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Mobility and solvent exposure of aromatic residues in bacteriorhodopsin investigated by ^{1}H-NMR and photo-CIDNP-NMR spectroscopy

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Proton-NMR studies of native bacteriorhodopsin revealed the existence of about nine relatively mobile aromatic residues out of the 32 totally present in the protein. These nine comprise approximately three tyrosines, two tryptophans and four phenylalanines. Photo-CIDNP data strongly suggest that, aside from phenylalanines, only one tyrosine and one tryptophan residue are exposed to the solvent. These data are discussed in terms of the current structural model for bacteriorhodopsin.

Bacteriorhodopsin; Membrane protein; Proton nuclear resonance; Photo-CIDNP; NMR; Side-chain mobility

1. INTRODUCTION

Bacteriorhodopsin is the only protein of the purple membrane from *Halobacterium halobium*. Upon light excitation, the protein generates an electrochemical proton gradient which is used by the bacteria for their energy requiring functions (reviews [1,2]). The prosthetic group of the pigment is retinal which is linked to the protein via a protonated Schiff base to a lysine residue. The purple membrane contains a single protein of 248 amino acids which spans the membrane in seven helices. Almost 80% of the protein is embedded in the lipid phase with only a few charged amino acid residues located within the hydrophobic core [3,4].

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Abbreviations: bR, bacteriorhodopsin; TSP, trimethylsilyl-1-propanesulfonate; FID, free induction decay; CIDNP, chemically induced dynamic nuclear polarization

Although models based on neutron diffraction and proteolysis studies [4] proposed bulk-water-exposed sequences and helix links, more experimental data are still needed to support or refute any such model. Recently, ^{2}H-NMR has been used to study the dynamics of amino acid residues in the purple membrane of *H. halobium* [5–9]. Results and interpretations presented in these ^{2}H-NMR studies vary with different sample preparations. Based on relative resonance intensities of narrow and broad spectral components, Keniry et al. [7] have attributed the narrow signals to residues on the bacteriorhodopsin surface and inferred that these amino acid groups are more mobile than those in the interior of the membrane. In contrast, Herzfeld et al. [8] conclude that few of the surface residues in bacteriorhodopsin are highly mobile on the ^{2}H-NMR time scale.

Here, the mobility and solvent exposure of aromatic residues in bacteriorhodopsin were investigated using ^{1}H-NMR and photochemically induced nuclear dynamic polarization (CIDNP)-NMR spectroscopy [10]. The data are compared with those obtained from ^{2}H-NMR experiments

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and discussed in terms of the current structural model for bacteriorhodopsin.

2. MATERIALS AND METHODS

All reagents used were reagent grade. Purple membranes from *H. halobium* were isolated according to Oesterhelt and Stoeckenius [11]. The final purification step included the use of a sucrose gradient [11]. Purity was checked by gel electrophoresis which gave only one band and by visible spectroscopy which showed the absence of extraneous carotenoid bands. The purple membrane sample was subsequently centrifuged and redispersed in an appropriate buffer. The apomembrane (apo-bR) was prepared by illuminating bR in the presence of hydroxylamine [12]. Retinal was removed from the freeze-dried apomembrane with toluene. All samples were dissolved in deuterium oxide containing perdeuterated SDS (Cambridge Isotope) from 0.1 to 1% (w/v). Apo-bR was dissolved in an appropriate volume of 1% perdeuterated SDS. Deuterated tyrosine was prepared according to Enei et al. [13] from perdeuterated phenol and incorporated into bR using a synthetic medium [14]. The incorporation yield was 90–95%, as judged by the simultaneous incorporation of ^{3}H-labeled tyrosine.

A sample of approx. 22 mg protein/ml (OD 40) was transferred into a NMR tube. The pH was adjusted by adding microtiter increments of NaOH or H_3PO_4 to the sample. All measurements were made at the pH value indicated in the text, read directly from the pH-meter, and not adjusted for isotope effects.

^{1}H-NMR spectra were recorded in the Fourier mode on a Bruker 500 MHz spectrometer at 303 K. The solvent deuterium signal was used as the field-frequency lock. All chemical shifts are quoted in parts per million (ppm) downfield from TSP.

The spin-lock HOHAHA two-dimensional NMR method of Davis and Bax [15] was used to help identify highly mobile aromatic ring proton spin systems. Axial peaks were suppressed by phase-cycling routines; quadrature detection was used in both dimensions and the carrier frequency was placed at the center of the spectrum. For the two-dimensional NMR spectral acquisition, water suppression was achieved by selective irradiation of the $^1H^2HO$ resonance for 2 s prior to the preparation pulse. The data set was collected at 512 FIDS containing 2 h words and was processed on a Digital micro Vax II computer system with software written in Fortran 77.

The bacterioopsin samples were used for the photo-CIDNP experiments to avoid competitive absorption of the retinal with the flavine dye used to induce the photo-CIDNP effect. Photo-CIDNP-NMR spectra were recorded on a Bruker HX-360 spectrometer. An argon ion laser (Digital Equipment Corp.) was employed as the light source. The 'neat' photo-CIDNP response was induced by irradiating the sample with laser light (0.5 s light pulse, 5 W; multiline) which was directed via an optical fiber into the NMR probe; a 0.05 s delay was used before the 90 rf observation pulse. The $^1H^2HO$ solvent resonance was saturated during the relaxation delay and the light pulse period. 8–12 'light' scans were accumulated for each spectrum. Although the photo-CIDNP enhancement was quite large, it was found useful to subtract four 'dark' transients normalized for each light scan to remove residual background magnetization.

3. RESULTS

The aromatic ring proton resonance region of 500 MHz NMR spectra of bacteriorhodopsin (bR) in 0.1, 0.4 and 1% SDS is shown in fig.1. For a protein the size of bR, i.e. 26800 Da, which is present in a 'condensed membrane phase', resonance linewidths are expected to be of the order of kilohertz. The appearance of some relatively sharp aromatic proton resonances, i.e. linewidths <50 Hz, suggests the presence of a number of relatively mobile aromatic residues. It should be noted that also in the aliphatic region sharp resonances can be observed (not shown). It is also apparent from fig.1 that as the SDS concentration is increased these relatively narrow resonances are associated with apparently broadened resonances arising from other aromatic ring protons in which motions are probably more dampened. Without the addition of SDS to bR samples, a few of these same narrow resonances are observed albeit with poor signal-to-noise ratios even after accumulation of some 20000 transients (not shown). If the bR samples are not fresh or are repeatedly freeze-dried prior to NMR experiments, resonance linewidths are not as narrow, although the same spectral patterns can be discerned.

The addition of SDS to purple membrane not only shifts the absorption maximum of the retinal chromophore but, at increasing concentrations, will eventually destroy the retinal-protein interaction and liberate free retinal [16]. SDS at 0.1% has virtually no effect on the chromophore and its stability, as judged by the circular dichroic spectrum which displays the characteristic bilobe indicative of a crystalline array [17,18]. At 0.4% SDS, bR is probably present in its monomeric form. Interestingly, similar SDS/bR ratios at lower concentrations have no effect on the circular dichroic bilobe pattern and, therefore, on stability even at a ratio of 100 SDS molecules/bR. Therefore, the rise in spectral area at 0.4 and 1% SDS compared to 0.1% SDS is most likely the result of a disruption in the purple membrane superstructure which allows for greater mobility of more residues in bR. It seems, moreover, that little area change is noted between 0.4 and 1% SDS and

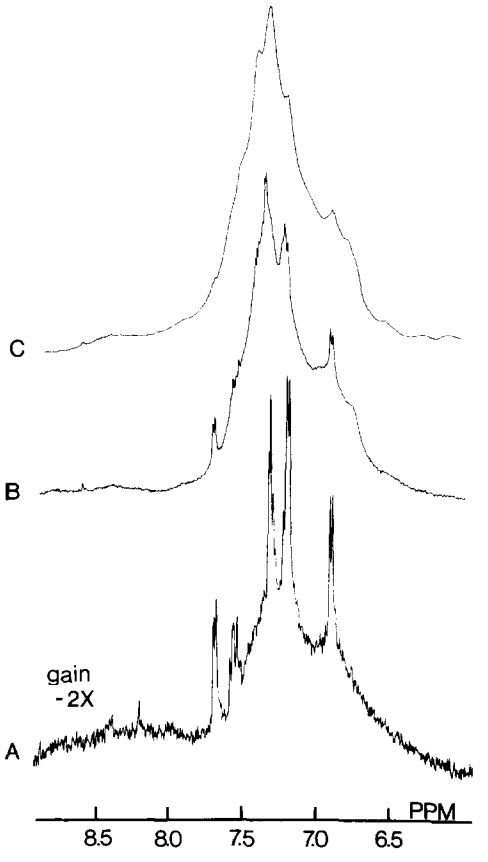

Fig.1. 500 MHz proton NMR spectrum of bacteriorhodopsin. Aromatic resonance region of native bR. Sample concentration was about 1 mM protein in 0.4 ml ^2H_2O; the freeze-dried bR sample had been redissolved in 0.1% (A), 0.4% (B) and 1% (C) perdeuterated SDS/deuterium oxide. The solution pH was 6.8. The vertical gain in (A) was reduced 2 × relative to (B,C).

that no chemical shift changes of the narrow resonances (0.1% SDS) are noted at the higher SDS concentrations. This could suggest that the gross conformation of bR is preserved under these conditions.

Under the assumption that these narrow resonances are in a fast exchange regime on the ^{1}H-NMR time scale ($>10^3$ s^{-1}), it should be possible to make a crude estimate of the number of aromatic ring protons responsible for these narrow resonances by calibrating the corresponding area intensity with that of a known concentration of internal TSP the protons of which resonate upfield away from the intense protein/lipid methyl resonances. This approach also assumes non-fractional populations. At 0.1% SDS, it can be estimated that about 15–20 highly mobile aromatic ring protons are present in native bR. This indicates that about 10% of the aromatic amino acid side chains are apparently mobile. At 0.4 or 1% SDS, where the membrane superstructure is already disrupted, these narrow resonances are still present at their characteristic chemical shift positions. It seems, however, that other aromatic residues have become more mobile by this transition, giving rise to an increase in spectral area. Due to a lack of resolution, linewidths are impossible to assess under these circumstances. Based on the area alone, it can be estimated that some 30–40 protons, corresponding to about 20–25% of the aromatic groups in bR, are relatively mobile.

Fig.2 gives a two-dimensional HOHAHA [15] ^{1}H-NMR contour plot of the aromatic resonance region of bR in 0.4% SDS. The HOHAHA experiment is sensitive only to well-resolved resonances, where, as in this case, motional narrowing is present. Therefore, narrow resonances can be differentiated from broad ones, and some aromatic ring spin systems can be identified. The 3,5 ring proton resonances of two, possibly three tyrosine residues resonate around 6.8 ppm. These are spin-coupled to the 2,6 proton resonances at about 7.1 ppm. Two tryptophan spin systems are also identified in fig.2. Other cross-peaks close to the diagonal are probably associated with phenylalanine spin systems which may account for about three or four additional aromatics. These resonances amount to highly mobile aromatic residues of approx. 3 Tyr, 2 Trp and 4 Phe, in agreement with estimates given earlier based on relative area intensities. As compared to 0.1% SDS (fig.1), most of the phenylalanine intensity and probably some of that of tyrosine seem to have been gained while the number of tryptophan residues involved still remains unclear.

The chemical shifts of these ring spin systems are found at normal random-coil positions (see fig.2), generally suggestive of solvent-exposed residues. To differentiate further between solvent-exposed and solvent-unexposed tyrosine and tryptophan residues, photo-CIDNP experiments were performed. Fig.3a gives a photo-CIDNP difference spectrum of apo-bR in 1% SDS which was required due to the sparse solubility of apo-bR in lesser amounts of SDS. But, as already pointed out

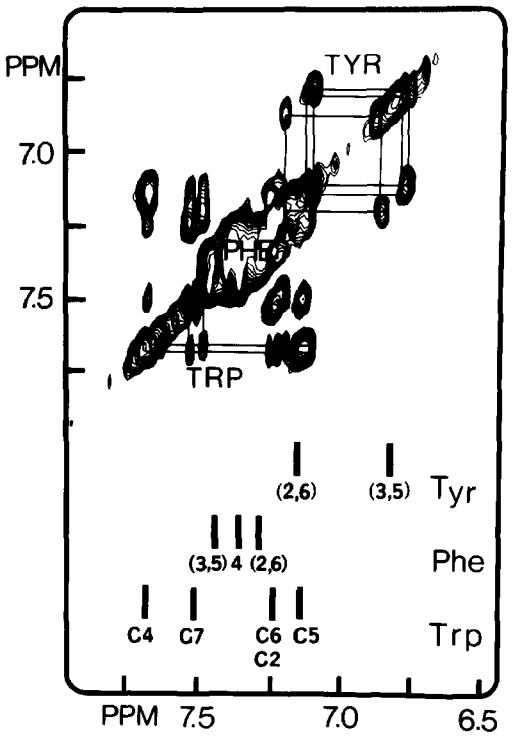

Fig.2. Spin-lock HOHAHA two-dimensional NMR contour plot. Aromatic resonance region of native bR with sample conditions as described in the legend to fig.1B. Labeling of resonances is described in the text. At the bottom of the figure, random-coil chemical shift positions for Trp, Tyr and Phe ring proton resonances are given from Bundi and Wüthrich [22].

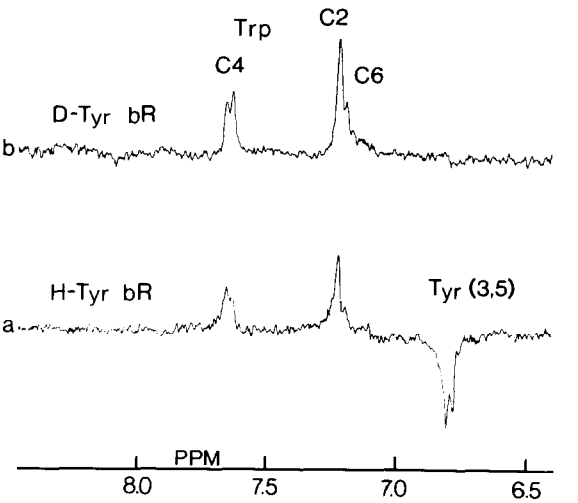

Fig.3. Photo-CIDNP difference spectra of bacteriorhodopsin. (a) Photo-CIDNP difference spectrum of downfield aromatic resonance region of apo-bacteriorhodopsin. (b) Photo-CIDNP difference spectrum of downfield aromatic resonance region of perdeuterated tyrosine–apo-bacteriorhodopsin. Sample conditions as in legend to fig.1C, except for the addition of 10^{-4} M carboxymethyllumiflavine.

earlier, spectral patterns are comparable between 0.1 and 1% SDS, suggesting that no gross structural changes occur in bR on addition of SDS up to 1%. Furthermore, extraction of the retinal chromophore also does not seem to affect the aromatic region spectral pattern. The photo-CIDNP difference spectrum signals are compatible with only one tyrosine (emission lines) and one tryptophan (absorptive resonances) being accessible to the hydrophilic, carboxymethyllumiflavine dye used to induce the photo-CIDNP effect. For tyrosine residues, this usually means that at least the hydroxyl group of the phenol ring is solvent-exposed, while for tryptophans at least the aromatic ring system would be exposed [19]. Phenylalanines give no photo-CIDNP effect, and bR contains no histidine residues.

As a control, the photo-CIDNP difference spectrum of the perdeuterated-tyrosine bR sample is shown in fig.3b. Clearly, the emissive tyrosine signal is absent. The solvent-exposed tyrosine residue correlates to the most downfield tyrosine spin system at 7.18 and 6.82 ppm (fig.2) for the 2,6 and 3,5 proton resonances, respectively. The signal at 7.65 ppm (fig.2) corresponds to the C4 proton resonance of the solvent-exposed tryptophan residue. Only the C2, C4 and C6 tryptophan proton resonances are enhanced by the photo-CIDNP effect. The absorptive resonances shown in fig.3, therefore, are associated with one tryptophan ring spin system of fig.2.

4. DISCUSSION

bR has a molecular mass of 26800 Da but in the membrane suspensions investigated here the membrane patch size will be at least one to two orders of magnitude higher. Proton resonance linewidths, therefore, are expected to be of the order of 1000 Hz. Indeed, an NMR spectrum of native purple membrane which contains the two-dimensional crystals of bR shows only very weak sharp bands; resonances are probably so broad as not to be observed. The addition of SDS, which at concen-

trations equal to or higher than about 0.4% (w/v) disrupts the crystalline array, leads to an increase in molecular motions in the bR/membrane complex and the appearance of more resonances in the form of an apparently broad band. These results indicate that the inherent mobility of certain side chains in bR is enhanced on the addition of SDS. On the other hand, even monomerisation which occurs at about 0.4% SDS does not lead to new resonances. It is, therefore, possible to compare the results from samples suspended in 0.1% SDS and those at concentrations up to at least 1% SDS as employed in the investigations outlined above.

The present data suggest the presence of about 9 relatively mobile aromatic residues, probably comprising 3 tyrosines, 2 tryptophans and about 3 or 4 phenylalanines. Aside from the phenylalanines, only 1 tyrosine and 1 tryptophan are solvent-exposed; others seem to be confined within the protein matrix or the membrane lipids. If the current model of membrane-bound bR is accepted, three possible alternatives or combinations thereof could account for the observed increased residue mobility: (i) aromatic rings themselves are highly mobile; (ii) one or more of the helices is/are highly mobile on the 500 MHz ^{1}H-NMR time scale; and (iii) the C- and N-terminal regions and/or chain-reversal domains which may well be solvent-exposed are flexible. Our data cannot yet differentiate among these possibilities.

Mobility of aromatic residues in bR has also been investigated by using solid-state ^{2}H-NMR on selectively deuterated protein samples [5–9]. In one study, the motion of all Trp residues in bR membrane preparations was concluded to be 'frozen out', i.e. to occur at rates $<10^5$ s^{-1} on a 8.5 T ^{2}H-NMR time scale, while all Tyr and Phe residues seemed to exhibit motions $>10^5$ s^{-1}. These were suggested to be freely mobile, undergoing fast ring flips [5]. In the present ^{1}H-NMR studies, 2 Trp residues seem to be freely mobile on an 11.5 T ^{1}H-NMR time scale. i.e. motions $>10^3$ s^{-1}. Comparison of these results might suggest motional rates between about 10^3 and 10^5 s^{-1} for these 2 Trp and less than 10^3 s^{-1} for the remaining Trp. In the same ^{2}H-NMR bR study [5], all 13 Phe and 9 out of 11 Tyr residues were assumed to undergo rapid ($>10^5$ s^{-1}) ring flips whereas the present results suggest that only about 3 or 4 Phe and 3 Tyr residues are highly mobile ($>10^3$ s^{-1}). This apparent conflict might be resolved by considering that the intensities in the ^{2}H-NMR spectra acquired via quadrupole echo sequences are not proportional to the number of spins except in the slow and fast limit motional regimes [9]. In the intermediate exchange regime (10^5 s^{-1}), calculated intensities are indeterminable and the ratio of mobile and immobile residues remains unknown. Therefore, there is little confidence in the estimates of Kinsey et al. [5]. In a later ^{2}H-NMR study, Kenery et al. [7] concluded that residues on the bR surface were more mobile than those in the protein interior.

Furthermore, due to questions of the purity of samples used in previous ^{2}H-NMR bR studies, Herzfeld et al. [8] suggest that these past results and interpretations on protein dynamics in bR should be reviewed. They claim that few if any aromatic residues in bR are highly mobile. Because the sample used in the present study was purified by a sucrose gradient and the purity was independently checked by SDS gel electrophoresis and visible spectroscopy, the bacteriorhodopsin investigated here can be confidently considered homogeneous.

Considering the limited number of mobile aromatic residues suggested in our ^{1}H-NMR studies, one might now search the bR structural model for possible assignment candidates. Analyses of X-ray diffraction patterns of purple membrane patches have suggested that native bR is structurally folded into seven closely packed helices which span the membrane interface [20]. Predictive structural algorithms using the amino acid sequence of bR also suggest the formation of seven helices of various lengths [4]. Two proximal tryptophans, i.e. Trp-10 and Trp-12, are present at the N-terminus where increased mobility is commonly observed in proteins while other tryptophans are supposedly buried in the protein and/or membrane. Trp-10 and Trp-12 may be those observed in the experiments outlined above. Phe-71, Phe-171 and Phe-230 are found in the chain-reversal domains and at the C-terminus, as are Tyr-131 and Tyr-133. Phe-42, Phe-156, Tyr-64 and Tyr-78 are located near the membrane surface of the structural model. Any of these might be assigned to highly mobile tyrosine and phenylalanine residues. However, tyrosine nitration experiments on native bR result in only Tyr-64

being derivatized [21]. This could suggest a tentative assignment for the only flavine-accessible (solvent-exposed) tyrosine residue identified in bR in the present study.

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