An Extended X-Ray Absorption Fine Structure Study of the High-Affinity Cation-Binding Site in the Purple Membrane

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ABSTRACT The structure of the high-affinity cation-binding site of bacteriorhodopsin was studied using extended x-ray absorption fine structure techniques. The results obtained for Mn^{2+} in aqueous solution and for the complex BR-Mn^{2+} (1:1 molar ratio) show great similarities, suggesting that Mn^{2+}, when bound to this site, is coordinated with six atoms of oxygen, forming an octahedral disposition. The interatomic distance between the atoms of oxygen and the Mn^{2+} was found to be 2.17 Å for the complex BR-Mn^{2+}, similar to Mn^{2+} in solution (2.15 Å). In addition, the absence of any other peak at greater distances in the Fourier-transformed spectrum indicates that neither phosphorus nor sulphur atoms are present in the second coordination shell. This suggests that this binding site is located in the protein, discarding the proximity of lipid polar headgroups.

INTRODUCTION

Bacteriorhodopsin (BR) is the photoreceptor protein in the purple membrane of *Halobacterium salinarium*. It acts as a proton pump, allowing the transformation of light into chemical energy (Oesterhelt and Stoeckenius, 1973). Purple membrane binds 4 mol of Ca^{2+} and 1 mol of Mg^{2+} per mol of BR (Chang et al., 1985). The extraction of these cations leads to a deionized form of the membrane (which is blue at pH 5 or below) that possesses characteristic features (Kimura et al., 1984; Ariki and Lanyi, 1986; Chang et al., 1986). The purple form can be regenerated by the addition of mono-, di-, or trivalent cations (Kimura et al., 1984; Ariki and Lanyi, 1986; Chang et al., 1986; Duñach et al., 1987). The spectral redshift observed when cations are removed has been explained by the protonation of the Schiff base counter-ion, Asp 85 (Subramaniam et al., 1990). Thus, the apparent pK for the protonation of Asp 85 appears to be increased by about 2 pH units by cation depletion (Duñach et al., 1988a).

The binding of divalent cations in BR has been explained by the existence of specific binding sites, probably to carboxylic groups (Chang et al., 1986) or, alternatively, by a nonspecific binding to the Gouy-Chapman bilayer (Szundi and Stoeckenius, 1989). Several experiments, however, seem to favor a specific binding of divalent cations in the purple membrane. ESR studies on the binding of the paramagnetic cation Mn^{2+} have indicated the existence of one high, four medium, and five low-affinity binding sites in bacteriorhodopsin (Duñach et al., 1987). On the other hand, cations are released when the purple membrane is heated (Chang et al., 1986), indicating that they are specifically bound. Furthermore, blue membrane possesses a decreased thermal stability as compared to the native membrane (Cladera et al., 1988).

In this work we present an x-ray absorption (extended x-ray absorption fine structure (EXAFS) and x-ray absorption near edge structure (XANES) study on the binding of Mn^{2+} to the high-affinity binding site of BR. EXAFS spectroscopy is currently used in a wide range of different systems, running from material science to biology, and it is acknowledged as a powerful tool for the structural characterization of the short- and medium-range order environment of the absorber atom. Its atomic selectivity and sensitivity to the first coordination shell of the atom of interest make it very suitable in our case because of the need to distinguish among the possible ligands of the Mn^{2+} cations. Two samples, Mn^{2+} in solution and the complex BR-Mn^{2+} (1:1 molar ratio), have been compared. In a previous work, Engelhard et al. (1987) carried out EXAFS measurements of Fe^{3+}-substituted BR, which provided an evidence of structural changes at the binding site, associated with changes in the photocycle.

MATERIALS AND METHODS

Purple membrane was isolated from the *Halobacterium salinarium* strain S9, as described previously (Oesterhelt and Stoeckenius, 1974). Cations were removed from purple membrane suspensions by passage through a cation-exchange Dowex AG-50W column (Duñach et al., 1987). A separate aliquot of deionized (blue) membrane was always used for pH and spectroscopic controls, to avoid any possibility of contamination. Regeneration was done by adding Mn^{2+} to the lyophilized blue membrane resuspended in water at a molar ratio of 1:1 (Mn^{2+}:BR).

The x-ray absorption experiments were carried out at the beam line 8.1 of the Synchrotron Radiation Source of the SERC Daresbury laboratory. The EXAFS and XANES spectra of the complex BR-Mn^{2+} and the model, consisting of 10 mM Mn^{2+} in water, were recorded in fluorescence mode. Acquisition times of about 24 h for the complex BR-Mn^{2+}, and about 4 h for Mn^{2+} in solution, were necessary to achieve a reasonable signal-to-noise ratio. A Si(111) channel-cut crystal monochromator was used, the storage ring operating at 1.8 GeV with an average current of 150 mA. The

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incident beam intensity was monitored by an argon-filled ionization chamber, and the fluorescence signal was detected by an array of 13 individual 11-mm-diameter Ge detectors working at 77 K.

The experimental EXAFS spectra were extracted from the raw data following standard techniques (Koningsberger and Prins, 1988). The EXAFS signals were extracted from the raw spectra measured in a wide range (i.e., up to about 600 eV above the absorption edge). The background subtraction was performed by means of a cubic spline that simulates the monotonic atomic absorption. The first shell contribution was extracted by Fourier-filtering the Fourier transform (FT) spectra between 0.8 and 2.8 Å in the case of the complex BR-Mn^{2+}, and between 0.7 and 2.2 Å for Mn^{2+} in solution. The structural parameters of interest (i.e., interatomic distances R, coordination numbers N, and Debye-Waller factors $\Delta\sigma^2$), were obtained by least-squares fitting of the filtered spectra with the signals obtained by using theoretical phases and amplitudes generated from the FEFF 3.11 code (Rehr et al., 1989). The sample containing Mn^{2+} in solution was used to check the reliability of the theoretical simulations.

RESULTS

Fig. 1 displays the raw absorption spectra corresponding to Mn^{2+} dissolved in water and the complex BR-Mn^{2+} recorded in the XANES region. Their shapes clearly indicate the presence of an octahedral oxygen symmetry (Bianconi et al., 1988). However, small differences are observed, namely, the different intensities of the main resonance, a less defined shoulder in the BR-Mn^{2+}, and the change of the pre-edge structure. The presence of a second shell effect or the loss of the perfect octahedral coordination (Bianconi et al., 1988) in the BR-Mn^{2+} complex could account for these differences in the coordination around Mn^{2+} in the two samples.

As shown in Fig. 2, the $\chi(k)$ signals of Mn^{2+} in water and the complex BR-Mn^{2+} are quite similar and are indicative of a unique oscillation frequency that can be related to the existence of a unique ordered coordination shell around the absorbing Mn atom.

The Fourier transforms of the spectra, performed in the range 2–10 $Å^{-1}$, are shown in Fig. 3. A main peak centered at about 1.7 Å is observed in both cases, which for Mn^{2+} in solution is originated by the first hydration coordination shell and by an analogous O coordination shell in the case of the complex BR-Mn^{2+}. No appreciable contribution beyond the first shell is observed in any case. We wish to underline that, to avoid artifacts in comparing two samples due to the mathematical treatment, the Fourier and best-fit analysis have been carried out in the same way and with the same values of ΔE_0 and mean electronic free path (λ). The results corresponding to the best fit for both the complex BR-Mn^{2+} and the model Mn^{2+} in solution are shown in Fig. 4 and summarized in Table 1. In the case of Mn^{2+} in solution we obtained a mean Mn-O interatomic distance of 2.15 Å and a coordination number of 6. These values are in good agreement with the expected octahedral water coordination of the Mn^{2+} and demonstrate the reliability of the theoretical values of phases and amplitudes. The results corresponding to the complex BR-Mn^{2+} show a similar

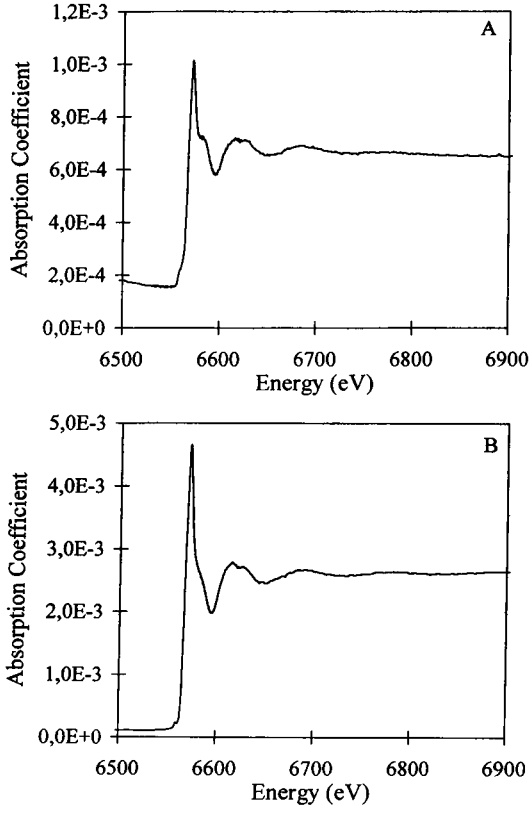

FIGURE 1 X-ray absorption spectra of the complex BR-Mn^{2+} at a molar ratio of 1:1 (*A*), and Mn^{2+} in water (*B*).

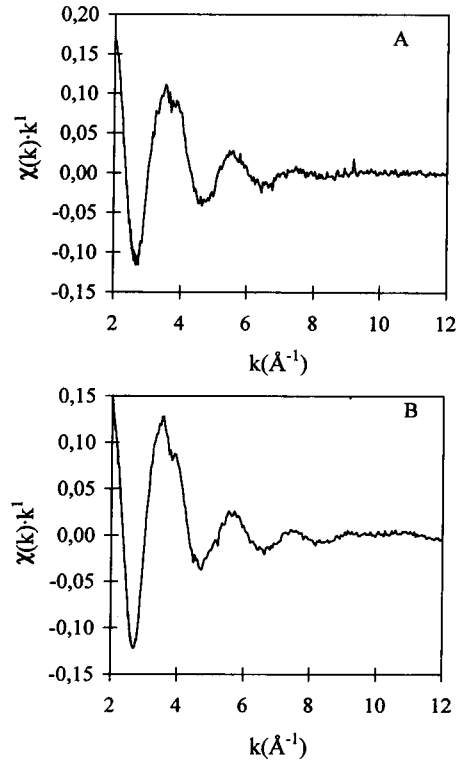

FIGURE 2 EXAFS spectra of the complex BR-Mn^{2+} at a molar ratio of 1:1 (*A*), and Mn^{2+} in water (*B*).

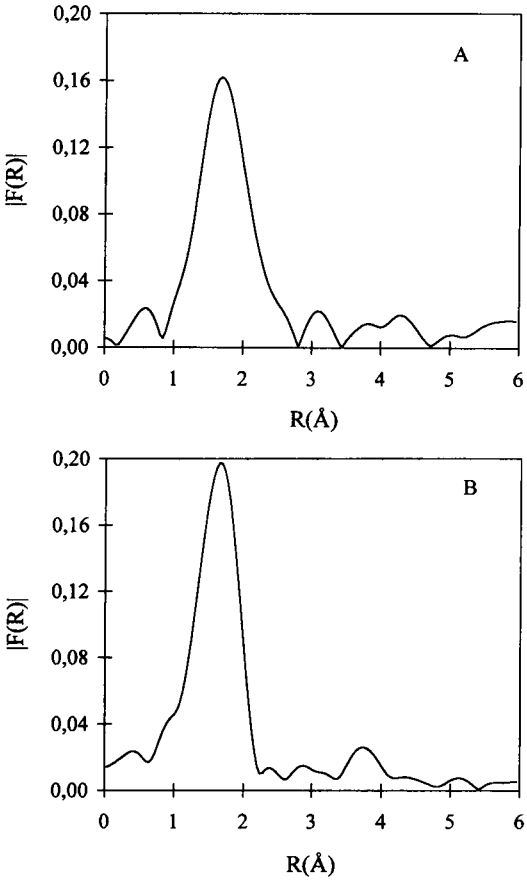

FIGURE 3 Fourier transforms of the EXAFS data of the complex BR-Mn^{2+} at a molar ratio of 1:1 (*A*), and Mn^{2+} in water (*B*).

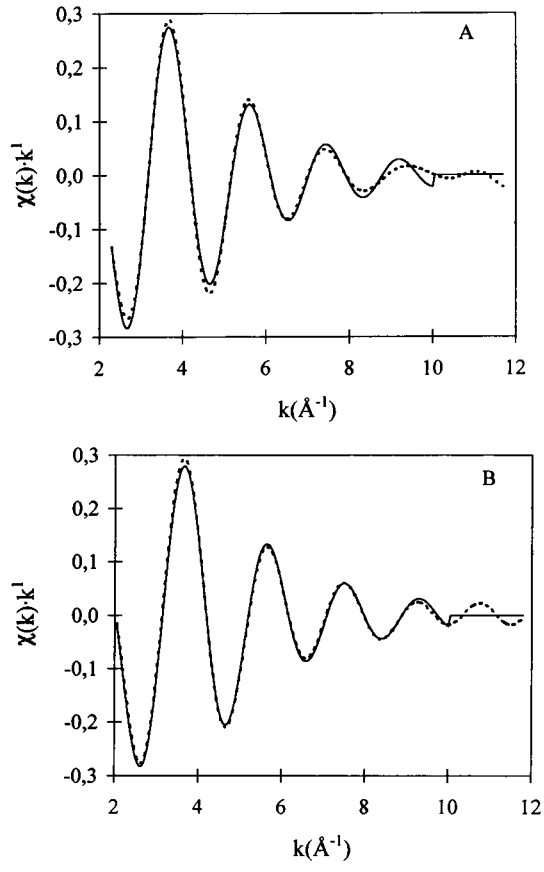

FIGURE 4 Fourier-filtered first-shell contribution of the complex BR-Mn^{2+} at a molar ratio of 1:1 (*A*), and Mn^{2+} in water (*B*).

coordination with oxygen and a similar mean interatomic distance between Mn and O (2.17 Å).

The fact that only one coordination shell is observed in the EXAFS spectrum makes it difficult to determine precisely the oxygen-containing groups that form the second coordination shell. To have a qualitative knowledge about the weight of the next nearest neighbors in the FT spectrum, theoretical simulations were performed, including either one or two atoms of sulfur or phosphorus and up to four carbon atoms. The resulting theoretical Fourier transform simulations are depicted in Fig. 5 and indicate that a second coordination shell of Mn^{2+} cannot contain phosphorus or sulfur atoms as, in this case, the FT spectrum would show a clear peak at about 2.45 Å (compare with Fig. 3).

On the other hand, theoretical simulations of the second shell, including up to three carbon atoms 3.21 Å away from Mn^{2+}, do not show any peak, but only a shoulder (not shown). This peak becomes evident, although it is weak, when four carbon atoms are included in the simulation (Fig. 5 *C*). In addition, the FT plot corresponding to EDTA-Mn^{2+} (which possesses four carbon atoms in its second shell) obtained under the same experimental conditions as the sample BR-Mn^{2+} presents a peak comparable to that obtained in the simulations when four carbon atoms were used (not shown).

DISCUSSION

In this work we intend to offer a description of the high-affinity cation-binding site in BR using Mn^{2+}. It was essential that the conditions used in our experiments permit us to extrapolate the conclusions to the physiologically important cations (e.g., Ca^{2+}). Therefore, to supplement the deionized membrane with Mn^{2+} we used in our experiments the same conditions used by Duñach et al. (1987). They found that both the stoichiometry and the apparent affinity constants for radioactive Ca^{2+} in deionized BR were almost identical to those found for Mn^{2+}. The relevance of these data is enhanced by the fact that they were obtained with different techniques (Duñach et al., 1987, 1988b).

Our results clearly show that the first coordination shell for Mn^{2+} is formed exclusively by oxygen atoms in a

TABLE 1 Interatomic distances (*R*) and coordination numbers (*N*) of the first cell of Mn^{2+} in water and bound to bacteriorhodopsin

Sample	*R* (Å)	*N*	$\Delta\sigma^2$ (Å^2)
BR-Mn^{2+} complex	2.17	6	0.005
$Mn(H_2O)$	2.15	6	0.004

$\Delta\sigma^2$ is the Debye-Waller factor.

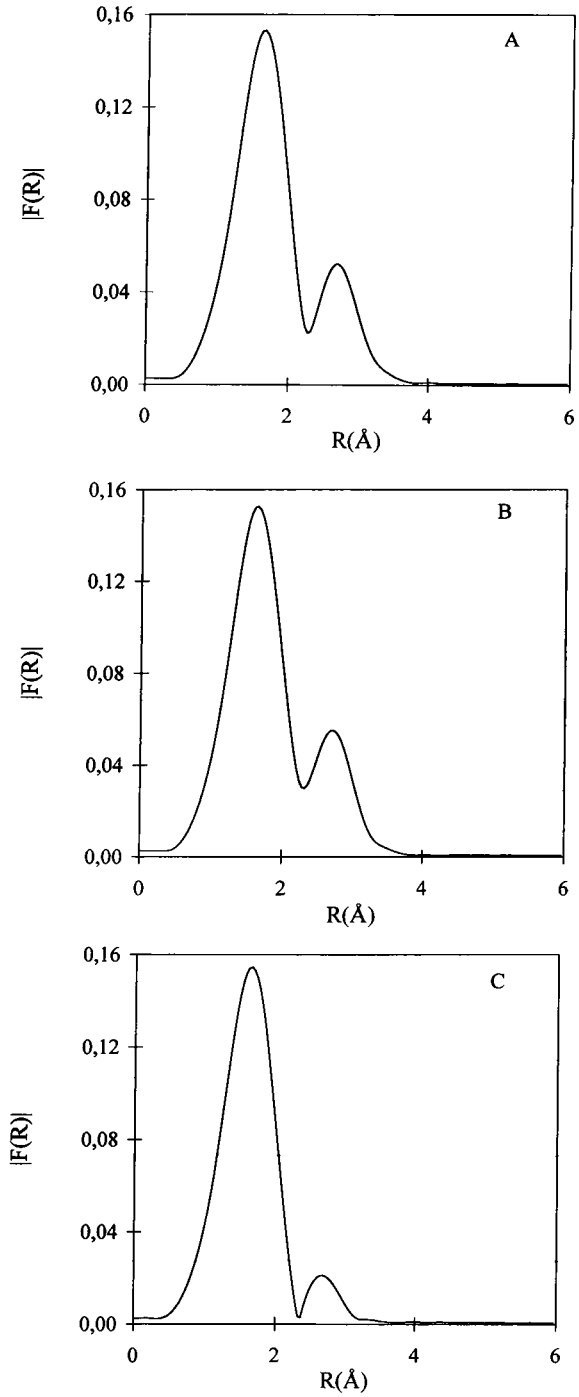

FIGURE 5 Fourier transforms of theoretical simulations corresponding to Mn^{2+} coordinated to six atoms of oxygen with two sulfurs (*A*), two phophorus (*B*), and four carbons (*C*) as next-nearest neighbors.

(distorted) octahedral coordination; similar results were obtained by using Fe^{3+} (Engelhard et al., 1987). However, the composition of the second coordination shell constitutes a more open question. Although in principle it could be formed by lipids (e.g., sulfates or phosphates), by the protein (carboxylic lateral chains) or even by water molecules, our theoretical simulations indicate that the presence of phosphate or sulfur atoms can be discarded. In turn, this rules out the possibility that the high-affinity site is located in the lipid region.

Although a previous EXAFS study on Fe^{3+}-substituted BR showed the presence of a relatively intense peak corresponding to the second shell at 2.4/2.8 Å in the Fourier-transformed plot (Engelhard et al., 1987), it has to be pointed out that this work was performed under different conditions, namely, i) very low temperature was used, which can a priori enhance the intensities observed in the FT plot, ii) a trivalent cation was used, which impairs the photocycle by an order of magnitude (Engelhard et al., 1990). This last observation suggests that Mn^{2+} and Fe^{3+} are in fact occupying different binding sites. This has been suggested for other cations. For example, the deionized bleached membrane shows a very low affinity for Ca^{2+} or Mn^{2+} (Chang et al., 1986; Duñach et al., 1986), whereas the affinity for Eu^{3+} is similar to that found in deionized membrane (Ariki et al., 1987). In addition, Duñach et al. (1988b) demonstrated that there are four binding sites for Hg^{2+} not shared with Ca^{2+}. Other investigators have also described specific properties for cations that would be compatible with their location in different binding sites (Jonas et al., 1990).

The involvement of carboxylic groups pertaining to lateral chains of amino acids (i.e., the presence of carbon atoms in the second shell) is more difficult to check because of the low scattering power of this element. The observations and theoretical simulations presented in the Results section rule out the presence of four or more carbon atoms in the second shell but indicate that the presence of up to three carboxylic groups in the Mn^{2+}-binding site can be possible. Another possibility is that Mn^{2+} does not bind to any of the carboxylic groups of the protein but exclusively to water molecules. Although the EXAFS results are not completely incompatible with this possibility (i.e., the interatomic distances and coordination numbers found in the complex BR-Mn^{2+} and for Mn^{2+} in water solution are very similar; see Table 1) different studies suggested a direct involvement of carboxylic side chains in cation binding (Jonas and Ebrey, 1991; Zhang et al., 1992). In any case, the water molecules ligated to Mn^{2+} should be tightly bound to the protein, because Mn^{2+} appears to be highly immobilized when bound to deionized BR (Duñach et al., 1987). Our results for BR-Mn^{2+} are in accord with this concept, because the different observed intensity of the main resonance as compared to Mn^{2+} in water, a less defined shoulder, and the change in the pre-edge structure are indicative of a second shell around the manganese atom (i.e., protein groups as ligands) or the presence of a distorted coordination around Mn^{2+} (i.e., fixed water molecules as ligands). In this regard, some tightly bound water molecules have been reported to be located near the Schiff base, as detected by neutron diffraction (Papadopoulos et al., 1990).

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