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Comparison of bacterial and animal rhodopsins by hydrogen exchange studies

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Rhodopsin is deeply embedded in a hydrocarbon membrane, yet hydrogen exchange (HX) studies show that about 70% of its peptide hydrogens are freely exposed to solvent water¹. This contrasts with other membrane-bound proteins and even aqueous proteins which generally involve about 70% of their peptide hydrogens in internal H-bonding. It has been suggested that the large fraction of disordered polypeptide chain indicated for rhodopsin resides in and acts to stabilise a channel of water penetrating into the membrane, and further that this channel must be quite large, perhaps 12 Å or more in diameter¹. Unwin and Henderson² described an electron diffraction model for the purple membrane protein of *Halobacterium halobium* (bacteriorhodopsin) which compares interestingly with the suggested structure of animal rhodopsin; membrane-embedded bacteriorhodopsin is arranged in a three-molecule circle surrounding an open space 20 Å in diameter. This space, however, is filled not with water but with lipid, and the protein walls of this blind channel are formed of a double rank of α helices which account for most of the bacteriorhodopsin polypeptide chain, so that bacteriorhodopsin, unlike animal rhodopsin, must be as extensively H-bonded as is the usual protein. We report here a HX study of bacteriorhodopsin. In agreement with the electron diffraction model, our HX results show that this protein, unlike animal rhodopsin, has about 75% of its peptides internally H-bonded. These and previous results, however, suggest a fundamental structural similarity between the two rhodopsins.

HX curves for bacteriorhodopsin in purple membrane are shown in Fig. 1. The slowly exchanging hydrogens measured come from internally H-bonded peptide groups of the membrane-bound protein (for discussion see ref. 1). To measure these, they were initially labelled, before the exchange-out experiment started, by incubating membrane suspensions in tritiated water for long periods at high pH to promote fast exchange. Exchange of the slow hydrogens that contribute to Fig. 1 was not characterised in any detail because we are more interested at this stage in the number of freely exposed peptide hydrogens in bacteriorhodopsin in order to compare this with the diffraction model and with animal rhodopsin.

To measure the faster exchanging, freely exposed peptide hydrogens, the earliest part of the curves in Fig. 1 must be studied. It is difficult to measure with good resolution these faster hydrogens above the large background of slow hydrogens. High resolution can be achieved by use of a limited exchange-in manipulation, described before (for example, ref. 3), which suppresses the background of slow hydrogens. Samples at pH 5 and 0 °C, where the free peptide half-time is close to 1 min, were initially exchanged-in for only 5 min, then quickly passed through a Sephadex column to terminate labelling and initiate exchange-out. This limited incubation period labels all the free peptides but only a greatly reduced number of the more slowly exchanging sites. The measured exchange-out data for the partially labelled protein (Fig. 2a) are fitted by a fast phase with the expected free peptide half-time and a slower phase with no predetermined parameters. The isolated fast phase is shown in Fig. 2b. Essentially the same procedure was repeated at pH 4.7 where peptide HX rate is twofold

slower (Fig. 3). In both cases the data indicate ~ 50 hydrogens per molecule exchanging at the expected free peptide rate. One can add 10 prolines to the unbonded residues. These amount to 25% of the total of 242 peptide groups⁴ in the molecule. This value is in line with the small fraction of free peptide hydrogens generally seen in other proteins and is consistent with the small fraction expected from the Unwin–Henderson model², but it is very different from the value of ~70% free peptides found for animal rhodopsin

The total number of hydrogens measured in these experiments can be obtained with best accuracy by considering Figs 1 and 2 together. Figure 2*a* gives the number that exchange in 5 min at *pH* 5.0 (since initial exchange-in time was 5 min). The number of hydrogens that exchange between 5 min (*pH* 5) and very much longer times can be obtained from the 5-min point on the *pH* 5.0 curve of Fig. 1. The sum of the faster and the slower hydrogens then yields the total number. Similarly, an independent estimate can be obtained from the *pH* 4.7 data (Figs 1 and 3*a*). The total number found, ~190 per bacteriorhodopsin molecule, is significantly lower even than the total number of amide hydrogens present (232 peptide H plus from 0 to 64 side chain amide H^{4,5}; degree of amidation in unknown). In similar studies with other proteins including membrane-bound animal rhodopsin, the number of hydrogens measured was experimentally identical with the number of amide hydrogens present. The resistant sites in bacteriorhodopsin which could not be labelled by the rigorous exchange-in conditions used here seem to carry the most slowly exchanging peptide hydrogens as yet indicated for any protein, with an exchange rate slower than the expected free peptide rate by a factor of 10¹⁰ or more. In view of the structure and placement of bacteriorhodopsin, this result is perhaps not too surprising.

These results indicate that bacteriorhodopsin has about 60 free peptides and therefore 180 internally H-bonded ones. This compares with the minimum number of 150 peptide H-bonds required by the Unwin–Henderson model² (to generate seven helices of seven turns each with the final turn non-bonded). Previous results¹ show the larger animal rhodopsin molecule to have about 100 H-bonded peptides, and just over 200 free peptides.

Some similarities between bacterial and animal rhodopsins have been pointed out by Osterholt and Stoeckenius⁵, including the use of a *cis*-retinaldehyde as a chromophore, the retinal to lysine Schiff-base linkage, and the effect of the protein on the retinal spectrum and on its chemical accessibility. Further, there is evidence that animal rhodopsin, like bacteriorhodopsin, has a quantity of α helix oriented predominantly normal to the membrane surface⁸, and there is the suggestion that animal rhodopsin, like bacteriorhodopsin, may exist as aggregates of several monomers⁹. HX results point to another mild coincidence; though animal rhodopsin is considerably larger than bacteriorhodopsin, the extra length of polypeptide chain in animal rhodopsin has no regular secondary structure so that the structured regions of these two proteins are of roughly comparable size.

Considering possible relatedness of these proteins, it is interesting to examine the bacteriorhodopsin model for clues to the structure and perhaps the function of animal rhodopsin. We note the coincidence involving the previously suggested large channel in animal rhodopsin and the 20-Å ‘channel’ found in bacteriorhodopsin. While the channel in bacteriorhodopsin is filled in with lipid², it has been suggested that animal rhodopsin maintains a functionally permeable channel by placing many of its extra amino acid residues within the channel with peptide groups exposed to maintain a polar milieu, therefore an aqueous filling¹. The apparent structural similarities mentioned above suggest a supporting structural framework like that in bacteriorhodopsin for the maintenance of the channel in animal rhodopsin.

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Fig. 1.

Hydrogen–tritium exchange-out data for purple membrane suspensions at *pH* 5.0 (○) and *pH* 4.7 (●). The bacteria were grown and the membranes were isolated according to Osterholt and Stoeckenius¹⁰. After exchange-in at *pH* 10.5 (2mM alanine buffer) for 5 d at 4 °C, the purple membrane suspension was adjusted to the appropriate *pH* and passed through a medium Sephadex-G25 column (at low hydrostatic pressure; 0 °C; *pH* 5.0, 10 mM cacodylate or *pH* 4.7, 5 mM citrate) to remove free tritiated water and initiate exchange-out. HX methods were detailed elsewhere¹¹. Two to three drop fractions collected from the final columns were diluted to 0.5 ml with 0.1 M phosphate buffer at *pH* 7.4, and maintained at 0°C in order to measure absorbance. (Absorbance measurements decrease slowly with time at room temperature and are slightly dependent upon *pH* and salt concentration.) Protein concentration in the samples was then computed using a molar extinction coefficient of 7.2×10^4 at 570 nm, determined using the retinaloxime method of Wald and Brown¹² and the thiobarbituric acid method of Futterman¹³. (Total range on 4 preparations was $7.0\text{--}7.5 \times 10^4$.) Bound tritium in the samples was assayed by liquid scintillation counting. These data were used to compute the number of hydrogens per molecule still unexchanged as a function of exchange-out time.

Fig. 2. Limited exchange-in/exchange-out data at pH 5.0 and $0^{\circ}C$. Purple membrane suspensions were labelled at $0^{\circ}C$ for only 5 min at pH 5 (10 mM cacodylate) and then passed quickly through a Sephadex column to initiate exchange-out. A semilog plot of the resulting data is given in *a* and the isolated free peptide phase, obtained by subtracting the slow phase from the early time data, is in *b*.

Fig. 3. Limited exchange-out data at pH 4.7 and $0^{\circ}C$. Exchange-in was for 10 min at pH 4.7 (5 mM citrate) and subsequent treatment was as for Fig. 2.