THE CONSEQUENCES OF A DEUTERIUM EXCHANGE TEST ON PROPOSED MECHANISMS FOR THE PURPLE MEMBRANE PROTON PUMP

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Received 19 June 1979
Revised version received 2 August 1979

1. Introduction

The capture of radiant energy from the sun by *Halobacterium halobium* and its ability to use this energy to power its proton pump [1–3] has received much attention recently. Their energy converters, which are purple patches that the bacteria develop in their cell membrane when the dioxygen concentration of the growth medium is reduced, are essentially 75% protein and the rest lipid [4]. The purple pigment, bacteriorhodopsin (bR), results from a combination of retinal and bacterioopsin (mol. wt 26 000), through a Schiff base linkage with a lysine residue [4,5]. It has been shown that the chromophore of light-adapted bR is all-*trans* retinal while that of the dark-adapted contains equal amounts of 13-*cis* and all-*trans* retinal [3]. Photoexcitation of bacteriorhodopsin (λ_{max} 570 nm) produces a red-shifted intermediate, bathobacteriorhodopsin (λ_{max} 610 nm), as the first detectable species [6]. A similar intermediate is formed in the rhodopsin system. Because this initial transformation in rhodopsin [7,8] and bacteriorhodopsin [8] has a low activation energy and exhibits a kinetic isotope effect in D_2O, it has been suggested that a proton transfer is involved. The Schiff base nitrogen has been proposed as the proton acceptor in rhodopsin [7–9] and implied for bacteriorhodopsin [8]. An alternative view, however, suggests that the formation of the red-shifted intermediate is due to a protein conformational change in the retinal binding region and that the isotope effect results from the attendant proton transfers within the protein [10].

Among the mechanisms proposed to account for aspects of the light-driven pumping cycle, three require carbon–hydrogen bond ruptures and reformations within the retinal moiety. This report describes a test of these proposals whereby the hydrogens of the apoprotein were exchanged in D_2O and the reconstituted and active pigment was subjected to light-driven pumping in D_2O. Retinal from these samples was found to have no deuterium incorporated.

2. Materials and methods

Halobacterium halobium R-1 strain was grown and their purple membranes were isolated and purified according to published methods [4]. Purified purple membranes (50 μM) were photobleached with 0.75 M hydroxylamine (pH 7.2) at room temperature. After complete bleaching (5 da) the membranes were dialyzed at 2°C against 5 × 110 vol. 10 mM potassium phosphate buffer (pH 7.4) to ensure that the NH_2OH concentration was < 75 pM. The membranes were then freeze-dried, incubated with D_2O for 24 h and this process repeated again. The characteristic A_{570} of the purple membrane was completely absent at this point but was regenerated by incubation with 1.5 equiv. all-*trans* retinal in D_2O. Of the original absorption > 85% was restored on prolonged incubation at 2°C with 75% formed in the first 5 min. Excess retinal was removed by washing twice with 2 vol. of *n*-hexane. The purple membrane, in D_2O, was illuminated with white light under different conditions (see table 1) prior to extraction with CH_2Cl_2 at 2°C. Extractions

Table 1
Excess atoms of deuterium in retinals isolated from bacteriorhodopsin illuminated in D_2O[a]

Source	all-*trans*[b]	13-*cis*[b]
Retinal from purple membrane reconstituted in D_2O	−0.01[c]	−0.01[c]
	0.00[d]	0.00[d]
Retinal from undisturbed purple membrane suspended in D_2O	0.01[c]	−0.02[c]
	0.01[d]	0.01[d]

[a] Our precision is ± 1% of one atom of deuterium
[b] Reagent all-*trans* retinal (Sigma Chemical Co.) was used directly and served as a standard for mass spectral analysis. $(M + 1)/M = 0.2179$ (corrected for the presence of 3.9% retinoic acid) and was assigned a value of 0.000 excess atoms of deuterium. Retinoic acid was absent from all of the samples above
[c] Illuminated continuously for 24 h
[d] Illuminated for 24 h cycling 4.5 s on and 10 s off

from D_2O, under dim red light, were repeated until 80% of the retinal was removed.

The extracted retinal was first purified by chromatography on a silica gel column, using *n*-hexane–ether (4:1), to remove any contaminating phospholipids and followed by highly pressure liquid chromatography (HPLC) using cyclohexane–ether as solvent and a microparticulate silica gel column capable of separating five *cis–trans* isomers of retinal [11]. 13-*cis* and all-*trans* retinal were collected separately and introduced into an Hitachi-Perkin Elmer RMU-7 mass spectrometer through its direct inlet.

We have used the method in [12] to demonstrate that the proton pumping action of dissociated and then reconstituted purple membrane, remains intact. The purple membrane, reconstituted as described, was incorporated by sonication into vesicles prepared from L-α-phosphatidylcholine dimyristoyl (Sigma Chemical Co.) and L-α-phosphatidylethanolamine dilauroyl (4:1) (A grade, Calbiochem). Temperature controlled vesicles were irradiated with light from a 500 W projection lamp filtered by a 3–69 Corning filter. The pH was monitored with an Orion (no. 801) pH meter and an A. H. Thomas 4094-L15 combination electrode. No pH change could be observed for vesicles treated in the same way but not containing purple membrane.

3. Results and discussion

To provide the most favorable conditions for testing whether reversible hydrogen transfer from the chromophore occurs, the protein's hydrogens, in the region of binding and those possibly shielded by the chromophore, were subjected to exchange with D_2O. The chromophore was dissociated from the purple membrane and the resulting colorless membrane incubated with excess D_2O for 2 × 24 h. Similar conditions have led to exchange of 35% of the total exchangeable polypeptide hydrogens present [13]. The pigment was then reconstituted in D_2O. The reconstituted purple membrane exhibited typical light-driven pumping behavior. As shown in fig.1, the purple membrane, incorporated into vesicles by the

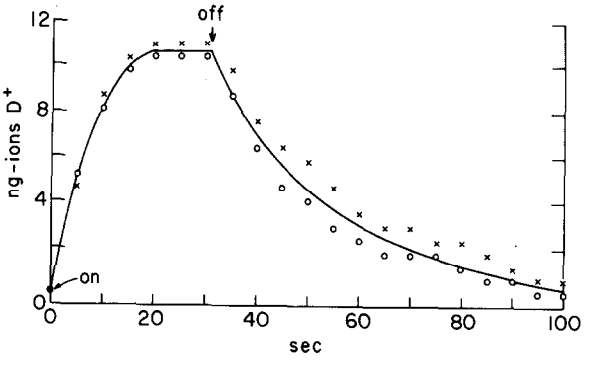


Fig.1. Light-driven pumping of H^+ from outside to inside phospholipid vesicles containing reconstituted deuterated bacteriorhodopsin. A total of 4.4 nmol bacteriorhodopsin were present. Crosses and circles represent two individual cycles. Light off and on are indicated.

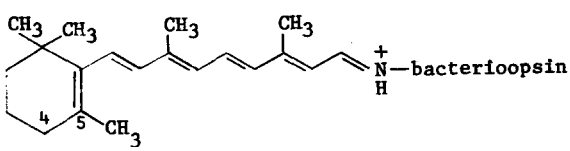
method in [12], pumped H^+ into their vesicles. The rate and extent was similar to that for vesicles containing unaltered purple membranes and similar to that already published [13].

The reconstituted purple membrane in D_2O was subjected to different conditions of illumination followed by dissociation and extraction of the chromophore. After extensive purification under non-exchanging conditions, retinals were analyzed by mass spectrometry. Spectra of standards agreed well with those in [14]. Retinal-d_0 ($M = 284$) exhibits an $M + 1$ peak [$(M + 1)/M = 0.2179$] which is almost solely due to the presence of 1.1% ^{13}C in the molecule. Retinal-d_1, if present, would lead to an increase in the $(M + 1)/M$ ratio. The results in table 1 indicate that hydrogen exchange in retinal does not occur during light-driven proton (deuteron) pumping in D_2O even though a substantial number of the exchangeable hydrogens on the protein are deuterated. Lack of peaks at higher masses indicate that more highly deuterated species are also absent.

The effect of a deuterated environment, in the context of the three mechanisms alluded to above, would suggest different degrees of proton exchange in the bound retinal. Mechanism 1 proposes that photoexcitation leads to charge separation within the retinal moiety and that proton translocation within the membrane occurs via a series of 1,2-shifts along the polyene chain [15]. This would lead to extensive exchanges with solvent and is therefore ruled out.

Mechanism 2 suggests that photoexcitation of bound, protonated retinal Schiff base promotes reversible hydrogen atom transfer between its C-5 methyl and an indole-nitrogen of a neighboring tryptophan [16]. This proposed retinal intermediate is suggested to be activated towards proton transfer from the Schiff base nitrogen to a neighboring acceptor and is pictured as one of the links in the proton relay chain.

A similar transfer was proposed to rationalize the red-shifted absorption of bathorhodopsin. A C-5 methyl proton was suggested to be transferred to an imino-nitrogen of imidazole in concert with transfer of a proton from the imidazole amino-nitrogen to the Schiff base [9a,b]. Although isotope exchange studies appeared to support this idea [9a], subsequent studies made C-5 methyl proton transfer untenable [9c]. Proton transfer from C-4 instead has now been suggested [7]. Similar prototropy for bathobacteriorhodopsin (mechanism 3) may seem reasonable [17].



Action via mechanism 2 or mechanism 3 could lead to the incorporation of 0–3 or 0–1 D-atoms, into retinal, respectively. The extent would depend on the accessibility of the acceptor groups to solvent D_2O and the relative rates of exchange of protons on acceptor nitrogen atoms (with solvent or neighboring residues) versus the rate of hydrogen transfer from nitrogen acceptor atoms back to retinal.

In our experiments the apoprotein was incubated in D_2O to exchange those protons which are near the chromophore but might be shielded by it from solvent. Subsequently the reconstituted purple membrane was photopumped continuously ($\sim 1.2 \times 10^7$ cycles) and intermittently for 24 h in D_2O. In view of the relatively long half-life (~ 4.5 μs) [6] of bathobacteriorhodopsin some exchange of its important acceptor NH group (imidazole or the newly created $\rangle N^+H_2$ of tryptophan) is expected each time it forms, provided that the group is accessible to D_2O or its surrogate, and consequently transfer of deuterium from an ND group back to a C-5 methylene (mechanism 2) or to C-4 (mechanism 3) would lead to deuterium incorporation. We feel that the present results cast doubt on these latter two mechanisms.

Acknowledgements

Research carried out at Brookhaven National Laboratory under contract with the US Department of Energy and supported by its Office of Basic Energy Sciences.

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