EUROPEAN SYNCHROTRON RADIATION FACILITY

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ESRF

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office using the **Electronic Report Submission Application:**

http://193.49.43.2:8080/smis/servlet/UserUtils?start

Reports supporting requests for additional beam time

Reports can now be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** EXAFS characterization of the cation binding site of the purple memebrane	**Experiment number**: SC/1776
Beamline: ID26	**Date of experiment**: from: 22-June-2005 to: 28-June-2005	**Date of report**: 28-Feb-2006
Shifts: 18	**Local contact(s)**: Dr. Marcin SIKORA	*Received at ESRF:*

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Report: 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. Purple membrane (PM) binds 5 mols of Ca^{2+} per mol of protein that play important structural and functional roles. The extraction of these cations leads to a deionised form of the membrane that possesses characteristic features. The purple form can be regenerate by the addition of mono-, di- or trivalent cations. The research concerning the cation binding to the purple membrane has mainly been directed towards the using of several indirect techniques as for example NMR, Scatchard plots, DSC, but these techniques are unable to give structural and geometrical information about the coordination of the metals. In this sense, X-ray absorption spectroscopy is a direct technique to obtain information about the local structure around a metal. It allows to study purple membrane in solution, i.e. in an environment which is much closer to its physiological state. Also has the advantage of being element-specific and does not require a crystalline sample. The nature and location of the cation binding site is currently in dispute and is the subject of intense investigations by a number of research groups. Several three-dimensional models of bacteriorhodopsin have been published, but so far none of them gives any evidence about the bound cations because they are in part or completely lost upon crystallization. In contrast, all of these three-dimensional models show different Asp and Glu groups that in terms of geometry and bound distances are compatible with cation binding sites. In this sense our previous studies have shown: i) the presence of one site of high affinity and four sites of medium affinity and five sites of low affinity for Mn^{2+}, ii) that the low-affinity sites are located on the BR C-terminal segment, and iii) by using EXAFS and XANES, that the binding sites are located in the protein and not in the lipidic region and more concretely that Ca^{2+} in the high-affinity binding site is coordinated with oxygen atoms from Asp85, Asp212 and three water molecules (please, see references on the new proposal form and on our proposal Ref. SC/1776).

The present work describes the results of a study aimed at identifying candidate cation binding sites on the extracellular region of bacteriorhodopsin, including a site near the retinal pocket. The approach used in our

work is a combined effort involving computational chemistry methods together with the Extended X-Ray Absorption Fine Structure (EXAFS) technique to obtain relevant information about the local structure of the protein in the neighborhood of Mn^{2+} ions in different affinity binding sites. The results of the present work permitted the identification of a high-affinity binding site for Mn^{2+} where the ion is coordinated simultaneously to $Asp212^-$ and $Asp85^-$. Moreover, the results demonstrate that extracellular glutamic acid residues are involved in cation binding.

With the aim of searching the local environment of the high and medium-affinity binding sites of Mn^{2+} in purple membrane we have measured the X-ray absorption spectra of deionized wild type (WT) and quadruple mutant bacteriorhodopsin (4Glu) regenerated with 1 Mn^{2+} and 5 Mn^{2+} per mole of BR, in order to fill only the high and medium affinity binding sites, respectively. In the quadruple 4Glu bacteriorhodopsin mutant, the four extracellular Glu residues have been mutated: E9Q+E74Q+E194Q+E204Q.

We have compared the cation binding in four different clusters of BR found in the GRID map and located in the extracellular region of the protein (external sites: $Glu74^-$; $Glu9^-$ and $Glu194^-$/$Glu204^-$) and a site near the retinal pocket ($Asp85^-$/$Asp212^-$). The local environment of Mn^{2+} in all these putative clusters is formed by carboxyl sidechains of Asp and Glu residues that had already been proposed as candidates for cation binding in BR). As a matter of fact, the EXAFS fittings results for PM regenerated with 1 Mn^{2+}/BR molecule are consistent with the high-affinity binding site being located between $Asp85^-$ and $Asp212^-$, for both pH values studied. For this site we find a quite similar average Mn-O distance for the two pH values of 7 and 10 (2.18 Å and 2.17 Å respectively), indicating that there is no change in the metal first coordination shell upon a pH increase. The average Mn-O distance found for Mn^{2+} in the protein shows a difference of 0.03-0.04 Å with respect to the Mn-O distance for Mn^{+2} in water solution found here, which is a significative difference considering the same coordination number of 6. This implies that the first coordination shell for the metal atom is different when it is bound to the protein or is solvated by water molecules.

Comparison of EXAFS results at pH values of 7 and 10 for all the studied samples ($WT1Mn^{2+}$; $WT5Mn^{2+}$; $4Glu5Mn^{2+}$) gives valuable information about the location of cation binding sites and on involvement of the mutated Glu residues. First, there is a strong similarity in the behavior of $4Glu5Mn^{2+}$ with $WT1Mn^{2+}$: the EXAFS spectra are similar and both show insensitivity on pH change. This suggests that both proteins have only one internal cation binding site. Second, EXAFS spectra of $WT5Mn^{2+}$ shows a strong change varying pH from 7 to 10, whereas $4Glu5Mn^{2+}$ is insensitive to this change. Therefore, the internal site of $WT1Mn^{2+}$ or of $4Glu5Mn^{2+}$ is not sensitive to pH, whereas some of the more external sites of $WT5Mn^{2+}$ are heavily affected by the pH change. Finally, the behavior of the 4Glu mutant is a strong evidence that some of the mutated Glu side chains are involved in the cation binding, in agreement with previous results, i.e. four of the 5 Mn^{2+} added to the 4Glu mutant are not fixed by the protein and remain in water solution. They are then eliminated in the centrifugation process used in the sample preparation.

Summarizing, these results delineate the presence of at leats two binding sites for Mn^{2+} in the purple membrane: one internal high-affinity site located in the vicinity of $Asp85^-$ and $Asp212^-$, and an external site of lower affinity located near $Glu194^-$ and $Glu204^-$. This hypothesis is in agreement with the results found in our previous works, where two different Mn-O distances (2.18 Å and 2.49 Å) for the $5Mn^{2+}$/BR sample are observed, in contrast with the distance of 2.17 Å found after the retinal extraction that induce tertiary structural changes in the protein. This implies the existence of at least two different geometries for cation binding sites, in agreement also with other authors.

	Clusters	Energy Mn^{2+} (kcal/mol)
1	$Glu194^-/Glu204^-$	0
2	$Asp85^-/Asp212^-$	30
3	$Asp36^-/Asp38^-$	40
4	$Glu9^-$	50
5	Asp115	55
6	Asp96	60
7	$Asp102^-/Asp104^-$	65
8	$Glu74^-$	65
9	$Glu166^-$	75

Relative energies of the metal binding sites predicted for Mn^{+2} GRID probes.

Figure 2A

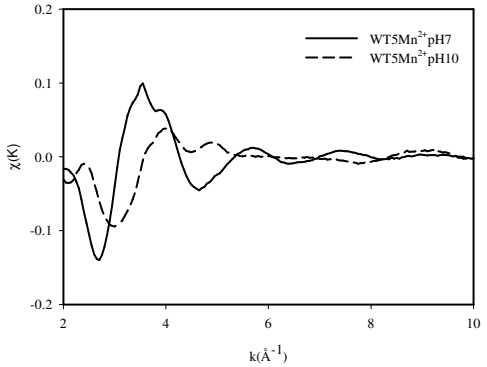

Figure 2B

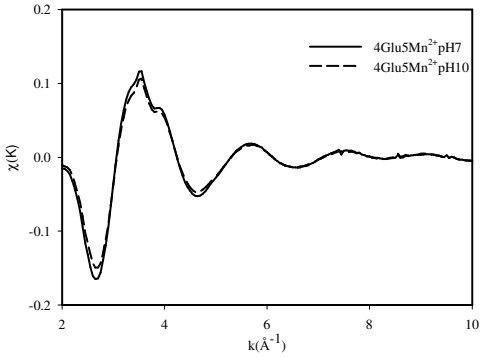

Figure 2. (A) EXAFS spectra χ(k) of WT bacteriorhodopsin-Mn^{2+} and (B) 4Glu mutant-Mn^{2+} at a molar ratio of 1:5, at pH 7 (solid line) and pH 10 (dashed line).

Figure 1

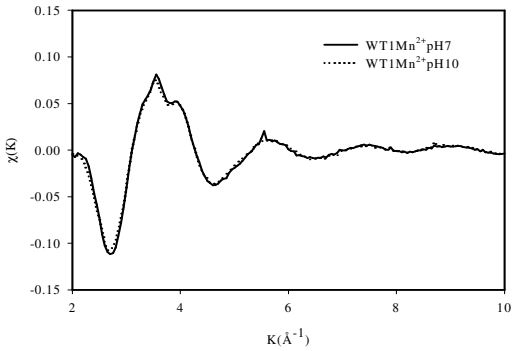

Figure 1. EXAFS spectra of the complex WT bacteriorhodopsin regenerated with $1Mn^{2+}$ per mole of protein, measured at pH 7 (solid line) and pH 10 (dashed line).

Figure 3

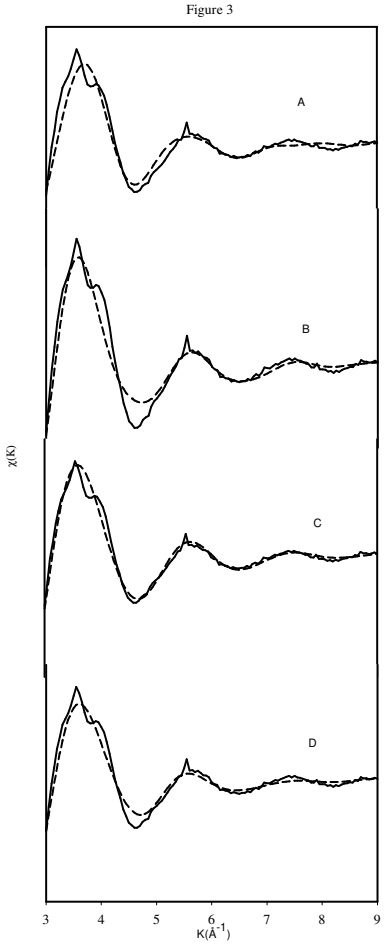

Figure 3. A comparison between the background-subtracted k-space EXAFS data of the $WT1Mn^{2+}$pH7 sample (solid line) and the best fit (dashed line) for clusters A:$Glu194^-/Glu204^-$; B: $Glu74^-$; C: $Asp85^-/Asp212^-$ and D: $Glu9^-$.

	R-factor (χ^2)	ΔE_0(eV)	ΔR(Å)	σ^2(Å^{2}x10^{-3})
$Glu74^-$	0.067	-6±3	-0.04±0.02	4
$Glu9^-$	0.053	-10±3	-0.07±0.02	4
$Asp85^-/Asp212^-$	0.029	-6±1	-0.02±0.01	4
$Glu194^-/Glu204^-$	0.078	-5±4	-0.04±0.02	4

Fit parameters corresponding to the fits on the putative BR-clusters (see Fig 3) and WT bacteriorhodopsin regenerated with $1Mn^{2+}$ per mole of protein, at pH 7.