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Structural changes in bacteriorhodopsin during proton translocation revealed by neutron diffraction

(purple membrane/H^+-transport mechanism/conformational changes)

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ABSTRACT **A neutron diffraction study of spectroscopic states for the light-energized proton pump bacteriorhodopsin (BR) is presented. The photocycle states BR-568 and M were generated at temperatures above 4°C and were measured after trapping at −180°C. In the BR-568 to M-state transition, which is known to be a key step in transmembrane proton pumping, reversible structural changes of the protein were detected. These structural alterations occur in the neighborhood of the cyclohexene ring and at the Schiff's base end of the chromophore retinal. They are interpreted as a 1–2° tilt of three or four of the transmembrane α-helices or as positional changes of four or five amino acids. The structural changes observed are inherent in the transport mechanism of bacteriorhodopsin.**

Membrane protein conformational changes are expected to be involved in transport mechanisms. For the light-driven proton pump bacteriorhodopsin (BR), however, the all-trans to 13-cis isomerization of the chromophore retinal, during the first steps of the photocycle, is the only structural alteration unambiguously determined to date (1, 2). A variety of spectroscopic and diffraction experiments have been performed to detect changes also in the protein moiety of BR connected with the transition from the BR-568 ground state to the photocycle intermediate M. These efforts resulted in contradictory ideas about the extent of the conformational changes occurring. They range from lattice disorder and dramatic changes in the tertiary conformation to only subtle alterations in the structure at high resolution (for review, see ref. 3). To settle this controversy, we have conducted a neutron diffraction study on different functional states of BR. The advantages of neutron diffraction for the study of biological membranes derive mainly from the natural contrast between protein and protein-associated water as well as between protein and lipid, which is higher for neutrons than for x-rays. Furthermore, since the coherent neutron scattering length of H (−3.74 fm) and ^2H (6.67 fm) is different not only in magnitude but also in sign, there are rich possibilities of ^2H labeling. By performing measurements in H_2O and ^2H_2O on the BR-568 ground state and the M intermediate, it should be possible not only to detect structural differences in the protein but also to observe any redistribution of associated water and exchangeable hydrogens related to the transition. The results obtained by neutron diffraction unambiguously show that in the BR-568 to M transition, which is known to be a key step in transmembrane proton pumping, reversible structural changes of the protein in the vicinity of the chromophore retinal occur. These light-induced conformational changes are proposed to trigger the vectorial H^+ translocation processes in the active center of BR.

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METHODS

Sample Preparation. Purple membranes were isolated from *Halobacterium halobium* (strain ET 1001). Neutron diffraction experiments require ≈100 mg of BR to perform the experiments in a reasonable time of ≈2 days for the sample in each state. Purple membranes were oriented on 14 thin quartz slides (65 × 15 × 0.3 mm) with their planes parallel to the support surface (mosaic spread of about 10° full width at half-maximum) by slowly drying aqueous suspensions at room temperature and 86% relative humidity. Each purple membrane film with an absorbance of 3–4 covered an area of 50 × 10 mm on the slide. To transfer guanidine hydrochloride into the films, they were soaked for ≈2 hr in a 1 M solution of the salt (containing in addition 50 mM $Na_2CO_3/NaHCO_3$ buffer) in H_2O (pH 9.4) or ^2H_2O (p^2H 9.4) and thereafter surplus solution was removed. The slides were incubated at 86% relative humidity for 2 days, resulting in a final guanidine hydrochloride concentration of ≈2 M, and then each film was sealed air-tight with a cover slide.

Purple membrane films were first converted to the light-adapted ground state BR-568 by illumination at temperatures between 20 and 7° C. Subsequently, the M state was generated by illuminating each film at 5–7°C and then it was quickly cooled to −180°C in the dark. Finally, the sample holder was inserted into the cryostat (−180°C) on the diffractometer. At this temperature the M state was stable for days. The ground state BR-568 was produced under equivalent conditions. After illumination of the films with the same amount of light as applied to the M state at 5–7°C, light exposure was terminated and upon relaxation of the M state to BR-568 at this temperature the sample was transferred into the specimen holder at −180°C.

The illumination and cooling procedure did not lead to any irreversible structural alterations in the purple membrane as can be inferred from data sets obtained at room temperature before and after the low-temperature experiments. In fact, all structural alterations described were completely reversible.

Neutron Diffraction. In-plane diffraction patterns were recorded in so-called Θ–2Θ scans at a wavelength of 4.52 Å on the D16 diffractometer at the Institut Laue-Langevin (Grenoble) (4), which required 2 days to obtain a statistical error of 1% for reflections of medium intensity. The amplitudes were derived from neutron diffraction intensities, phases, and intensity ratios for overlapping reflections from electron microscopy (5), as described (4). An analysis of the validity of the difference Fourier method as applied in this investigation was reported earlier (6). A statistical approach for splitting the overlapping reflections as suggested in ref. 7 was also applied and led to similar difference maps.

Abbreviation: BR, bacteriorhodopsin.

RESULTS AND DISCUSSION

Trapping of Photocycle States. To trap the M intermediate of the photocycle (lifetime at room temperature, ≈10 ms), the joint action of low temperature and the chemical guanidine hydrochloride was required. In the absence of guanidine hydrochloride, because of the high absorbance of each film, light of very high intensity was required for the complete conversion of BR-568 to the M state. This led to artificial local dehydration in the film with associated structural changes of BR according to our diffraction data. In the presence of 2 M guanidine hydrochloride at alkaline pH, the decay rate of the M state is slower by 2–3 orders of magnitude. Therefore, complete conversion into this intermediate occurs at temperatures above 4°C even at the relatively low light intensity applied (20 mW/cm^2, $490 < \lambda < 680$ nm). The trapping of the M state above 4°C excludes the possibility that structural changes of BR are inhibited due to freezing of the conformation.

It is important to note that at 20°C we could demonstrate light-induced reversible proton release during the BR-568 to M transition for purple membranes in the presence of 1 M guanidine hydrochloride, at pH 9.4 and at pH 6.0. At 7.6 M guanidine hydrochloride and alkaline pH, 1 mol of protons per mol of bleached BR is released (8), a stoichiometry also found in the absence of this salt at neutral pH (9). Although 6 M guanidine hydrochloride has been reported not to induce changes in the secondary structure of BR (10), we have performed control neutron diffraction measurements with samples containing 200 mM or no guanidine hydrochloride (and 10 mM $Na_2CO_3/NaHCO_3$, $p^2H = 9.4$). The same general results were obtained as compared to the 2 M guanidine hydrochloride samples; however, M formation was incomplete. On the other hand, the purple membrane film in 2 M guanidine hydrochloride turned completely yellow during the illumination procedure described above and developed no purple color when kept at −180°C for 70 hr. The absorbance maximum at around 410 nm (determined for control samples covered with less purple membrane) and other features reported [unprotonated 13-*cis*-retinal chromophore with planar 6-s-trans bond and C=N anti-Schiff-base bond; ref 11] for the pigment species trapped in guanidine hydrochloride under illumination are characteristic for the M intermediate of the physiological photocycle of BR (12). Therefore, the light-induced structural changes reported below are occurring during the transition from the ground state BR-568 to the "true" M intermediate but are not caused by the M-like photointermediate of the N state (11–13). Our data in the absence of guanidine hydrochloride support this conclusion.

Light-Induced Structural Changes in BR Detected by Neutron Diffraction. Neutron diffraction patterns were recorded from oriented purple membrane stacks at low temperature (−180°C) in the absence or in the presence of guanidine hydrochloride with BR trapped in the BR-568 and M state, respectively (Fig. 1). The reflections were indexed according to a hexagonal lattice with lattice constants of **a** = 61.1 Å for the BR-568 and of **a** = 61.4 Å for the M state. No broadening of the reflections was observed, neither between room and liquid nitrogen temperatures nor comparing the BR-568 and the M state, thus showing no significant change in crystalline order. This is in agreement with electron diffraction results (3) but contradicts previous conclusions drawn from spectroscopic (14, 15) and time-resolved x-ray diffraction (16) experiments. In the present resolution range, the Debye–Waller factor remains unchanged, excluding an increase of large-scale fluctuations in the unit cell during M formation. Inspection of the intensity variations of the neutron diffraction patterns reveals, however, significant differences between both BR states (Fig. 1). The overall intensity change $\sum \Delta I / \sum I$ between the two states is 9%. The differences in the reflection intensities are completely reversible, and reproducible with various samples. This is expressed, for example, by a high correlation ($c = 0.96$) of the difference structure factors $\Delta F = F(\text{M}) - F(\text{BR-568})$ for two independent measurements in ^2H_2O with the same specimen. The differences in the neutron diffraction intensities (Fig. 1) could have been caused by either redistribution of protein associated water molecules and exchangeable hydrogens or by displacements of protein mass. To distinguish between these possibilities, measurements were repeated with samples equilibrated in H_2O. The M to BR-568 intensity differences in H_2O show high similarity in magnitude and sign in the ΔF values compared to those in ^2H_2O. This result is reflected by a high correlation coefficient of $c = 0.87$ for the ΔF values in H_2O and ^2H_2O, indicating that the intensity differences observed are mainly caused by conformational alterations in the protein.

From the neutron diffraction difference amplitudes and the phases from electron microscopy (5), the difference Fourier map $\Delta\rho = \rho(\text{M}) - \rho(\text{BR-568})$ was calculated to an 8-Å resolution for the H_2O and ^2H_2O samples (Fig. 2 *a* and *b*). The phases used were determined from glucose-embedded purple membranes in a cryoelectron microscope at −268°C, where the lattice constant of **a** = 62.5 Å was preserved (5). In our

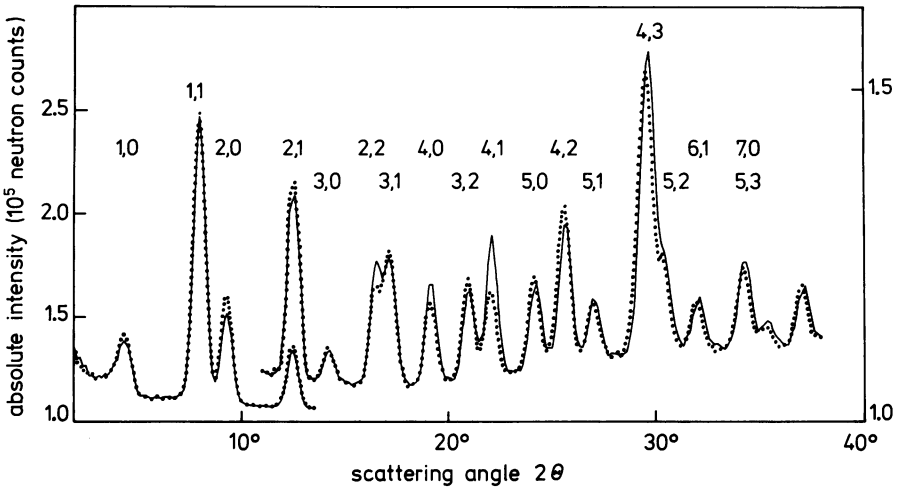

FIG. 1. Neutron diffraction intensities of purple membrane stacks in ^2H_2O of the light-adapted ground state BR-568 (—) and of the M intermediate of the photocycle (· · · ·) at −180°C. Neutron counts as a function of the detector angle 2Θ are shown. Reflections are indexed according to an hexagonal lattice.

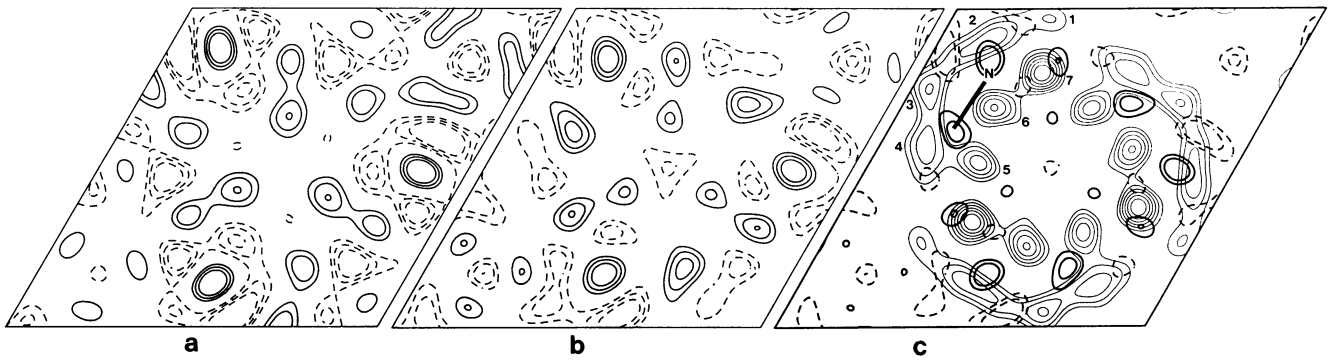

FIG. 2. Two-dimensional difference Fourier maps, $\Delta\rho = \rho(\text{M}) - \rho(\text{BR-568})$, showing changes in the projected structure between the M state and the ground state BR-568 of the purple membrane in H_2O (*a*) and in ^2H_2O (*b*). Only the three highest of the five positive (—) and five negative (- - - -) equidistant contour levels are drawn. A single contour interval corresponds to a density difference of 0.7% of the peak density of helix 7 in the BR structure. (*c*) Superimposed on the projected BR structure in the M state at −180°C (^2H_2O), the highest positive and negative levels of the difference map in ^2H_2O are drawn as well as the in-plane orientation of the chromophore according to refs. 4, 17, and 18 (N indicates the position of the Schiff's base nitrogen at the end of the polyene chain of retinal).

study the lattice constant **a** decreased by 1.2 Å from 62.3 Å at room temperature to 61.1 Å at −180°C in accordance with previous measurements (19). Application of these phases to the slightly smaller unit cell at low temperatures is justified, since a constant shrinkage of both the lipid and the protein region in the elementary cell occurred. This is proven by the fact that the reflection intensities do not change in our resolution regime between these two temperatures (see also ref. 19). Model calculations show that the small difference in the lattice constant **a** of 0.3 Å between BR-568 and M does not change appreciably amplitudes and phases at this resolution.

Superimposed on the BR structure in the M state at −180°C, Fig. 2*c* displays the highest positive and negative contour levels of the difference map in ^2H_2O and an outline indicating the in-plane position of the retinal (4, 17, 18). This projected M-state structure in Fig. 2*c* shows no prominent alteration relative to the BR-568 state at −180°C (data not shown) and resembles the projected structure of the dark-adapted BR obtained by electron microscopy (20). This proves that there is no large-scale global structural change in the BR monomer and that the orientation of the BR molecule in the trimer as well as the orientation of the trimer in the unit cell are identical for both photocycle states, in disagreement with previous conclusions (14, 21). Only the scattering density maximum of helix 2 is shifted by ≈1 Å toward the protein center. This shift corresponds to the strongest pair of a positive and negative peak next to helix 2 in the difference density map. Another strong difference density pair emerges near to helix 4 and 5, again with a positive density shift toward the retinal area. A less-prominent pair flanks helix 7. Obviously, both predominant positive-density peaks, which develop during M formation, are in projection localized in the vicinity of the cyclohexene ring and the Schiff's base end of the chromophore.

What is the structural basis of the observed alterations in scattering density during the BR-568 to M transition? As already indicated by the high correlation of difference structure factors of samples in ^2H_2O and in H_2O, the features in the difference Fourier maps are very similar (Fig. 2 *a* and *b*). This confirms that the ΔF values are not dominated by a redistribution of water or exchangeable hydrogens but by alterations in the conformation of the protein. The scattering length of the most prominent positive difference peak at helix 2 corresponds to ≈7% of the scattering length of helix 7—i.e., to the density of ≈1.5 amino acids. The overall structural alterations per BR monomer are explained either by a small tilting of three or four helices (helices 2, 7, 4, and/or 5) or by positional changes of four or five amino acids. Our results cannot exclude shifts of amino acid residues over distances of 3–5 Å. However, we favor a small tilt in α-helices of 1–2°—i.e., in the limit set by recent Fourier transform infrared (FTIR) measurements (22, 23). It should be noted that our results refer to changes in the projected structure parallel to the plane of the membrane.

Comparison of our data with those of high-resolution electron diffraction of the M intermediate by Glaeser *et al.* (3) is of special relevance. Their diffraction data show significant changes only in the resolution regime of 5.0–3.3 Å but not in the resolution zone of our data sets, and they are of smaller magnitude than in the present investigation. This discrepancy might be due to differences in the sample preparation. The electron microscopy was performed in the vacuum on glucose-embedded membrane sheets. Only the M sample was illuminated during cooling to −90°C, different from our procedure where for both states the sample was exposed to the same amount of light above 4°C. The difference Fourier maps of both investigations agree only with respect to the prominent positive density peak at helix 2. Light-induced small alterations of the protein backbone were proposed from FTIR spectroscopy (22, 24, 25).

We assume that the localized changes in the protein moiety of BR are of relevance for its function as a proton pump. Photon absorption leads to a trans to 13-cis isomerization of the retinal in the primary photoproduct K (26), which might induce the observed structural alterations of the protein in the vicinity of the cyclohexene ring and the Schiff's base. During M formation, the initially protonated nitrogen of the Schiff's base is forced into different interactions with charged amino acids. The induced deprotonation of the nitrogen is followed by the observed release of one proton into the external aqueous medium (9). Thereafter, the reversible changes in the protein structure result in the reprotonation of the Schiff's base nitrogen and initiate the 13-cis to trans isomerization of the retinal. It is worth mentioning that these transient conformational changes detected by our neutron diffraction study occur at those positions where according to the "external two-point-charge model" (27) negative counterions are located at the chromophore. The rate of the structural changes in the protein shall determine M formation and the deprotonation of the Schiff's base (28). The elimination of the positive charge on the nitrogen by deprotonation is responsible for the large blue-shift of the absorption maximum for BR during M formation. Our findings are also in line with the transient protonation changes of various amino acid residues (28, 29) and support the "C–T model" of isomerization-driven protein conformational changes as the reprotonation switch (12). The main conformational displacement toward the center of the protein visible in the strong positive differ-

ence peak at helix 2 (Fig. 2*c*) in combination with the data of refs. 17 and 18 suggest that this helix corresponds to the transmembrane helix G containing the lysine-216 to which the retinal is covalently attached.

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