THE HYDROPHOBIC HEART OF RHODOPSIN REVEALED BY AN INFRARED $^{1}H-^{2}H$ EXCHANGE STUDY

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1. Introduction

Rhodopsin, the visual pigment and also the major protein of vertebrate retinal rod outer segments, is directly involved in the early molecular events of vision. It is now generally accepted that it is a transmembrane protein with more than 50% of its volume embedded in the lipid bilayer [1–3]. Spectroscopic studies have shown that the conformation of rhodopsin's polypeptide chain is extensively α-helical (about 50%) [4,5] with no detectable β-structure [6]. A significant fraction of the tyrosine residues also appear to be hydrogen bonded possibly to aspartic acid or glutamic acid [6]. However the way in which the protein is arranged in the membrane has not yet been clearly defined.

Hydrogen isotope exchange methods are sensitive tools to study protein structure [7]: exchange rates are reduced when solvent accessibility is hindered [10], which may be an important factor for an integral membrane protein. In addition it is known that peptide hydrogens which are hydrogen bonded either to peptide carboxyl groups in α-helices or to side chain residues exchange slower than non-hydrogen bonded or water-hydrogen bonded peptide hydrogens [7–9].

$^{3}H-^{1}H$ exchange studies on rod outer segment (ROS) membranes have already been reported [11,12]. However, although it is generally considered that the tritium technique only visualises the exchange of peptide hydrogens [9], it detects potentially the exchange of all the labile protons, peptide and side chains.

In order to obtain unambiguous information about the solvent accessibility of rhodopsin's peptide hydrogens, we have studied by infrared spectroscopy the degree of deuterium incorporated into membrane bound rhodopsin. This method offers the advantage of measuring directly and quantitatively the $^{1}H-^{2}H$ exchange of the peptide protons only [8].

Our infrared results show that rhodopsin has a very low solvent accessibility and that a considerable number of its amide protons are buried in a 'heart' shielded from the solvent water.

2. Experimental

All experiments were carried out in the dark or under dim red light.

Fresh ROS membranes were purified as described (A_{280}/A_{500} ratios between 2.0 and 2.2) [13]. Exchange was initiated by diluting 25 times in $^{2}H_2O$ buffer (isotopic purity 99.8%) a 0.5 ml sample of the membrane suspension, this avoids any lyophilisation step. The membranes were then collected by centrifugation (20 min, 100 000 × *g*) and the pellet resuspended in the appropriate volume of supernatant. The final

rhodopsin concentration was approx. 10 mg/ml. ^1H–^2H exchange, at 26°C unless otherwise stated, was performed in two buffers of equivalent ionic strength:

(i) 10 mM imidazole, 100 mM sodium phosphate, pH 7 (Im–P_i buffer).

(ii) 10 mM imidazole, 225 mM NaCl, pH 7 (Im–NaCl buffer).

The rate of ^1H–^2H exchange in peptide NH groups was followed by infrared spectroscopy [14]. Infrared spectra of ROS membranes at appropriate time intervals were recorded on a Perkin-Elmer 180 double beam spectrophotometer equipped with matched, thermostated cells (CaF, 0.1 mm pathlength), the reference cell contained supernatant from the ^2H_2O buffer washing step. Other aspects of this method have been described [15].

The percentage of peptide hydrogens exchanged at a given time was calculated from the corresponding infrared spectrum: the amide II / amide I absorbance ratio, after background correction, is proportional to the fraction of unexchanged peptide protons with the proportionality coefficient $w = 1/0.45$ [15,16]. Complete deuteration was only achieved by heating the sample of rhodopsin either to 60°C for 6 h, or to 100°C for 3 h, or by treatment with 5% sodium dodecyl sulfate at 60°C for 3 h.

3. Results and discussions

The amide region of the infrared spectra of ROS membranes and hence of rhodopsin, since it represents at least 85% of the ROS membrane proteins [17], is shown in fig.1. Except for the band at 1736 cm^{-1} which is due to the phospholipid ester carbonyl [6], the general spectrum has the well-known shape for proteins dissolved in ^2H_2O buffer. The amide I band (due to the C=O stretching vibration of the peptide bond) has its maximum in spectrum 1 at 1655 cm^{-1} which indicates considerable α-helical structure [18,19], the shoulder at 1643 cm^{-1} may represent a small amount of random coil. The amide II band (due to the coupled N–^1H and C=O vibrations) is centered at 1546 cm^{-1}; its intensity decreases as amide protons become deuterated to be replaced by a band at

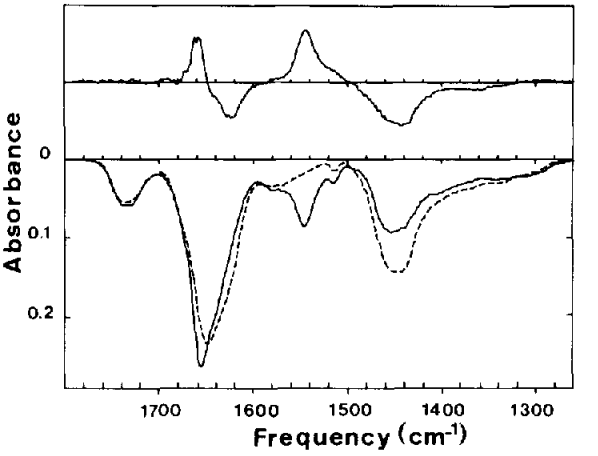

Fig.1. The amide region of the infrared spectrum of ROS membranes in ^2H_2O buffer pH 7. Spectrum 1 (——): ROS membranes after 24 h incubation at 26°C in 10 mM imidazole, 100 mM sodium phosphate pH 7, ^2H_2O buffer. Spectrum 2 (– – – – –): the same sample after heating for 6 h at 60°C in the spectrometer cell and then cooling to 26°C. The computed difference between these two spectra is presented in the upper graph.

1455 cm^{-1}. Its disappearance in spectrum 2 indicates that complete deuteration has been achieved after heating for 6 h at 60°C. Complete exchange was only achieved under conditions where structural modifications of the protein occurred as judged by the change in shape of the amide I band [18,19]. The 1515 cm^{-1} peak is due to tyrosine residues. The computed difference absorption spectrum (upper graph of fig.1) shows that upon denaturation there is no change in the amide I band integrated intensity, since the sum of the differences between 1700 cm^{-1} and 1587 cm^{-1} is zero

In previous work we have determined the kinetics of tritium incorporation into ROS membrane proteins for two different ionic conditions [12], phosphate ions being found to increase the number of slowly-exchanging tritium atoms incorporated. Infrared ^1H–^2H exchange results, obtained for several different incubation conditions are summarized in fig.2. Three major points should be noted:

(i) When the exchange is performed in Im–NaCl buffer at 26°C, only 35% of the peptide hydrogens were deuterated even after incubating for up to 40 h.

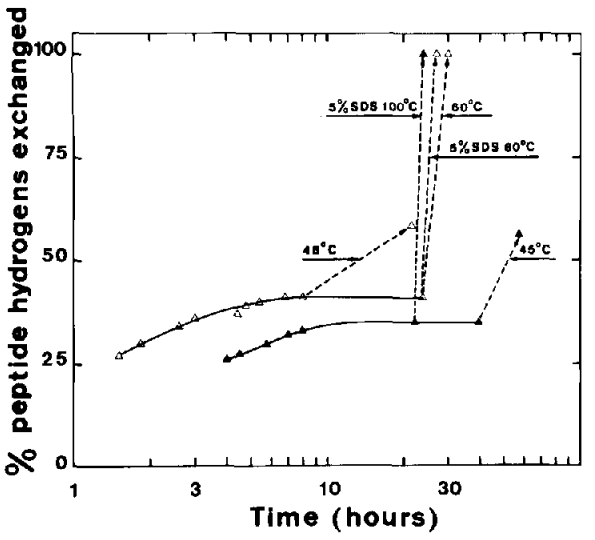

Fig.2. Effect of the incubation conditions on the equilibrium $^1H-^2H$ exchange of ROS membrane peptide hydrogens. Exchange was performed in two buffers of equivalent ionic strength: (△) 10 mM imidazole, 100 mM sodium phosphate pH 7 (Im–P_i buffer) or (▲) 10 mM imidazole, 225 mM NaCl, pH 7 (Im–NaCl buffer). The incubation temperature was 26°C unless otherwise indicated in the figure. SDS, sodium dodecyl sulphate.

(ii) Heating the sample to 45°C for 18 h, which does not cause denaturation as judged by the fact that the amide I band does not change in shape, only resulted in the deuteration of an additional 20% of the peptide hydrogens; in both buffer systems, about 40% of the peptide hydrogens remain unexchanged. These protons only become exchangeable after denaturation of the protein secondary structure.

(iii) Illumination did not affect the number of peptide hydrogens which exchange in either buffer used.

These infrared $^1H-^2H$ exchange results should be compared with those reported from $^3H-^1H$ exchange studies. The present results corroborate our findings [12] on the effect of phosphate ions; at 26°C an additional 6% of the peptide hydrogens exchange when the incubation is performed in Im–P_i buffer instead of Im–NaCl buffer. However, in the presence of phosphate ions, the plateau of tritium incorporations was attained after 36 h incubation [12]. In contrast, the deuteration of ROS peptide hydrogens (under identical incubation conditions) reaches a plateau after only 7 h. That kinetic isotope effects are not responsible for this difference is indicated by the fact that in Im–NaCl buffer the plateaux of incorporation of tritium atoms and of the deuteration of peptide hydrogens are both attained between 8–12 h exchange. The most probable explanation seems to be that the tritium atoms which are incorporated between 7 h and 36 h incubation in Im–P_i buffer are very slowly-exchanging non-peptide hydrogens.

Indeed Downer and Englander [11], using the tritium exchange technique, measured a total of 1.05 exchangeable hydrogens/peptide. We have shown [12] that under their exchange in conditions (Ringer, 0°C, 50 h) the most slowly exchanging protons of rhodopsin were not tritiated, and that the plateau tritium incorporation in ROS membranes was similar in Ringer at 0°C and in Im–NaCl buffer, pH 7, at 26°C. Thus the present peptide exchange data implies that no more than about 40% of the peptide hydrogens, 0.40 H/peptide, would have exchanged in their sample. It appears therefore that a considerable number of side chain hydrogens, in the interior of the protein molecule, are slowly exchanging; their exchange rates being similar or slower than that of freely solvent exposed peptide hydrogens. These side-chain hydrogens would be measured in tritium exchange experiments and could be misidentified as peptide hydrogens. Although slowly-exchanging side-chain hydrogens have been observed in lysozyme [20], cytochrome *c* [15] and angiotensin II [21], their proportion in rhodopsin appears to be considerably more important.

It is worth mentioning in this context that apparently all other proteins (exept bacteriorhodopsin) which have been studied under similar conditions [22] exchange their amide protons to a greater extent than rhodopsin. In bacteriorhodopsin only about 30–60% of the exchangeable polypeptide hydrogens are exchanged in a 48 h incubation period [23]. It seems therefore that rhodopsin and bacteriorhodopsin have structures with unusually low solvent accessibilities. This may be a general property of integral membrane proteins.

The results presented here have certain implications for the possible structure fo rhodopsin. The frequency

of the amide I band maximum, 1655 cm^{-1} (fig. 1, spectrum 1), is slightly higher than that generally observed for a right-handed α-helix, 1650 cm^{-1} [18]. Similar amide I band displacements have been observed for insulin [18,19] and collagen [24] where strong interchain interactions exist [24,25]. This suggests that a considerable part of rhodopsin's α-helical segments are closely packed, consequently reducing their solvent accessibility and thus the exchange rate of the corresponding peptide hydrogens. If in addition these α-helical segments were to be embedded in the hydrophobic part of the membrane, they would be shielded even more. In fact, the infrared ^1H–^2H exchange data show that about 40% of the peptide hydrogens, those which do not exchange at 45°C (see fig.2), constitude a 'heart' of extremely low solvent accessibility. These peptide hydrogens only become exchangeable after denaturation of the protein secondary structure. Such an exchange behaviour is qualitatively what would be expected for compact α-helical segments deeply embedded in the hydrophobic lipid bilayer.

The transmembrane region of at least one other intrinsic membrane protein [26] has been shown to consist principally of α-helical segments orientated perpendicular to the plane of the membrane. To suggest that in rhodopsin these α-helices are orientated perpendicular to the membrane would be pure speculation at present. It is worth noting however that such an arrangement would explain the diamagnetic anisotropy of rod outer segments [27].

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