X-ray Structure of Bacteriorhodopsin at 2.5 Ångstroms from Microcrystals Grown in Lipidic Cubic Phases
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23. S100 was generated by dilution of LSS with nine volumes of XB– with 500 mM KCl, 0.1 mM CaCl₂, 10 mM Heps (pH 7.7), and 10 mM MgCl₂, and centrifuged at 100,000 g for 1 hour. Beads were washed with 15 volumes each of XB– supplement (previously denoted as a buffer for Xic1/pCS2 genes). Plasmid template ssM13 DNA was added to 10 mM final concentration. DNA replication was measured by trichloroacetic acid precipitation of radiolabeled fragments (4), and by x-ray analysis before publication; J. Ruderman, D. Finlay, E. Pebay-Peyroula, Institut de Biologie Structurale/CEA-CNRS/Université Joseph Fourier, 41 Avenue des Martyrs, F-38027 Grenoble Cedex 1, France.

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X-ray Structure of Bacteriorhodopsin at 2.5 Angstroms from Microcrystals Grown in Lipidic Cubic Phases

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Lipidic cubic phases provide a continuous three-dimensional bilayer matrix that facilitates nucleation and growth of bacteriorhodopsin microcrystals. The crystals diffract x-rays isotropically to 2.0 angstroms. The structure of this light-driven proton pump was solved at a resolution of 2.5 angstroms by molecular replacement, using previous results from electron crystallographic studies as a model. The earlier structure was generally confirmed, but several differences were found, including loop conformations and side chain residues. Eight water molecules are now identified experimentally in the proton pathway. These findings reveal the constituents of the proton translocation pathway in the ground state.

Bacteriorhodopsin (bR) is a light-driven proton-translocating pump that converts the energy of photons into an electrochemical potential (1). In the plasma membrane of Halobacterium salinarium (previously known as H. halobium), this integral membrane protein, which is located within the bilayer boundary, is tightly packed in two-dimensional crystals termed purple patches (2). The pioneering studies of Hendrix and co-workers (2) applied a range of techniques including electron microscopy (3) and x-ray analyses (4), provided the first insight into the structural organization of a membrane protein at an intermediate resolution of 7 Å. Over the past 20 years, major advances in electron crystallography resulted in considerable improvements in resolution (5), which currently reaches 3.5 Å in the plane of the membrane and 4.5 Å perpendicular to it (6). These studies revealed that the structure of bR consists of seven membrane-spanning α-helices that are connected by three external and three cytoplasmic loops (7). The pigment, retinal, is bound covalently (through a protonated Schiff base) to Lys216. It is buried in the interior of the protein and is stabilized by the binding of the β-ionone ring in a hydrophobic pocket.

The function of bR has been studied extensively by a variety of structural, genetic, and spectroscopic methods and by molecular dynamics calculations (6, 8–10). The primary event, absorption of a photon, causes isomerization of the retinal from the all-trans to the 13-cis configuration. A series of intermediate events follows, including deprotonation of the Schiff base and proton transfer to Asp85. Another proton is subsequently released, the Schiff base is re-protonated from Asp86, and a proton is taken up from the cytoplasmic side. Thermal isomerization of the retinal to the ground state completes the photocycle.

A detailed understanding of this mechanism requires knowledge of its structure at atomic resolution, an endeavor for which well-diffracting, highly ordered three-dimensional (3D) crystals appear most promising. Several attempts have been reported (11), but the crystals obtained were either too small or exhibited extensive mosaicity as a result of poor order along the c axis. To overcome these limitations, we devised a concept for the crystallization of membrane proteins by exploiting the properties of continuous lipidic cubic phases (12). Membrane proteins, once inserted into this continuous, 3D curved lipid bilayer matrix, diffuse laterally to nucleate and eventually to yield well-ordered crystals. Our initial results with microcrystals of bR, which diffracted x-rays to 3.7 Å resolution (12), have

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been extended to 2.0 Å. Here, we present the 3D structure of this protein at a resolution of 2.5 Å, based on the analysis of data obtained with highly intense synchrotron radiation.

Preparation of monomeric bR and its crystallization from monoglyceride-based cubic phases were done as described (12, 13). Thin hexagonal plates with dimensions of 20 to 40 μm by 20 to 40 μm by 5 μm formed readily and reproducibly within days from a monoolein-water cubic phase, yielding hundreds of crystals in each charge. The thinnest crystals consisted of about 500 unit cells along the c axis and exhibited the least mosaicity (a few tenths as compared with several degrees for larger crystals). The very intense microfocus beamline ID13 at the European Synchrotron Radiation Facility (ESRF) was required for data collection at high resolution. The best crystals (10% of all crystals examined) diffracted to 2.0 Å (Table 1). The data from flash-frozen (100 K) crystals were integrated to 2.4 Å (Fig. 1). The space group was determined to be $P6_3$, with a unit cell of $a = b = 61.76$ Å, $c = 104.16$ Å, $\alpha = \beta = 90^\circ$ and $\gamma = 120^\circ$, and one monomer per asymmetric unit. The packing is dense; 62% of the volume is occupied by protein, whereas the remainder is estimated to be distributed about equally between water and lipids. The monoolein-based cubic phase itself undergoes a reversible cubic-to-lamellar phase transition upon cooling (14). This transition of the phase surrounding the immobilized bR crystals does not affect the crystal quality.

Our current x-ray model of bR comprises 82% of the 248 amino acid residues (residues 7 to 156 and 167 to 225), the retinal, and 144 positions of atoms that correspond to water or lipid molecules (see below). Residues 1 to 6, 157 to 166, and 226 to 248 appear disordered and could not be reconstructed. The crystal packing shows protein layers stacked along the c axis with molecules related by a twofold screw axis (Fig. 1). The interactions along this direction are rather limited. Distinct protein-protein interactions exist only between loops AB (defined as the loop connecting helices A and B) and BC of two symmetry-related molecules (inserts in Fig. 1). The crystal contacts thus appear rather weak, consistent with the diffraction between 5 and 10 Å. Stereochemical values are all within the expected ranges for a 2.5 Å structure. All residues are in allowed regions of the Ramachandran plot. Coordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (entry code 1AP9).

Table 1. Crystallographic data collection, and processing. Preparation of monomeric bR and its crystallization from monoolein-water cubic phases were as described (12, 13). Data were collected at ESRF using the D2AM beamline for the characterization of crystals and space group determination. A data set to 4 Å resolution was collected on a single crystal over a range of 140° of rotation. High-resolution data on crystals cooled to 100 K were collected on beamline ID13 ($\lambda = 0.69$ Å) using a MARResearch imaging plate. The monoolein lipids composing the cubic phase provided cryoprotection. Data were integrated to 2.4 Å (Table 1). The monoolein crystals examined) diffracted to 2.0 Å. Here, we present the 3D structure of this protein at a resolution of 2.5 Å, based on the analysis of data obtained with highly intense synchrotron radiation.

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the observation that about 9 crystals out of 10 exhibit a large mosaicity along this axis. The packing of bR in the (a,b) plane (Fig. 2) is similar to that observed in the purple patches: The molecules are arranged in trimers with comparable unit cell dimensions. Loops CD, DE, and FG are well defined in the electron density maps (2F_{o}-F_{c}) and are also reflected by the refined temperature factors. Loops AB and BC are less well defined, and loop EF is not detected. Loops AB and CD account for the intratrimmer contacts. No intertrimer protein contacts can be seen in the (a,b) plane (Fig. 2). Comparison between the x-ray and EM models (Fig. 3) reveals a high degree of similarity in the helical regions, as shown from the root-mean-square (rms) deviations (15). Markedly different conformations in loops AB and BC are observed (Fig. 3). Whether these differences are induced by the formation of crystal contacts is unknown.

The retinal, located deep within the protein (Fig. 4C), is the heart of bR. It is positioned between the two nearly parallel helical regions, as shown from the root-mean-square deviations (15). This finding is at variance with the EM model (Fig. 4E). This residue, located at the onset of helix G, is close to the extracellular side and has been suggested as a possible proton releasing group (18, 19), but see below. The orientation of the side chain of Tyr^{57} is different as well, as is the hydrogen bond network around Asp^{212}, which in turn is hydrogen-bonded to Tyr^{57} and Tyr^{185}. A hydrogen bond to Trp^{86} is not observed (Fig. 4F).

For protons to overcome the distances between the amino acid residues involved in proton translocation, water molecules in the interior of bR appear to be inevitable constituents (20). We searched the electron density map for the presence of water molecules and identified 144 possible positions, of which 93 have a temperature factor (B value) smaller than 60 Å². Among them, at least eight water molecules are located in the putative proton channel between helices B, C, and G. Four of these could be identified unequivocally (Fig. 4A); of the remaining 136, some are located within bR.

![Fig. 2. bR trimers in the (a,b) plane. The view is along the c axis from the cytoplasmic toward the extracellular space. The schematic upper right sector defines the positions of the helices and connecting loops, drawn as solid lines on the cytoplasmic side and as dashed lines on the extracellular side. The upper left sector depicts the backbone of the helices in green. Loops AB and CD, which participate extensively in the intratrimmer interactions, are highlighted in the lower sector, which reveals the backbone and side chain residues in stick models (color code as defined in Fig. 1).](image-url)
boundaries but not in the putative proton channel, and thus they may represent structural water molecules. The other water molecules are distributed among two populations. The first is located on the surface of the trimer and is exposed to bulk solvent, which at this stage of the refinement may either be ordered water or missing loops. The second is located laterally around the trimers and may correspond to lipids, but an unequivocal identification requires further investigation. Higher resolution and better quality data should allow water to be distinguished from lipid molecules in that location.

Within the channel, the photoexcited protonated Schiff base releases its proton to Asp85 toward the extracellular side. Its car...

Fig. 4. (A) Schematic representation of the proton pathway with selected structural details. Residues involved in, or in the vicinity of, the proton pathway are abbreviated as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; K, Lys; L, Leu; M, Met; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Carbon, nitrogen, and oxygen are shown in italics. The scheme is drawn to facilitate correlations with the electron density maps, and is consequently distorted. Water molecules indicated in solid blue circles are well-defined in the model (with $B$ factors less than the $\langle B \rangle$ over the protein); open blue circles represent molecules that refine with high $B$ factors ($>100$ Å$^2$). The quality of the well-defined water molecules was confirmed with difference electron density maps ($F_{obs} - F_{calc}$) in which the four water molecules were omitted from the model. At a resolution of 2.5 Å, distances from 2.7 to 3.3 Å are considered as potential hydrogen bonds. The distances from water molecule 5 to the Schiff base nitrogen, from Asp38 to Asp96, and from Thr205 to Glu9 are shown with arrows. Water molecule 7 is 4.2 Å from Asp85. The temperature factor of the oxygen refines to 43 Å$^2$. This water molecule is 6.7 Å from the Schiff base on the cytoplasmic side (seen in the background). In (C), continuous density represents the retinal in the all-trans configuration. Its molecular environment is similar to that described in the EM model [compare figure 2D in (6)] and consists of Met20, Ala53, Tyr83, Trp86, Thr89, Leu93, Met118, Ile119, Trp138, Ser141, Thr142, Met145, Trp182, Tyr185, Pro186, Trp189, and Asp215, all of which are within van der Waals distances to the retinal. In (D), the largest water pocket in the extracellular channel is shown. Two water molecules are modeled in this pocket and refined with temperature factors of 37 and 55 Å$^2$, respectively. Water molecule 4 is within hydrogen-bonding distance to Arg82 as well as to water molecule 3. The orientation of the side chain of Arg82 is well defined in the electron density map. The water pocket is close to Asp212, which is the acceptor of the proton from the Schiff base during the photocycle, and to Asp212. Water molecule 8 may participate in a hydrogen bond chain from Asp212 to Arg82. In (E), Glu204 is located at the extracellular side of helix G. It has been suggested to be the proton releasing group (19). The EM model of this residue, superimposed in green, shows a large difference in the side chain orientation. In (F), Asp212 is within hydrogen-bonding distance to Tyr57 and Tyr185. In (G), water molecule 2 is close to helix G, within hydrogen-bonding distance to the carbonyl of Lys216 and the amide of Val217. This molecule may thus account for the deformation of helix G near residue 216.
boxyl group remains protonated until the last step of the photocycle (21). Asp^{85} resides within hydrogen-bonding distance of both Trp^{86} and Thr^{88}. We did not find any water molecules within hydrogen-bonding distance of Asp^{85}. Retinal isomerization seems to cause rearrangement of the neighboring residues, in particular Trp^{86}, thereby destabilizing its binding to Asp^{85}. Consequently, it is likely that the latter residue resembles the Schiff base, allowing direct proton transfer to occur. The water pocket near Asp^{85}, Tyr^{57}, Asp^{212}, and Arg^{52}, including water molecules 3 and 4, forms a hydrogen bond network that facilitates proton transfer from Asp^{85} to Arg^{52} and Thr^{205} (Fig. 4D). Such a delocalized proton transfer was indeed observed on the basis of Fourier transform infrared experiments (22). Because Glu^{8} is situated near the exit of the proton channel (Fig. 4E), with its carboxylate group 5 Å from Thr^{205}, a possible hydrogen bond network that facilitates proton transfer was indeed observed on the basis of Fourier transform infrared experiments (22). Because Glu^{8} is situated near the exit of the proton channel (Fig. 4E), with its carboxylate group 5 Å from Thr^{205}, a possible hydrogen bond network that facilitates proton transfer to occur.

The well-defined water molecule 2, located in the vicinity of the backbone of helix G, forms hydrogen bonds to the backbone carbonyl of Lys^{216} and the amide of Val^{17} (Fig. 4G). The deviation of the geometry of helix G from standard values between residues 216 and 217 may be a result of the binding of this water molecule, which would compete with an intrahelical hydrogen bond. Molecular dynamics simulations (10) have predicted an unusual conformation of helix G and the binding of a water molecule to the backbone carbonyl of Lys^{16}. A water network in an intermediate state (M state) has been suggested on the basis of spectroscopic analyses (23).

In the cytoplasmic segment of the channel, water molecule 1 forms a hydrogen bond to Trp^{182} (Fig. 4B). Asp^{86} is known from time-resolved studies with bR mutants to reorient the Schiff base (9, 21, 24). From our structure, this residue is located 10 Å from the Schiff base, a distance too large for a proton transfer step (Fig. 4A). We found that water molecule 5 is hydrogen-bonded to Asp^{86}, but unequivocal positions of additional water molecules could not be detected in this region. The proton pathway in the cytoplasmic segment of the channel is therefore not uniquely defined at this time. The portion of the channel from Asp^{96} to the cytoplasmic surface is delimited by Asp^{86} and could be accessible to bulk water. Asp^{86} is the first residue in the putative cytoplasmic proton uptake pathway (25). A pK value of Asp^{86} higher than 11, determined previously (26), could be explained by limited solvent accessibility caused by the position of Phe^{42}. In summary, the positions of the water molecules in the extracellular part are well defined and support our proposed proton pathway, whereas the mechanism of translocation in the more hydrophobic cytoplasmic channel remains to be elucidated.

Our high-resolution 3D structure of bR identifies the locations of water molecules within this membrane protein. Thus, the structural basis for the mechanism of proton translocation, hitherto largely speculative, is set, at least in the extracellular part of the channel. The x-ray structure is biologically relevant, because bR in the crystalline state undergoes the main steps in the photocycle, as shown by Fourier transform infrared spectroscopy in the fully transparent cubic phase (27). Also, the high degree of similarity between the x-ray structure and the EM model demonstrates the complementarity of these methods: Most of the side chains that were previously modeled can now be established, whereas several exhibit distinct differences. Our structure of the ground state represents a snapshot in the photocycle; thus, solving the high-resolution structure of the M state(s) should reveal differences in the protein structure and altered positions of some of the water molecules. This should eventually provide a basis for the understanding of the complete photocycle at atomic resolution, and should allow the plethora of earlier studies to be interpreted in structural terms.

Solving the structure of bR to 2.5 Å from very small crystals affirms the potential of the latest generation of synchrotron microfocus x-ray sources. Moreover, the concept of crystallization of membrane proteins from 3D membrane-mimetic matrices, afforded by lipidic cubic phases (28), permits the crystallization of membrane proteins from the bacterial rhodopsin, L. (1980). Also, the high degree of similarity between the x-ray structure and the EM model demonstrates the complementarity of these methods: Most of the side chains that were previously modeled can now be established, whereas several exhibit distinct differences. Our structure of the ground state represents a snapshot in the photocycle; thus, solving the high-resolution structure of the M state(s) should reveal differences in the protein structure and altered positions of some of the water molecules. This should eventually provide a basis for the understanding of the complete photocycle at atomic resolution, and should allow the plethora of earlier studies to be interpreted in structural terms.

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Protein folding in the endoplasmic reticulum (ER) often involves the formation of disulfide bonds. The oxidizing conditions required within this organelle were shown to be maintained through the release of small thiols, mainly cysteine and glutathione. Thiol secretion was stimulated when proteins rich in disulfide bonds were translocated into the ER, and secretion was prevented by the inhibition of protein synthesis. Endogenously generated cysteine and glutathione counteracted thiol-mediated retention in the ER and altered the extracellular redox. The secretion of thiols might link disulfide bond formation in the ER to intra- and intercellular redox signaling.

The ER is the port of entry and main folding compartment for proteins destined for the central vacuolar system (1). Nascent proteins are translocated into the ER in the reduced state and rapidly form disulfides in the suitable redox environment of this organelle (2, 3). Oxidizing conditions are likely to be generated by the import of oxidized glutathione (GSSG) and cysteine (3) and must be continuously maintained to counteract the vectorial import of oxidized glutathione (GSSG) through the release of small thiols, mainly cysteine and glutathione. Thiol secretion increased when proteins rich in disulfide bonds were translocated into the ER, and secretion was prevented by the inhibition of protein synthesis. Endogenously generated cysteine and glutathione counteracted thiol-mediated retention in the ER and altered the extracellular redox. The secretion of thiols might link disulfide bond formation in the ER to intra- and intercellular redox signaling.

**Fig. 1.** Thiol release through the secretory pathway requires protein synthesis. (A) J558L cells were cultured in a water bath for the indicated times at 20° or 37°C. After 3 hours, aliquots of cells cultured at 20°C were further cultured for 2 hours at 37°C [A and (B)]. J558L cells were cultured in an incubator at 37°C with or without BFA (5 μg/ml) or 500 μM cycloheximide (CHX) [(C) and (D)]. The spent media were harvested by centrifugation and the accumulation of thiols [A and (C), mean of three experiments], and λ chains [(E)] were quantitated by the 5,5′-dithio- bis-(2-nitrobenzoic acid) (DTNB) assay (20) and immunoblotting [(F)], respectively. (E) Thiol secretion from *Xenopus* oocytes. Groups of five oocytes (21) were cultured with or without monensin (20 μg/ml) or 500 μM CHX for the indicated times. The average of two experiments is shown. The results are expressed as nanomoles of thiols secreted by single oocytes or 1 × 104 J558L cells, as derived from a standard curve obtained with purified cysteine. What seems to be a stronger inhibitory effect of monensin in the extracellular space (Fig. 1). As observed for other cell types (3), both J558L myeloma cells (Fig. 1A, A and C) and *Xenopus laevis* oocytes (Fig. 1E) released thiols. The export of thiols and of constitutively secreted proteins [immunoglobulin (Ig) λ chains] was compared in J558L cells after perturbation of vesicular traffic by treatment at low temperature (20°C) or in the presence of brefeldin A (BFA) (5). After the first hour of treatment, the extracellular accumulation of both λ chains and thiols was inhibited (Fig. 1), which suggested that a functional secretory apparatus was required for their release. When cells were kept at 20°C for 3 hours and then transferred to 37°C, the secretion of both thiols and proteins was promptly restored (Fig. 1A and B). The similar behavior of thiols and λ chains suggests that they did indeed utilize the same transport systems. In agreement with previous observations that J558L cells secrete λ chains that are completely oxidized (4), virtually all secreted thiols were soluble in acetone (6). Differences in the equilibrium constant (Keq) may explain the coexistence of reduced glutathione and cysteine with oxidized proteins (7). Similar findings were also obtained with isolated amphibian oocytes, in which monensin, another inhibitor of protein secretion, inhibited thiol release (Fig. 1E). Monensin was used because BFA is ineffective in *Xenopus* oocytes (8).

We also predicted that thiol release should be influenced by the synthesis of secretory proteins containing disulfide bonds. In agreement with this, cyclohexi-