HYDROGEN EXCHANGE OF DARK-ADAPTED AND ILLUMINATED BACTERIORHODOPSIN

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1. Introduction

Bacteriorhodopsin (BR) in the purple membrane regions of the cell membrane of halobacteria functions as a light-driven proton pump resulting in the formation of proton gradients across the cell envelope membrane [1–3]. According to the model of Henderson and Unwin [4], of the purple membrane derived from X-ray diffraction analysis and electron micrographs, bacteriorhodopsin molecules consist of seven alpha-helical polypeptide segments oriented perpendicular to the plane of the membrane, the helical content being estimated to be 70–80%. From this model, and also from the lack of change of the dichroic ratio during the decay of polarized flash photolysis [5], it has been generally considered that bacteriorhodopsin molecules are organized in a fairly rigid structure which does not change conformation or rotate in the membrane. If conformational changes of bacteriorhodopsin are involved in proton translocation this would have importance for the mechanism of energy transduction.

Dark-adapted bacteriorhodopsin exhibits a maximum absorption band at 558 nm which upon illumination shifts within several seconds to 568 nm [6]; this species then undergoes a photochemical reaction cycle leading to the formation and decay of a number of other distinct spectral intermediates [6–8]. Previous studies have indicated that retinal itself undergoes changes in conformation during photochemical cycling [8], but little direct information exists on the nature or extent of conformational change in the protein moiety. Our previous studies [9] on chemical modification of dark-adapted and illuminated purple membrane showed different inhibitory effects by certain imidoesters (dimethyl adipimidate) in these two states.

In the present investigation tritium and deuterium exchange studies have been used as a more precise method to obtain evidence of different conformations of bacteriorhodopsin in dark-adapted and illuminated purple membrane preparations. Our results reveal small but distinctly different exchange rates, hence the molecule has a different conformation in these two conditions.

2. Materials and methods

Purified purple membrane preparations from *Halobacterium halobium* were kindly provided by Dr J. Lanyi (NASA-Ames Research Center).

For tritium exchange-in studies, purple membrane preparations were suspended in buffer, 20 mM phosphate at either pH 6.6 or pH 8.2 and 200 mM KCl in rubber stoppered vials. Samples were covered with aluminium foil and kept at 2°C for 24 h. The samples were then removed, one kept in the dark and the other under illumination at 28°C for 30 min, then 10 mCi of ^3H_2O (ICN Co.) was added. At various time periods 0.1 ml aliquots were withdrawn into 10 ml buffer and immediately centrifuged at 15 000 × *g* for 10 min. The precipitate was rinsed three times with H_2O and finally suspended in 1 ml H_2O. Tritium radioactivity was measured in 0.2 ml of this solution and the remainder of the solution was used for determining the concentration of bacteriorhodopsin by 570 nm absorbance measurements [10]. This procedure was satisfactory for longer-term studies of tritium exchange, but for short-term studies the method

of Englander and Englander [11] was used in which samples could be processed more rapidly by passing them through a Sephadex column.

Deuterium exchange-in studies were performed by incubating purple membrane preparations in 99.5% D_2O/200 mM KCl, p*D* 5.8. At various time intervals aliquots of the solution were withdrawn, frozen at −50°C and then lyophilized. The infrared absorption spectrum of dried purple membranes was recorded using a Nujol–NaCl plate. The ratio of amide I : II absorbance was used as a measure of deuterium exchange according to the method of Susi [12].

Tritium exchange-out was carried out with purple membrane preparations kept in the presence of 10 mCi ^3H_2O for 24–48 h at 28°C. Since purple membrane fragments tend to aggregate after long-term incubation, exchange-out studies were carried out by the dialysis method. Purple membranes were collected by centrifugation, resuspended in 3 ml of buffer (20 mM phosphate/200 mM KCl), transferred to a dialysis tube and dialyzed against 2 liters of buffer. Aliquots, 50 μl, were removed from the dialysis bag for counting. Initial rates of tritium exchange-out were measured by the Sephadex column method [11].

3. Results and discussion

Since tritium exchange is a sensitive method for detection of protein conformational changes, we compared the rates and extent of such exchange in dark-adapted and illuminated purple membrane preparations. Typical tritium exchange-in results show two phases of exchange as shown in fig.1A,B. In the early phase of incubation about 16–25% of total exchangeable polypeptide hydrogen was exchanged, thereafter exchange occurred more gradually (fig.1A). In this experiment about 89 and 78 hydrogen/molecule, which corresponds to 37% and 32%, respectively, of the total of 242 exchangeable polypeptide hydrogens [13], were exchanged in 48 h by the dark-adapted and illuminated samples, respectively. In three independent experiments the average difference between the dark-adapted and illuminated samples was 6.7 ± 2.3 (5–9%) after 48 h. When tritium exchange was performed at 4°C exchange rates were slowed down, but the initial rapid exchange was not

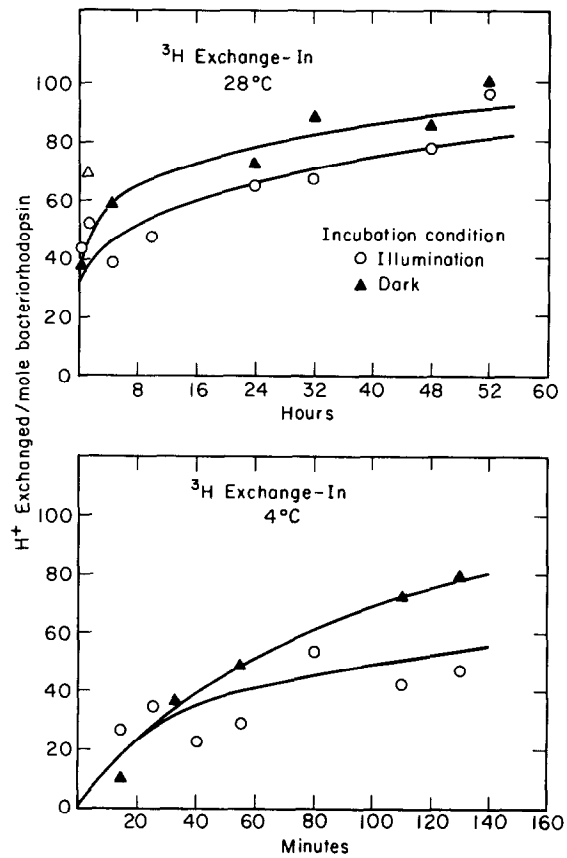

Fig.1. Kinetics of exchange-in of ^3H_2O into dark-adapted and illuminated bacteriorhodopsin in purple membranes. (A) Long-term exchange carried out by dialysis method. (B) Initial phase of exchange-in.

significantly affected. The experiments carried out at 4°C (fig.1B) were made by the Sephadex column method [11] which permitted a more precise measurement of the initial kinetics. In this case about 12% of the total exchangeable hydrogen were exchanged in the first 40 min in both dark-adapted and illuminated samples. After the initial phase, the second phase of exchange, although much lower than at the higher temperature, was still 1.5 times more rapid in the dark-adapted than in illuminated samples. Thus a small but reproducible higher tritium exchange rate is observed in dark-adapted as compared to illuminated samples.

Experiments were also carried out using D_2O; measurements of the ratio of the amide II and amide I bands indicated that the exchange-in profiles were almost identical to those observed in the studies with tritium. About 20% of the total exchangeable polypeptide hydrogen exchanged in the first 2 h was followed by a gradual exchange over the next 40 h. Over the 40 h period, the extent of exchange-in (about 45%) by the dark-adapted sample was about 7% greater than the illuminated sample.

The kinetics of tritium exchange-out in dark and illuminated purple membranes was also investigated (fig.2, table 1). An initial rapid decrease of tritium is observed in both dark and illuminated samples. This may be attributed to the free ^3H_2O adsorbed as confirmed by the dialysis profile of the ^3H_2O control (not shown). The second phase of exchange-out, which is not resolved from the first phase, is due to hydrogen exchange from exposed polypeptide segments and a third phase of exchange is a slower rate due to buried and/or hydrogen bonded hydrogens. The latter show a half-time for exchange about 2.5-fold faster for the dark-adapted than for the illuminated samples. Similar differences between dark and illuminated samples were observed in several other conditions as summarized in table 1.

Hydrogen exchange by tritium or deuterium shows consistent differences between dark-adapted and illuminated samples. In all cases dark-adapted

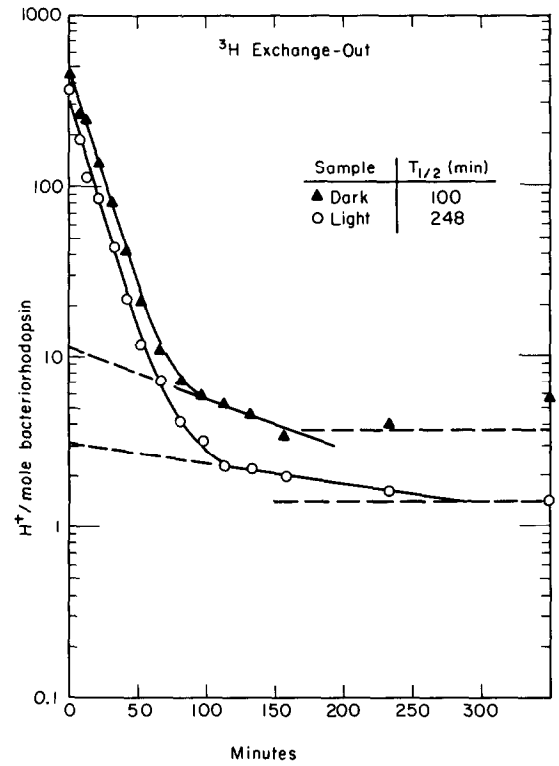

Fig.2. Kinetics of ^3H_2O exchange-out from dark-adapted and illuminated bacteriorhodopsin in purple membranes. Horizontal dashed lines show the equilibrated level of tritium after prolonged dialysis.

Table 1
Tritium exchange-out rates for bacteriorhodopsin

Sample	Initial phase (t = 1/2 min)	Slow phase (t = 1/2 min)	Conditions
Dark-adapted	39.3	228	pH 6.6 buffer, 4°C
Illuminated	44.5	328	Sephadex column method
Dark-adapted	7.4	80	pH 6.6 buffer, 4°C
Illuminated	11.7	267	Sephadex column method
Dark-adapted		147	pH 6.6 buffer, 4°C
Illuminated		256	Dialysis method
Dark-adapted		100	pH 6.6 buffer, 4°C
Illuminated		248	Dialysis method
Dark-adapted		138	pH 8.2 buffer, 4°C
Illuminated		325	Dialysis method

purple membranes can more freely exchange hydrogen (both kinetics and extent) indicating a more open or rigid conformation of bacteriorhodopsin in the dark-adapted state. It may be that a more compact conformation of the illuminated preparation is a more favorable condition for facilitating proton translocation and for chromophore–protein interaction. In contrast to the situation with mammalian rhodopsin [14], where complete exchange occurs in a 10–12 h period, the exchange rate of bacteriorhodopsin is fairly slow and only about 30–60% of the exchangeable polypeptide hydrogen is exchanged in a 48 h period. These results seem consistent with the three-dimensional model of Henderson and Unwin [4] for the structure of bacteriorhodopsin in the purple membrane in that the initial phase of rapid exchange-in may be due to the exposed moiety of the protein at the membrane interface, and the secondary slower phase due to hydrogen bonded in the interior of the alpha helix or buried more deeply in the protein moiety within the plane of the membrane. Our results (fig.1) and also those of Englander and Englander [13] who reported tritium exchange in purple membranes indicate that the exposed moiety of the protein is probably not more than 25% of the total protein, and that the residual protons are not only hydrogen bonded, but deeply buried in the lipid of the membrane because the secondary phase exchange-in rate is too slow to be explained by usual rates observed for hydrogen of soluble proteins. Although these studies confirm that bacteriorhodopsin is probably fairly rigidly positioned in the membrane based on the slow rate of exchange, it is also equally clear that small but reproducible conformational changes indeed do exist between the dark-adapted and illuminated samples. The dark–light difference in the number of hydrogens being exchanged over a 48 h period is about 7% of the total exchangeable polypeptide hydrogen. Becher and Cassim [15] found no apparent difference between dark-adapted and illuminated bacteriorhodopsin by circular dichroism measurements and concluded the conformational change is limited and localized, involving only a few amino acid residues. Recent studies by Kagawa et al. [16] on circular dichroism of purple membranes in guanidine hydrochloride solution showed marked differences in conformation, involving aromatic amino acid groups between dark and illuminated samples but not of alpha helical content. Indeed our results suggest that the illumination of dark-adapted bacteriorhodopsin induces conformational changes which we estimate involves 5–10 amino acid residues based on extrapolation to zero time of the slow phase of hydrogen exchange-in (fig.1A,B) and out (fig.2) which shows that the net difference of exposed polypeptide hydrogens is between 5 and 10. This change may be essential for facilitating chromophore–protein interaction and for directing the vectorial translocation of protons through bacteriorhodopsin.

Acknowledgement

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