of bO containing one and two Yb^{3+} atoms per bO reconstructed from the two-dimensional correlation analysis. (Inset) Schematic diagram showing the possible configuration of the ligands of Yb^{3+} suggested by the results of this work.

Table 1. VSBs observed in the Yb^{3+}/bR and Yb^{3+}/bO spectra, and their proposed assignments (cm^{-1})

VSB, cm^{-1}	Assignment
775	ν_{sym} P–O
1112	ν_{sym} PO_2^-
1192	ν_{asym} PO_2^-
1268 (w)	ϕ C–O
1445	ν_{sym} CO_2^-
1510	C=C
1570	ν_{asym} CO_2^-
1890	ν_{sym} P–O + ν_{sym} PO_2^-
1970	ν_{sym} P–O + ν_{asym} PO_2^-

w, weak; ν , stretching vibration; sym, symmetric; asym, anti-symmetric; ϕ , phenolic ring.

mental conditions, even for YbCl₃ saturated H₂O solutions. However, in D₂O, the Yb³⁺ VSBs are not quenched.

A proposed schematic diagram of the Yb³⁺ binding site deduced both for bO and bR consistent with the observed results is shown in Fig. 4. The essential feature of this model site is that the Yb³⁺ is fixed in a site that includes at least one protein carboxylic acid residue, at least one tyrosine residue, and at least one phospholipid head group, suggesting that the Yb³⁺ is near the surface of bR.

A number of spectroscopic (26, 27) and potentiometric titration (21, 22) studies of bR and some of its mutants lead to the conclusion that Ca²⁺ binds to amino acid residues not far from the retinal. Furthermore, Ca²⁺ in the second high-affinity site (7, 8, 22) is responsible for the color change in regenerated bR. Lanthanides are expected to have greater binding affinities for phospholipid head groups than do Ca²⁺ or Mg²⁺. 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 PO₂⁻ headgroups, whereas Ca²⁺ did not (38).

Yb³⁺ regenerated bR is purple in color. If Yb³⁺ occupies only one type of surface (or near surface) site having the structure shown in Fig. 4, it is possible that this could lead to color change by controlling the surface pH (43). If another Yb³⁺ occupies another protein site near the retinal, as proposed (21, 22, 26) for Ca²⁺, it must not strongly radiate due to either a weak ligand field or quenching by H₂O molecules or other OH groups.

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