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^{19}F NMR study of 5-fluorotryptophan-labeled bacteriorhodopsin

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^{19}F NMR and CD spectra reveal that bacteriorhodopsin as well as its 5-fluorotryptophan-labeled analog solubilized in a CH_3OH-$CHCl_3$ mixture (i) retains a secondary structure of the fully active chromoprotein in the purple membrane and (ii) possesses a folded structure in which modifications at the Lys-216 bound retinal are sensed by sequentially remote tryptophan residues. Individual fragments isolated after limited proteolysis and $NaBH_4$-cleavage of bacteriorhodopsin keep the spatial structure of the intact polypeptide chain in the organic solvent.

Bacteriorhodopsin; Membrane protein solubilization; Protein structure; ^{19}F-NMR; CD

1. INTRODUCTION

Although bacteriorhodopsin is one of the best characterized membrane proteins [1], its polypeptide backbone folding models remain to be experimentally refined. In recent years NMR spectroscopy has become a very efficient technique for spatial structure evaluation of peptides and small proteins in solution. A prerequisite for attaining this goal is separation and identification of individual signals. When studying membrane proteins, one faces an obstacle just at this stage: even small peptides incorporated into bilayer membranes display very broad signals [2], thus the power of modern high resolution NMR spectroscopy cannot be fully utilized. One of the ways to overcome this problem is to use an artificial milieu wherein the protein retains its spatial structure providing at the same time reasonable NMR spectra. In principle, milieus of two different types should be considered: (i) micelles (e.g., see our recent results for the transmembrane ion channel of gramicidin A [3]) and (ii) organic solvents. A detailed NMR study of the solubilized membrane protein is justified if its structure in an artificial milieu is identical or at least similar to the native one. As a first and quick check of that, CD spectroscopy appears very attractive. Here we report on ^{19}F NMR and CD studies on the conformation of biosynthetic fluorine-labeled bR solubilized in a CH_3OH-$CHCl_3$ mixture. The advantage of ^{19}F NMR spectroscopy is evident: the spectra of fluorine-labeled proteins are composed of a small number of signals, the positions of which are very sensitive to a microenvironment of fluorine-

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Abbreviations: bR, bacteriorhodopsin; bR(F), 5-fluorotryptophan labeled bacteriorhodopsin; RetbO(F), 5-fluorotryptophan labeled retinylbacterioopsin; Ret′-bO(F), 5-fluorotryptophan labeled 1,1,5-tridesmethyl-1,2,3,4-tetrahydro-3-fluororetinylbacterioopsin; C1, Gly-72–Ser-248 peptide of RetbO(F); C2, <Glu-1–Phe-71 peptide of RetbO(F); B1, <Glu-1–Gly-155 peptide of RetbO(F); B2, Phe-156–Ser-248 peptide of Retbo(F); NMR, nuclear magnetic resonance; CD, circular dichroism

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bearing groups [4]. These features greatly simplify spectral analysis and make possible characterization of the protein spatial structure.

2. MATERIALS AND METHODS

bR(F), RetbO(F) and Ret'bO(F) were prepared as in [5]. The extent of Trp(5F) incorporation was determined by amino acid analysis. 1,1,5-Tridesmethyl-1,2,3,4-tetrahydro-3-fluororetinal was a generous gift of Dr B.I. Mitsner (Moscow). Schiff base reduction and $NaBH_4$ cleavage of the polypeptide chain were performed as in [6] and α-chymotrypsin cleavage as in [7]. C1 (residues 72–248), C2 (1–71), B1 (1–155), and B2 (156–248) fragments of RetbO(F) (fig.1) were isolated by Sephadex LH-60 chromatography in C_2H_5OH-HCOOH (7:3) [8].

CD_3OH (99% deuterium, Isotop, USSR), HCOOH (Merck), Triton X-100 (Sigma) and freshly distilled $CHCl_3$ and C_2H_5OH were used throughout.

^{19}F NMR (470.56 MHz) spectra of 0.2–0.03 mM solutions were recorded using a Bruker WM 500 NMR spectrometer at 32°C. Prior to Fourier transformation all free induction decays were multiplied by $\exp(-10t)$ in order to increase the signal-to-noise ratio. Chemical shifts are given with reference to 5 mM solution of H-LD-Trp(5F)-OH in CD_3OH-$CHCl_3$ (3:1), 0.1 M $LiClO_4$.

CD spectra were obtained on a Roussel-Jouan Dichrograph III CNRS (France) at room temperature.

3. RESULTS AND DISCUSSION

Three different media were used in the present work: (I) CD_3OH-$CHCl_3$ (1:1), 0.1 M $LiClO_4$; (II) CD_3OH-$CHCl_3$ (1:1), 0.1 M $LiClO_4$, 1.2 M HCOOH; and (III) CD_3OH-HCOOH (3:7). Fig.2 shows the CD spectra of bR solubilized in the media (I–III) and of bR in Triton X-100, where a monomeric bR molecule keeps spectral as well as many structural features of the native chromoprotein [9]. It should be noted that in media (I) and (II) not only the CD pattern of the native bR is preserved but also the retinal-protein aldimine bond is retained. If a small amount of HCOOH is added to a bR solution in medium (I), a typical bathochromic shift of 370→455 nm occurs due to Schiff base protonation. The CD_3OH-$CHCl_3$-$LiClO_4$ mixture seems to preserve the native-like structure of the solubilized bR.

In order to probe the spatial structure of bR in medium (I), ^{19}F NMR spectra were investigated for bR(F) which contains eight Trp(5F) residues instead of Trp residues (see fig.1). The bR(F) in membrane suspensions retains the CD spectrum, chromophore absorption maximum (575 nm as compared to 570 nm for the light-adapted bR), as well as the capacity for light-driven proton

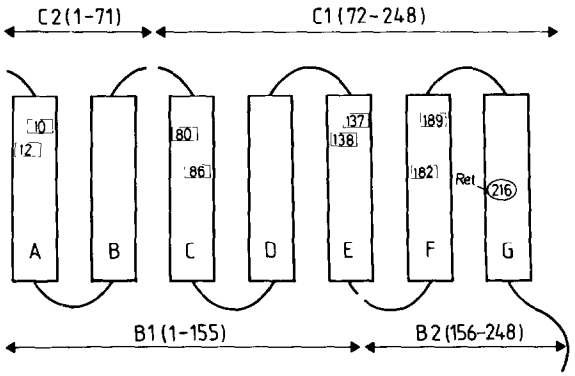

Fig.1. Scheme of bR polypeptide chain folding [1]. Seven transmembrane segments are designated by A–G. Numbers indicate the positions of eight tryptophan residues and the lysine residue with the attached retinal (Ret) moiety. C1, C2 and B1, B2 denote the peptides produced by α-chymotrypsin and $NaBH_4$ cleavage, respectively.

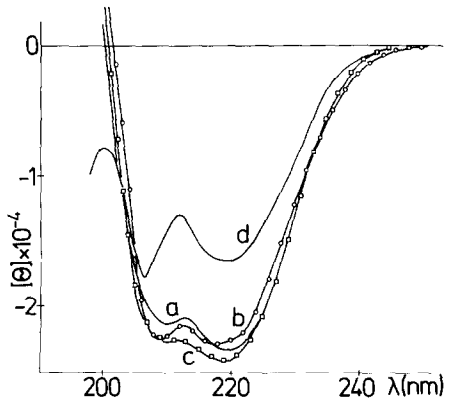

Fig.2. CD spectra of bR (a) in Triton X-100, (b) in medium (I), (c) in medium (II) and (d) in medium (III).

transport typical of native bR [5]. ^{19}F NMR spectrum of bR(F) in medium (I) (fig.3A) consists of six singlets of equal intensity (peaks 1–4, 7 and 8) and one singlet (5,6) of double intensity, the evidence of uniform incorporation of Trp(5F) residues into polypeptide chain. In medium (I), ^{19}F NMR spectra of bR(F) with different (50, 70, and 95%) contents of Trp(5F) residues were identical. Apparently, there is no mutual chemical shift effect and no close spatial proximity of Trp(5F) residues. A significantly different spectrum is obtained for bR(F) in medium (III) (fig.3D). This is in accord with the CD spectra (fig.2) which indicate a substantially lower content of an ordered secondary structure in this system.

We expected that the ^{19}F spectrum should respond to changes in the chromophore structure, such as aldimine bond reduction (see [10]) or β-ionone ring modification, and might shed light on the bR spatial structure in medium (I). Indeed, although the ^{19}F NMR spectra of bR(F) and RetbO(F) in medium (I) are similar (fig.3A and B), aldimine bond reduction causes the chemical shift changes of signals 3 and 4 and to a lesser degree of signal 7. Since the aldimine bond of Ret'bR(F) in medium (I) is unstable, ^{19}F NMR spectra of RetbO(F) and Ret'bO(F) were compared (fig.3B and C). These closely resemble each other, except that β-ionone ring modification changes the peak amplitude ratio in the group of signals 1, 2 and 3 (most probably, due to narrowing of signal 3). Thus two distinct modifications of the chromophore attached to the Lys-216 residue affect signals of distinct Trp(5F) residues, which are remote in the amino acid sequence from Lys-216 (fig.1). This clearly demonstrates the presence of a specific spatial structure of bR in medium (I).

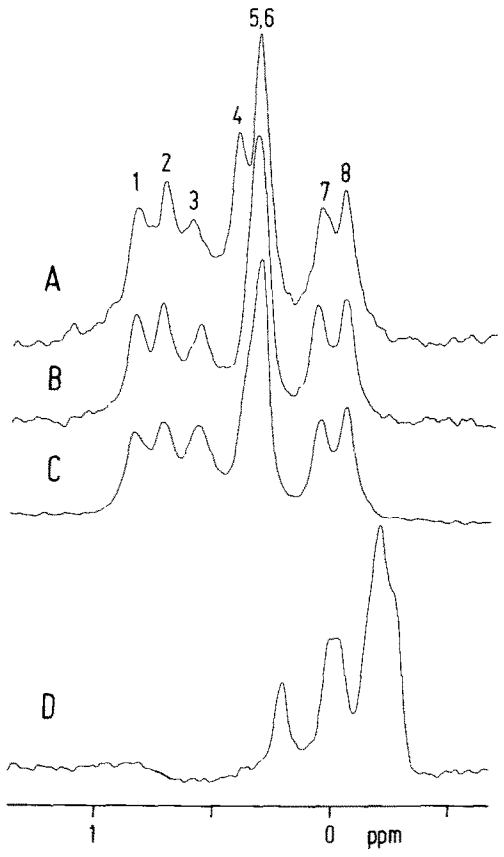

Fig.3. ^{19}F NMR spectra. (A) bR(F) in (I), (B) RetbO(F) in (I), (C) Ret'bO(F) in (I) and (D) bR(F) in (III).

Analysis of influence of ionogenic group titration on NMR spectra is a well established approach for detecting intramolecular contacts in water-soluble proteins [11]. With this aim in mind, we used titration of bR(F) by formic acid in organic solvent (fig.4). Chemical shifts of signals 4 and 5 and, to a lesser degree, of signals 1 and 2 have noticeable dependence on the acid concentration. It seems likely that the observed dependences are associated with protonation of ionogenic groups which are spatially close to Trp(5F) residues. Signals 4 and 5 shift in accord with Schiff base

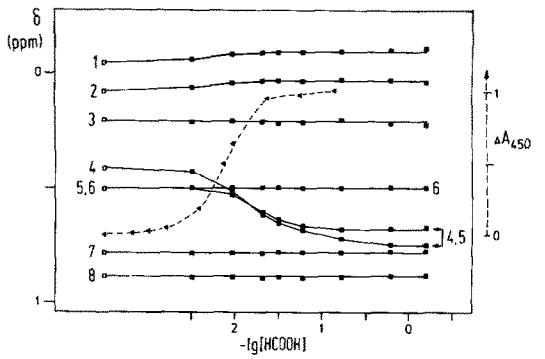

Fig.4. [HCOOH] concentration dependences of ^{19}F chemical shifts (solid lines) and of light absorption monitored at 450 nm (dashed line) for bR(F) in medium (I). Open squares and triangle correspond to spectra obtained without HCOOH.

protonation as followed from the 370→455 nm transition of the absorption maximum (fig.4).

The effects of chromophore modifications and HCOOH titration reveal a set of intramolecular interactions. To interpret the results in terms of the specified Trp(5F) residues of the amino acid sequence, the assignments of the ^{19}F signals have to be made. For this purpose we used peptides obtained by RetbO(F) cleavage: C1 and C2 as well as B1 and B2 (see fig.1). A comparison of the ^{19}F NMR spectra of intact RetbO(F) and $NaBH_4$-cleaved preparations in media (I) and (II) revealed that several signals are sensitive to the cleavage (fig.5). Nevertheless, general features of the spectrum were retained. Thus separated B1 and B2 peptides were used to gain signal assignments. From fig.5D–F it is obvious that the spectrum of cleaved RetbO(F) comprises a sum of B1 and B2 spectra and thereby signals 1–5 and 7 belong to six Trp(5F) residues of B1 peptide (residues 1–155) and signals 6 and 8 belong to Trp (5F) 182 and 189 residues of B2 peptide (residues 156–248).

Similar comparison of the spectra for RetbO(F) and its chymotryptic fragments C1 and C2 in media (II) and (I) (fig.6) makes possible the assignment of signals 1–3 and 6–8 to Trp(5F) residues of C1 (72–248), and signals 4 and 5 to Trp(5F) 10 and 12 of C2 (1–71). Titration of fragment C2 by formic acid in medium (I) reveals the concentration dependence similar to that of signals 4 and 5 of bR(F) in medium (I) (fig.4). Thus fluorine chemical shifts of Trp (5F) 10 and 12 are most likely affected by the carboxyl group of Glu-9 residue adjacent in the sequence. For intact bR(F) the

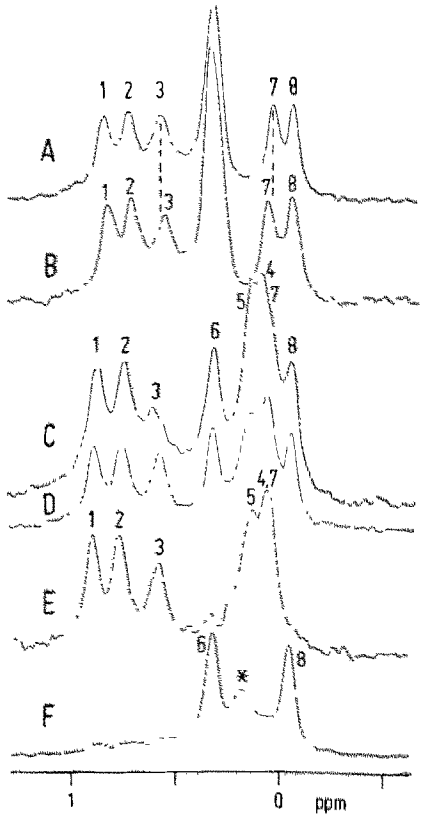

Fig.5. ^{19}F NMR spectra. (A) $NaBH_4$-cleaved RetbO(F) in (I), (B) RetbO(F) in (I), (C) RetbO(F) in (II), (D) $NaBH_4$-cleaved RetbO(F) in (II), (E) B1 peptide of RetbO(F) in (II) and (F) B2 peptide of RetbO(F) in (II). * Origin of this signal in spectrum (F) remains obscure.

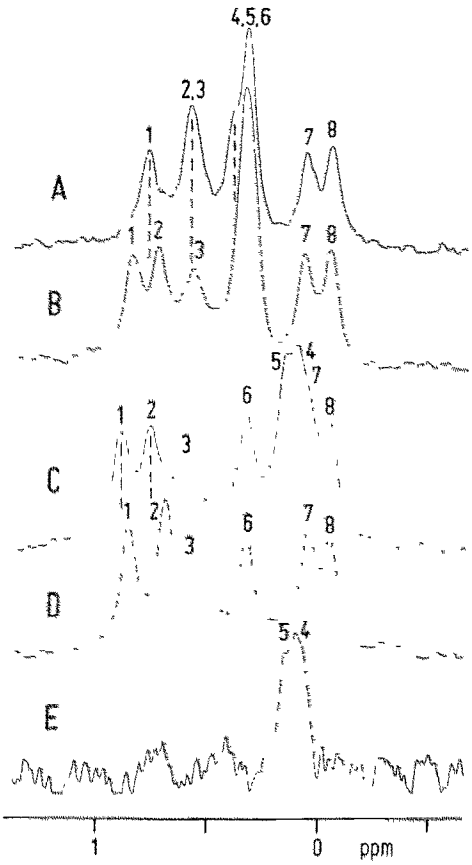

Fig.6. ^{19}F NMR spectra. (A) α-Chymotrypsin cleaved RetbO(F) in (I), (B) RetbO(F) in (I), (C) RetbO(F) in (II), (D) C1 peptide of RetbO(F) in (II) and (E) C2 peptide of RetbO(E) in (II).

titration of Schiff base should be considered as well.

The analysis of ^{19}F NMR spectra of intact and cleaved polypeptide chains leads to the assignment of ^{19}F signals for Trp(5F) 10, 12, 182 and 189 residues shown in fig.7 and to the conclusion that the isolated C1, C2, B1 and B2 peptides retain spatial structure which is inherent to them in intact RetbO(F). The conclusion agrees with the results of CD measurements for these peptides [12]. The secondary structure of bR(F) in medium (I) is similar to that of bR in Triton X-100 (fig.2) or in the aqueous suspension of purple membranes. Assuming that the generally accepted model of seven rods (fig.1) is also valid for medium (I), a tentative assignment of signals 1, 2, 3, and 7 within the Gly-72–Gly-155 sequence can be made. The corresponding four Trp(5F) residues are located in the amino acid sequence in pairs (fig.1): Trp(5F) 80 and 86 in segment C and Trp(5F) 137 and 138 in segment E. On the other hand, the four signals are also subdivided in two pairs: (i) signals 1 and 2 are sensitive to both α-chymotrypsin cleavage of peptide bond Phe-71–Gly-72 (fig.6) and an ionogenic group titration (fig.4); (ii) signals 3 and 7 are sensitive to $NaBH_4$ cleavage at Gly-155–Phe-156 (fig.5) and to aldimine bond reduction (fig.3A,B). Apparently Trp(5F) 80 and 86 should be sensitive to Phe-71–Gly-72 cleavage and subsequent distortion of the segment B and C interactions, while Trp(5F) 137 and 138 should respond to Gly-155–Phe-156 cleavage and to concomitant perturbation of the segment E and F interactions. Therefore, signals 1 and 2 were tentatively assigned to Trp(5F) 80 and 86 and signals 3 and 7, to Trp(5F) 137 and 138, respectively.

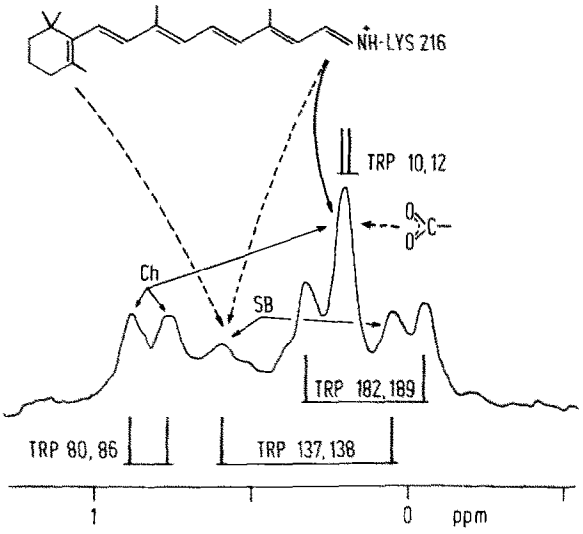

Fig.7. Schematic presentation of the ^{19}F signal assignments of bR(F) solubilized in medium (I) at 21 mM HCOOH. Solid arrows designate interactions between the identified partners, and dashed arrows are used if one of the interacting groupings is assigned tentatively. Ch and SB indicate effects of α-chymotrypsin and $NaBH_4$ cleavage, respectively.

In summary, the detected intramolecular interactions (fig.7) demonstrate that bR(F) solubilized in medium (I) or (II) has a specific tertiary structure. Its characteristic feature is the proximity of segments A and F, as deduced from the effect of Schiff's base reduction on signal 4 of Trp(5F) 10 or 12. If the tentative assignment of signal 3 is correct, the β-ionone ring should have contacts with segment E. A comparison of our conclusions with the earlier proposed models for bR folding [13,14] and data on spatial disposition of the retinal residue [15,16] reveals no contradiction between the bR tertiary structure in medium (I) and in the purple membrane. Thus further ^{19}F NMR and two-dimensional ^{1}H NMR studies of bR and its fragments solubilized in medium (I) appear promising for elucidating the folding motif and detailed secondary structure.

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