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Light- and dark-adapted bacteriorhodopsin, a time-resolved neutron diffraction study

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Recently, neutron diffraction experiments have revealed well-resolved and reversible changes in the protein conformation of bacteriorhodopsin (BR) between the light-adapted ground state and the M-intermediate of the proton pumping photocycle (Dencher, Dresselhaus, Zaccai and Buldt (1989) Proc. Natl. Acad. Sci. USA 86, 7876–7879). These changes are triggered by the light-induced isomerization of the chromophore retinal from the all-*trans* to the 13-*cis* configuration. Dark-adapted purple membranes contain a mixture of two pigment species with either the all-*trans*- or 13-*cis*-retinal isomer as chromophore. Employing a time-resolved neutron diffraction technique, no changes in protein conformation in the resolution regime of up to 7 Å are observed during the transition between the two ground-state species 13-*cis*-BR and all-*trans*-BR. This is in line with the fact that the conversion of all-*trans* BR to 13-*cis*-BR involves an additional isomerization about the C15=N Schiff's base bond, which in contrast to M formation minimizes retinal displacement and keeps the Schiff's base in the original protein environment. Furthermore, there is no indication for large-scale redistribution of water molecules in the purple membrane during light-dark adaptation.

Introduction

Dark-equilibrated purple membranes (PM) contain a large proportion of 13-*cis*-bacteriorhodopsin (BR_{548}), i e, a bacteriorhodopsin (BR) species with the 13-*cis* isomer of retinal as chromophore covalently linked to the protein moiety via a protonated Schiff's base [1] Depending on the experimental conditions, values of about 50% [1–6], 55% [7,8], 60% [9], or 66% [10] 13-*cis* BR_{548} in dark-adapted PM have been reported 13-*cis* BR_{548} undergoes a photocycle lacking an M-like intermediate [2] and exhibits internal charge motions [8,11], however, no vectorial proton translocation has been observed [5,6,12]

The chromophore 13-*cis*-retinal does not only exist in the ground-state species 13-*cis* BR_{548}, but is also the chromophore configuration in most of the photocycle intermediates originating from the ground-state species all-*trans* BR_{568} [7,13,14] During illumination with moderate light intensities (light adaptation) all ground-state 13-*cis* BR_{548} molecules are converted into all-*trans* BR_{568} [1,2,15], the ground-state species of the proton translocating photocycle Recently, by application of neutron diffraction, we have localized reversible structural changes of the protein in the vicinity of the chromophore occurring during the transition from all-*trans* BR_{568} to the M-intermediate [16,17] This transition, which is accompanied by vectorial proton release, is triggered by the photochemical isomerization of all-*trans*-retinal to the 13-*cis* configuration Therefore, it is interesting to know if the photochemical 13-*cis* (BR_{548}) to all-*trans* (BR_{568}) as well as the thermal all-*trans* (BR_{568}) to 13-*cis* (BR_{548}) isomerization of retinal involved in light-dark adaptation also leads to structural changes in the protein moiety

In the present study, however, no conformational difference in the projected structure of the protein between light- and dark-adapted BR could be detected by neutron diffraction Furthermore, in contradiction to a previous suggestion [18], there is no redistribution of water molecules in BR during light-dark adaptation

Materials and Methods

Purple membranes were isolated from *Halobacterium halobium* (strain ET 1001) according to standard proce-

Abbreviations BR, bacteriorhodopsin, PM, purple membrane

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dures Neutron diffraction experiments require approx 70 mg BR for in-plane data acquisition in a reasonable time Purple membranes were oriented on 14 thin quartz slides (65 × 15 × 0 3 mm) with their planes parallel to the support surface by slowly drying aqueous suspensions at room temperature and 86% relative humidity in D_2O Before the neutron diffraction measurements, these PM samples were reequilibrated to 100% relative humidity (see below) Each PM film had an optical density of less than 3 at 568 nm Control experiments have shown that films prepared at a relative humidity below 76% could not be reequilibrated to 100%, even after several days and therefore did not undergo complete light-dark adaptation A second set of 17 slides covered with PM were prepared in the same way, reequilibrated at 100% relative humidity in D_2O, and thereafter glued together with a cover slide of the same dimensions These sealed samples do prevent any evaporation of water This fact was spectroscopically verified Sealed PM film samples, and as control also unsealed samples, were illuminated with an intensity 40-times higher than applied for the actual diffraction experiment Thereafter, the visible absorbance spectrum as well as the spectral changes during light-dark adaptation were monitored The width of the absorbance band (e g , the FWHM) is a reliable indicator for both the degree of hydration of BR and the ability of light-dark adaptation (Dencher, unpublished result) Upon illumination, only the sealed PM films revealed the spectroscopic properties and the light-dark adaptation features characteristic for PM in aqueous suspensions, excluding any evaporation of water due to temperature changes Preparation of samples, reequilibration at either 100% D_2O or H_2O relative humidity, light- or dark-adaptation, and mounting of the non-sealed PM films into the sample container were performed in a specially designed glove box that avoids D_2O/H_2O exchange with the environment

The kinetics of light-dark adaptation for the various PM film samples were measured at 585 nm, the maximum in the difference spectrum for dark- and light-adapted BR, in a temperature- and humidity-controlled compartment of a UV-visible recording spectrophotometer (Shimadzu UV 240) Care was taken that the intensity of the measuring beam was sufficiently weak so that even at 5°C the dark adaptation reaction was not influenced Both, at 5 0 and 20 0°C, the rate constants determined for PM film samples were very similar to those of PM in aqueous suspensions, as long as about 100% relative humidity was sustained (compare also Ref 19) At 5 0°C (measured at the surface of the PM film) and 100% D_2O relative humidity, the dark adaptation reaction followed first-order kinetics with a lifetime of 26 6 h

The neutron diffraction experiments were carried out at 5°C, just above the freezing point of D_2O, for two reasons At this temperature the light-adapted state decays with a lifetime of 26 6 h Therefore, the relaxation of the light- to the dark-adapted state could be measured during several neutron runs each consisting of an in-plane run of 4 h followed by a short lamellar scan of 0 5 h The second advantage using this temperature was that complete light-adaptation was obtained by a very weak illumination over 4 h (18 watt Osram L18W/25 lamp with Schott heat filter KG1, intensity on the sample surface 500 $\mu W/cm^2$; simultaneous illumination of both sides of the PM films) Thus, any heating of the films, that results in artificial local dehydration with associated structural changes of BR [17], was avoided This is important because it has been demonstrated that at a relative humidity of less than 95% only partial conversion to the light-adapted state can be achieved [19] All the diffraction experiments were performed with a sample container well isolated in order to minimize temperature gradients in the sample compartment which would lead to a dehydration of PM films Illumination and subsequent mounting of the stack of 14 sample slides were carried out in a glove box at 100% D_2O or H_2O atmosphere in a cold room at 5°C near to the diffractometer The transport of the sample container to the thermostat on the diffractometer was finished within 1 min After a quick alignment of the sample container, the data collection runs started immediately Further details concerning the neutron diffraction technique are described elsewhere [17,20,21]

Results

All except one [17] of the neutron diffraction experiments previously described have been performed in the dark-adapted or in an undefined state of BR Fig 1 presents the projected structure of PM in the light-adapted state in H_2O at 5°C No noticeable differences compared to maps of the dark-adapted state are visible (data not shown) As outlined in Materials and Meth-

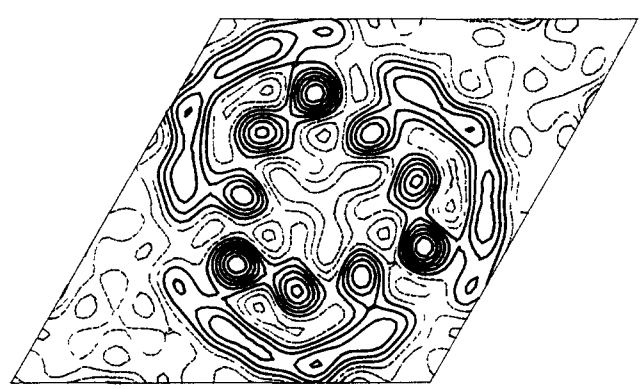

Fig 1 Projected structure of purple membrane in the light-adapted state at 5°C (H_2O, 100% relative humidity, unit cell dimension 62 5 Å)

ods, the kinetics of the light-to-dark relaxation was followed by a series of runs each consisting of a 4 h long in-plane θ–2θ scan and a short lamellar scan of 0 5 h In order to obtain a counting statistic in background points of better than 0 5%, the light to dark relaxation runs were repeated several times and the data of the same measuring periods were averaged Fig 2a shows in the lower graph a diffraction pattern for the first 4 h of this relaxation (starting 0 5 h after the end of illumination) The upper graph represents the difference pattern between these averaged in-plane runs (0 5–4 5 h after termination of illumination) and a series of 4 h runs 40 h later, i e , after the dark-adapted state was approached The only changes above the noise level occur in the two low-order reflections (1,1) and (2,0) This would indicate that during light-dark relaxation small changes between protein and lipid domains took place in the in-plane structure of PM However, an analysis of the differences, showing an increase in intensity of these reflections in the light-adapted state, indicates a small dehydration in the lipid domains of the purple membranes This was deduced from the observed intensity changes in these reflections upon a systematic variation in the hydration of PM films [33] These

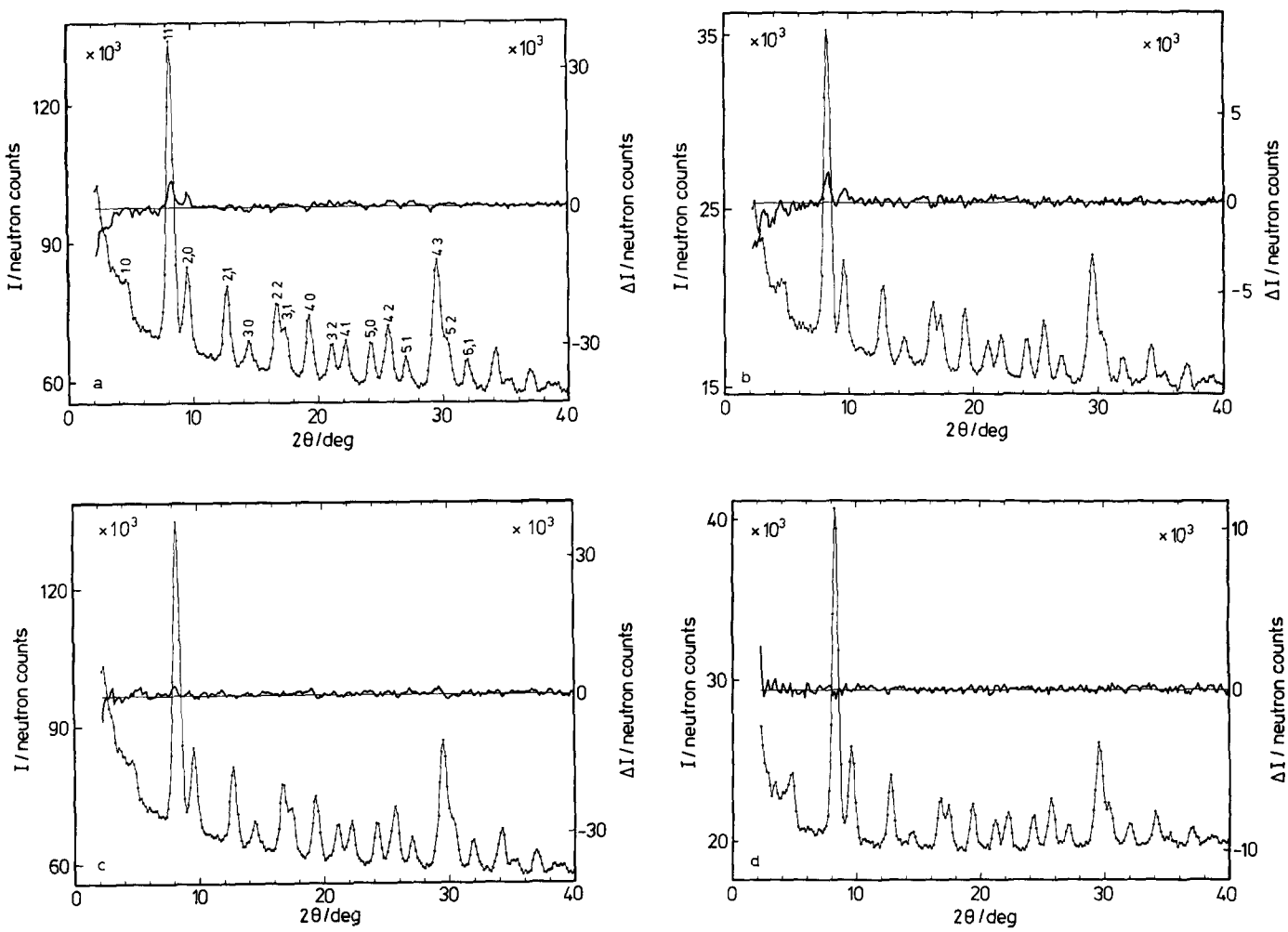

Fig 2 (a) Difference diffraction pattern (upper graph) of purple membrane films in D_2O between the light-adapted state (time window from 0 5 to 4 5 h after the end of illumination) and after relaxation to the dark-adapted state 40 h later (time window from 38 to 42 h after termination of illumination) In the lower graph neutron counts of the light-adapted state (first time window) are plotted against diffraction angle 2θ and indexed according to an hexagonal lattice (b) Difference pattern obtained for a dark-adapted sample (same PM films in D_2O as used in Fig 2a) directly after transfer into the sample compartment (time window from 0 5 to 4 5 h) and 17 5 h later (from 15 5 to 19 5 h) The lower graph depicts the neutron counts as a function of the detector angle 2θ monitored during the initial time period (0 5–4 5 h) after loading the sample compartment The total measuring time was shorter than in (a) resulting in a smaller number of neutron counts (c) Difference pattern between the light- (illuminated) and dark-adapted (not illuminated) specimen (difference of the lower traces in (a) and (b) after scaling) Both data collection runs (average of several 4 h measuring periods) were started 0 5 h after loading the sample container with the same sample either in the light-adapted or dark-adapted state The lower graph shows the reflections for the light-adapted sample in D_2O (same as lower trace of Fig 2a) (d) Difference diffraction pattern from a stack of sealed PM films (D_2O) in the light-adapted state (time window from 0 5 to 4 5 h) and 11 8 h later (time window from 9 8 to 13 8 h after the termination of illumination) in the relaxation towards the dark-adapted state The diffraction pattern of the second time window is shown in the lower graph

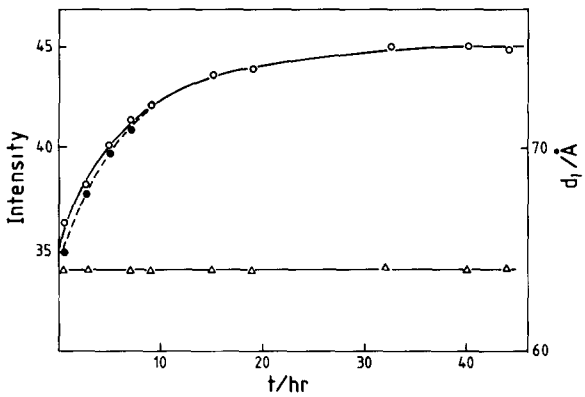

Fig 3 Lamellar diffraction of purple membranes in D_2O at 5°C Relaxation of the intensity (relative units) of the first lamellar order ($l = 1$) of the specimen in the light-adapted state (○———○) and in the dark-adapted state (●- - -●) The lower trace (△———△) shows the very small increase of the lamellar spacing d_1 during relaxation of the light-adapted sample

hydration changes, however, are not related to light-dark adaptation, since their time dependence is much faster (about 8 h)

As control, time-dependent alterations in a dark-adapted (not illuminated) specimen (same sample as Fig 2a) were monitored after its transfer into the sample container (Fig 2b) The difference pattern (upper graph) of the dark-adapted sample between in-plane scans for the first 4 h after the start of the measurement and 4 h runs after 17 5 h has the same increase in intensity for the reflections (1,1) and (2,0) as detected in the light-dark transition (Fig 2a) This result demonstrates that the changes observed in Fig 2a do not originate from changes in the structure between the light- and dark-adapted states, but probably from small temperature effects during the transfer of the sample container from the cold room to the diffractometer It seems reasonable that the small temperature increase has produced temperature gradients in the sample compartment, leading to the small dehydration effect of the PM films and the observed subsequent relaxation to full hydration It is interesting to note that lamellar diffraction shows the same behaviour The temporal changes in intensity of the lamellar first order are the same whether the measurement is started with a light- or with a dark-adapted specimen (Fig 3) The time constant of the observed relaxation is about 8 h and therefore much smaller than the lifetime of the light-adapted state under these conditions ($\tau = 26\,6$ h) The lamellar spacing of the light-adapted state (Fig 3) shows only a very small increase with time from 64 8 Å at 1 h to 64 9 Å at 40 h after the end of illumination These values demonstrate that the dehydration effect occurring during transfer of the sample container is very small as determined by the slight change in lamellar spacing, but leads to a dramatic increase in the intensity of the first lamellar order of about 25%

In order to correct for the dehydration artefact, the diffraction patterns of the same PM films, being in the light-adapted or dark-adapted state, respectively, at the start of the measurement, were compared at identical time intervals after transfer of the sample compartment to the diffractometer The difference pattern obtained in this way for the first time period (Fig 2c upper trace, corresponding to the difference of the diffraction pattern shown in the lower traces of Figs 2a and 2b) and also for subsequent time periods reveals no differences between the light- and the dark-adapted state in the resolution regime of up to 7 Å (statistical error in intensity background points of 0 5%) We are describing this effect in detail since most changes during light-dark adaptation and also the large-scale structural alterations in the PM during formation of the intermediate M previously reported in the literature might be caused by similar artefacts In order to confirm the result of Fig 2c, an additional experiment with air-tightly sealed specimen was performed The difference pattern of a light-to-dark relaxation between the initial time period after starting the experiment and runs carried out 11 8 h later (Fig 2d) does not reveal any difference peaks larger than the noise level, in full agreement to the result of Fig 2c With sealed films no dehydration can occur in the presence of small temperature gradients in contrast to the observation with non-sealed films (Figs 2a and 2b)

Discussion

The present neutron diffraction investigation clearly demonstrates that the transition from all-*trans* BR_{568} to 13-*cis* BR_{548} (light-dark adaptation) is not accompanied by detectable structural changes in the protein moiety of BR and in its hexagonal lattice arrangement in the purple membrane It should be noted that only in-plane changes of the structure would be detectable in the present study Since the light-dark equilibration critically depends on the humidity of the sample [8,19,22] and since small variations in the hydration also affect neutron diffraction data (Figs 2a and 2b), special care was taken to study the samples under the most appropriate conditions such as 100% relative humidity, temperatures above 5°C, and light adaptation with low light intensities Furthermore, all necessary controls were performed to avoid or to detect artefacts, e g , heat-induced structural changes during light adaptation [23]

The absence of substantial conformational changes in BR's protein moiety during light-dark adaptation is unambiguously demonstrated for the first time by a diffraction technique and is in line with previous suggestions based on indirect spectroscopic methods [24,25] Also the very small enthalpy and entropy changes for the thermal isomerization of the chromo-

phore during dark adaptation exclude larger changes in the protein conformation [2,6] This is corroborated by high-pressure experiments with the calculated small molar volume change of -7.8 ± 3.2 ml/mol for the reaction all-*trans* BR_{568} to 13-*cis* BR_{548} (a smaller volume for 13-*cis* BR_{548}), corresponding to the formation of about two hydrogen bonds or to the ionization of no more than two residues [4] The difference in the permanent dipole moment of light- and dark-adapted PM corresponds to a change of about 0.5 electric charges at one surface of BR that could originate from the alteration in the dissociational state of a few surface amino acids [26]

Why is the all-*trans* to 13-*cis* isomerization of the chromophore retinal accompanied by localized structural changes in the protein only for the transition from all-*trans* BR_{568} (light-adapted ground state) to the M-intermediate [17], but not for the all-*trans* BR_{568} to 13-*cis* BR_{548} (light-dark adaptation) reaction? The answer seems to be obvious if one considers the fact that the latter, but not the former process involves an additional isomerization around the C15=N Schiff's base bond, i.e., the chromophore in all-*trans* BR_{568} has a 13-*trans*,15-*anti* (= *trans*) configuration and in the photocycle intermediate M 13-*cis*,15-*anti*, but is 13-*cis*,15-*syn* (= *cis*) in 13-*cis* BR_{548} [9,27–29] As is stated in Ref 27 and depicted in Fig 1 of Ref 29, the 15-*syn* bond in the 13-*cis* isomer of BR_{548} allows the chromophore to be accommodated in an approximately linear binding pocket of about the same dimensions as required for the all-*trans*,15-*anti* isomer Therefore, no pronounced change in the protein conformation will be induced by this double isomerization In accordance with this view, the change in retinal angle with respect to the membrane normal is $\leq 0.8°$ between all-*trans* BR_{568} and 13-*cis* BR_{548} [26,30,31] Furthermore, the interaction of the chromophore with neighboring aromatic amino acid residues is reported to be very similar for these two BR species [32] On the other hand, isomerization solely around the C13=C14 bond occurring during M formation has to lead to a large steric force on the protein with the observed structural changes in the vicinity of the chromophore binding pocket [17]

This structural difference in the retinal geometry and not a rearrangement of the protein moiety seems to be responsible for the lacking proton pumping activity of 13-*cis* BR_{548} It could also be speculated that a displacement of one of the strongly bound water molecules or exchangeable hydrogens, recently localized at the Schiff's base end of the chromophore in the projected structure of BR [33], causes the ineffectiveness of 13-*cis* BR_{548} in H^+-translocation For the function of BR as a light-driven proton pump it should also be considered that during the all-*trans* BR_{568} to M-transition with its protein conformational changes, the Schiff's base becomes transiently deprotonated, which is not the case for the all-*trans* BR_{568} to 13-*cis* BR_{548} reaction

One specific advantage of neutron diffraction is that due to the large difference in the coherent neutron scattering length of H and ^2H, redistribution of associated water and exchangeable hydrogens related to any molecular event can be studied By conducting experiments in H_2O and D_2O, we could exclude any large-scale redistribution of water molecules or exchangeable hydrogens related to light-dark adaptation, both in the lipid and in the protein domains of the PM Especially, the previous postulation, based on infrared spectroscopy, of an increase in sorbed water of 200 molecules per BR upon light adaptation [18] can be rejected This large amount should lead to an increase in the lamellar spacing d_1 of about 4.8 Å, if these water molecules would be aligned as a homogeneous interlamellar layer Our results depicted in Fig 3 (lower trace) are in clear contradiction to this calculated large spacing increase If these water molecules would redistribute in the membrane phase, they could have been easily detected due to the resulting contrast variations However, our data exclude this possibility, too

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