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X-ray absorption studies on bacteriorhodopsin

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Extended X-ray absorption (EXAFS) measurements of iron-substituted bacteriorhodopsin revealed for the light-adapted state (bR^{LA}) a homogeneous distribution of the first-shell ligands of iron. In Contrary to this result, the dark-adapted (bR^{DA}) and the M-state (bR^{M}) display significant heterogeneity at the iron site with bR^{DA} intermediate between bR^{M} and bR^{LA} in average distance. These data provide direct evidence of structural changes at the membrane associated with photocycle changes at the cation-binding site.

Bacteriorhodopsin; Metal-substituted bacteriorhodopsin; Photocycle; M-intermediate; EXAFS

1. INTRODUCTION

The purple membrane of *Halobacterium halobium* consists of a single protein, bacteriorhodopsin (bR), which upon light excitation converts the light energy into a proton concentration gradient (reviews [1,2]). The purple color of the chromophore is derived from the specific interaction of a protonated retinylidene Schiff base with the protein. Recently, it was shown that the removal of cations from the membrane results in a 'blue membrane' with an absorption maximum at 604 nm. The purple color can be regained by the addition of cations [3–12]. This observation made it possible to study the influence of different cations on the structure and function of bR. It was shown that they interfere with the photocycle and proton pump [5]. These data [5] and data from the literature [4,6] showed that there are two high-affinity sites, whose dissociation constants are separated by one to two orders of magnitude. On the basis of these results it became possible to saturate one binding site only. Here we report the results of an analysis of this binding site using extended X-ray absorption fine structure (EXAFS) spectroscopy.

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2. MATERIALS AND METHODS

Purple membrane from *H. halobium* was isolated according to Oesterhelt and Stoeckenius [13]. All reagents used were reagent grade. The blue membrane was obtained by passing purple membrane through an ion-exchange column (AG-50WX-8, BioRad) using the method of Kimura et al. [4]. The iron-substituted purple membrane was prepared by adding 0.6 equiv. $FeCl_3$ to a suspension of 150 mg blue membrane. The pH was adjusted to 9.0 with diazabicycloundecene (DBU). Subsequently, the purple sample was centrifuged. The pellet was further concentrated to a wet paste by freeze-drying. Atomic absorption spectra of this sample revealed a ratio of 0.56 iron per bR molecule.

A sample of approx. 30 mg (10 mM, OD 126) was transferred into a sample holder (2 × 4 × 10 mm) for the EXAFS measurements which was covered by a Mylar film on the two open sides. The

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dark-adapted bR (bR^{DA}) was obtained by maintaining the sample for 12 h in the dark at room temperature. The light-adapted bR (bR^{LA}) was prepared by illuminating the sample alternately from both sides for 2 h at room temperature. The light came through a light guide from a projector and was filtered below 500 nm. Because a sample with an absorbance of 2 is light-adapted in less than 1 min the 2 h of illumination of the EXAFS sample are sufficient for light adaptation. The M state (bR^{M}) was obtained by illuminating the sample at −30°C for 5 h using the same experimental set-up. The extent of M formation in the highly dense sample was determined visually by its yellow color with its known absorption maximum at 410 nm. Illumination was continued for 4–5 h, until the sample turned almost completely yellow. The sample was stored at −200°C. After EXAFS data were collected, the M intermediate, on warming the sample to 0°C, decayed back to the purple light-adapted bR indicating minimal chromophore damage during irradiation.

The EXAFS data for the Fe-bR samples were collected at the Stanford Synchroton Radiation Laboratory (SSRL) on beamline II-2 as described [14–16]. All samples were kept at −100°C during data collection. The X-ray absorption raw data were corrected for the iron absorber, multiplied by the wave vector cubed, Fourier transformed, and fitted according to standard methods [14–19]. The errors were calculated by varying the parameter in question, while holding the other parameters fixed. The raw M state data were corrected for the fraction of bR^{LA} by a subtraction procedure.

3. RESULTS

The incorporation of 0.56 equiv. Fe^{3+} into the blue membrane shifts its absorption maximum from 604 to 600 nm. From binding studies it is known that at this concentration all iron is bound to one of the high-affinity binding sites of bR [5]. Raising the pH above 7 restores the native purple membrane with an absorption maximum at 568 nm.

Fig.1 shows the X-ray absorption curve with the iron edge (I, 7135 eV), the ligand field indicator region (II, 7170 eV) and the EXAFS peaks at 7275 eV (III) and 7400 eV (IV), the latter being faintly visible. The data were processed according

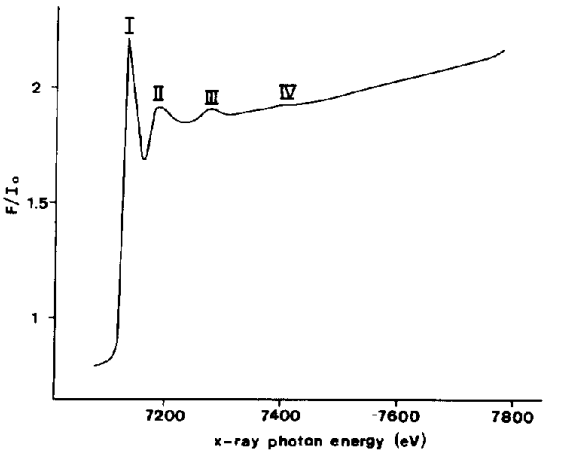

Fig.1. X-ray absorption spectrum of light adapted bacteriorhodopsin with linear background subtraction (I_0 = incident intensity, F = X-ray fluorescence intensity).

to standard methods [14–19] to determine the distances between the iron atoms and the nearest neighbours. The Fourier transform data of bR^{LA} (solid line) and bR^{M} (broken line) in fig.2 show main peaks at about 1.5 Å (bR^{LA}) and 1.6 Å (bR^{M}). These peaks represent back-scattering from the ligands directly bound to iron (first-shell ligands). Corresponding shifts between the bR^{LA} and M state are found as contributions from the higher shells, although with different intensities. Lower intensities scattering at higher distances are within the average noise level of the individual data

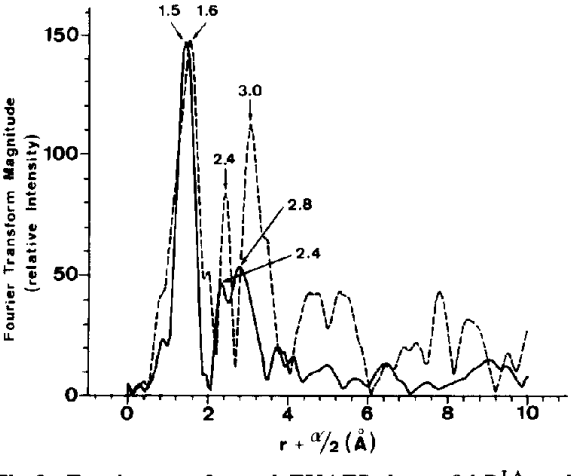

Fig.2. Fourier-transformed EXAFS data of bR^{LA} and bR^{M} (α = absorber scatterer phase shift).

set. Strong back-scattering from the higher shells is found at apparent distances of 2.4, 2.8, 2.4 and 3.0 Å, respectively. It should be noted that the Fourier transform peaks are shifted from their true distances by the absorber-scatterer phase shift α as defined elsewhere [17].

Table 1 shows the results of fitting a single atom type (FeO model) to the unknown sets of bR data. A single atom type approximates the light-adapted bR data well, with a minimum solution of 5.8 ± 1.5 ligands at 1.97 ± 0.02 Å from iron. The bR^{DA}- and bR^{M}-subtracted data cannot be adequately calculated with a single atom type as shown by the poor R^2 values. But, approximating the bR^{DA} and bR^{M} data with two atom types improved the R^2 only by a factor of 1/2, hardly significant enough to justify the addition of the extra parameters (not shown). The bR^{LA} data also yielded a slightly better result using the two-atom procedure, however the improvement (30%) is also insufficient to justify the additional parameters. Therefore, only one-atom-type approximations are reported. The results for bR^{DA} are 5.5 ± 1.7 ligands at 1.99 ± 0.02 Å.

The bR^{M} state results were calculated based on the average of two separate experiments. To test the results against the assumption for M occupancy several subtracted data sets were evaluated. When no correction for bR^{LA} is made, the results are only slightly different from the light-adapted form, with 5.6 ± 1.3 ligands at 1.99 ± 0.02 Å. As more and more of the light-adapted form is subtracted from the M state data the minimum solution is a longer average distance with a slight reduction in coordination number. This solution is 4.7 ± 1.5 ligands at 2.02 ± 0.02 Å (table 1). When more than 60% of bR^{LA} is subtracted from the bR^{M} state data the minimum R^2 starts increasing rapidly. The increase in average ligand distance for the M state is reproducible for the two separate experiments and not critically dependent on the exact amount of M state produced by the experimental protocol described. This is because the differences between M and bR^{LA} are so large that they are greater than the uncertainty in the estimation of the concentration of M.

Table 1

Fit of EXAFS data of bR^{LA}, bR^{DA} and bR^{M} (r = distance between Fe^{3+} and its first-shell ligands in Å; N = number of ligands; R = sum of residuals)

	r	N	R^2
bR^{LA}	1.97 ± 0.02	5.8 ± 1.5	7.0
bR^{DA}	1.99 ± 0.02	5.5 ± 1.7	43.0
bR^{M}	2.02 ± 0.02	4.7 ± 1.5	26.0

Control experiments indicate that the EXAFS of bR^{LA} spectra was regenerated to its purple state from the M state sample by warming to 0°C, showing that the integrity of the bR had been preserved.

4. DISCUSSION

Since EXAFS data reflect the average structure of all metal sites of the sample it is difficult to deconvolute the individual structures if multiple sites are present. It was therefore desirable to use a metal-substituted bR with only one cation per bR molecule. On the other hand, even with a single cation/bR, heterogeneity among the bR molecules could result in heterogeneities in the distance solutions. This is clearly of concern, since it has already been reported by several authors that the M state represents multiple species (e.g. [20,21]).

There exists corroborating evidence that the samples used in these experiments contain only one occupied ferric binding site. The equilibrium kinetics of the binding of Fe^{3+} to the blue membrane show that in substoichiometric amounts the iron is only attached to site I ($K_d^I = 4.7 \times 10^{-7}$ M, $K_d^{II} = 2.0 \times 10^{-5}$ M) (Kohl et al., in preparation). Atomic absorption analysis of the supernatant demonstrates that virtually all iron is bound to the protein. Unpublished observations of solid-state NMR of [4-^{13}C]Asp-labelled bR show that the first binding site is connected to an Asp residue (Engelhard et al., in preparation). Furthermore, crystallographic studies [12] showed that the binding site is close to helix 7. Under these conditions there are no indications for a second occupied binding site.

The results for EXAFS provide direct evidence for structural changes associated with photocycle change at the cation-binding site. The M state is quite different from the bR^{LA} in average distance, and possibly different in coordination number. However, high-resolution edge studies in progress might answer this particular question more precise-

ly. The bR^{DA} is intermediate in iron-ligand distance between the bR^{LA} and bR^{M} state forms, and it might well be that the orientation of the retinal is linked to changes at the cation site.

Fig.2 shows strong back-scattering from the higher-shell ligands, indicating a highly ordered site. The data reveal two distinct distance contributions, at apparent distances of 2.4 and 2.8 Å for bR^{LA}. The larger distance for the M state data has clearly increased suggesting a reorganisation of those ligands alone.

The EXAFS data are compatible with an octahedral configuration with 5.8 ± 1.5 ligands for bR^{LA}. We propose a cation site of octahedral structure, and six coordinates for bR^{LA} and bR^{DA}, with the iron bound to oxygen atoms of the carboxylate groups. This octahedral configuration is also supported by Mössbauer experiments using ^{57}Fe-purple membrane samples (Parak et al., in preparation). However, from the EXAFS data alone it is difficult to distinguish nitrogen ligands from oxygen ligands. However, additional evidence from sequence and NMR data (Engelhard et al., in preparation) as well as the higher-shell distances indicate oxygen atoms as first-shell ligands. Therefore, a possible model of the cation site might involve the carboxyl groups from aspartic acids and other protein and/or water groups.

Our conclusions for the bR^{DA} and the M state must be tempered by the fact that we have not found unique solutions for these structures and that our estimate of M occupancy is only qualitative. There may exist heterogeneities in the M state and the bR^{DA} structures that need further elucidation.

It is known from the photocycle kinetics of cation-substituted bR that the cations affect mainly the M-bR transitions [4]. These steps involve the picking up of a proton from the cytoplasm. This implies that the steps from bR to M in the cation-substituted form are unaffected and represent the physiological reaction. Our results indicate that the homogeneous structure of bR^{LA} is disrupted in the M state, perhaps by the release of a protein group, which may become protonated.

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