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Sequence-specific resonance assignment and secondary structure of (1–71) bacterioopsin

Alexander G. Sobol, Alexander S. Arseniev*, Galina V. Abdulaeva, Larisa Yu. Musina and Vladimir F. Bystrov

Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow, V-437, Russia

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SUMMARY

The conformation of chymotryptic fragment C2 of bacteriorhodopsin (residues 1–71) was studied by 2D ^{1}H NMR. The fragment was solubilized in a mixture of chloroform/methanol (1:1), 0.1 M $LiClO_4$. Most of the resonances in ^{1}H NMR spectra of fragment C2 were assigned using phase-sensitive DQF-COSY, TOCSY, and NOESY techniques. To simplify the assignment procedure for overlapping regions of NMR spectra, an analog of fragment C2 with leucines deuterated in β-positions was used. Deuterium exchange rates for amide protons were measured in a series of TOCSY spectra. Two right-handed α-helical regions Pro^8–Lys^{30} and Lys^{41}–Leu^{62} were identified on the basis of NOE connectivities and deuterium exchange rates. The N-terminal part of the fragment (Ala^2–Gly^6) adopts the helical conformation stabilized by 3 hydrogen bonds.

INTRODUCTION

Bacteriorhodopsin (BR), a light-driven proton pump, is the main component of the purple membrane of *Halobacterium halobium*. Its polypeptide chain consists of 248 amino acid residues (Ovchinnikov et al., 1979; Khorana et al., 1979) and the chromophore retinal bound to Lys^{216} (Bayley et al., 1981). The light absorption by the chromophore triggers the photocycle, which leads to translocation of a proton through the cell membrane.

Bacteriorhodopsin has been under investigation in many laboratories during the last 15 years and the results obtained have been thoroughly reviewed (Ovchinnikov, 1982; Dencher, 1983; Stoeckenius, 1985; Khorana, 1988). In 1975, electron microscopy of bacteriorhodopsin two-dimensional crystals revealed seven (A,B,C,D,E,F,G) electron-dense membrane-spanning segments

* To whom correspondence should be addressed.

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oriented almost perpendicularly to the membrane surface (Henderson and Unwin, 1975). Information on the bacteriorhodopsin structure and functioning was also obtained by different techniques such as chemical modification, proteolysis, immunochemistry, and site-directed mutagenesis (Ovchinnikov, 1982; Dencher, 1983; Stoeckenius, 1985; Khorana, 1988). It was used for a rough correlation of electron-dense regions and the amino acid sequence of bacteriorhodopsin (Engelman et al., 1980; Khorana, 1988). Mobility of aromatic side chains was investigated by solid-state ^2H NMR spectroscopy of selectively deuterated samples (Keniry et al., 1984) and by high-resolution ^1H NMR (Mayo et al., 1988). Most detailed information on the BR spatial structure was provided by electron cryo-microscopy (Henderson et al., 1990). However, the low resolution (≈ 10 Å) along the direction perpendicular to the membrane surface did not allow for obtaining exhaustive details.

In our laboratory we use a high-resolution NMR approach to determine the membrane protein spatial structure. It was shown using CD and ^{19}F NMR spectroscopy (Arseniev et al., 1987) that bacteriorhodopsin solubilized in a chloroform/methanol mixture retains at least the 'native-like' secondary structure and has the specific tertiary structure. Furthermore, even the fragments of bacteriorhodopsin obtained by proteolytic cleavage retain their secondary structure in the artificial milieu (Arseniev et al., 1987; Abdulaeva et al., 1991). Using 2D ^1H NMR techniques we studied spatial structures of membrane-spanning synthetic segments of bacterioopsin: 34–65 (segment B) (Arseniev at al., 1988), 102–136 (segment D) (Maslennikov et al., 1991) and 205–231 (segment G) (Maslennikov et al., 1990), as well as proteolytic fragment (163–231)bacterioopsin (BP2) (Barsukov et al., 1990). Regions of the stable α-helical structure were identified in all of these peptides.

This paper deals with the conformation of fragment (1–71)bacterioopsin (C2) obtained by chymotryptic treatment of the purple membrane and solubilized then in a chloroform/methanol (1:1), 0.1 M $LiClO_4$ mixture. Most of the resonances in ^1H NMR spectra of fragment C2 were assigned. NOE connectivities and deuterium exchange rates undoubtedly indicate 2 right-handed α-helical regions corresponding to membrane-spanning segments A and B.

MATERIALS AND METHODS

Bacteriorhodopsin (BR) was obtained as described (Oesterhelt and Stoeckenius, 1974).

[^2H]Leu-bacteriorhodopsin ([^2H]Leu-BR) was prepared biosynthetically by growing *H. halobium* in a synthetic growth medium (Gochnauer and Kushner, 1969) containing deuterated DL-[α,β-$^2H_3$]Leu (1.6 g/l) instead of L-Leu (0.8 g/l). Biosynthesis was carried out at 37 C on a rotary shaker equipped with 8 (20 W each) fluorescent lamps. The procedure included 3 steps. At first, 100 ml of [^2H]Leu synthetic medium was autoclaved at 121 C for 40 min in a 250 ml flask and then inoculated with 50 ml of a 2.5-day-old culture of *H. halobium* grown in an oxoid peptone medium (Colab Laboratories, USA) as described (Oesterhelt and Stoeckenius, 1974). After 2–2.5 days this culture was used to inoculate 1.2 l of the sterile [^2H]Leu medium in a 2 l conical flask. After 2–2.5 days, the second culture was added to 13 l of the deuterated medium in ten 2 l conical flasks. After 4 days the culture was harvested.

DL-[α,β-$^2H_3$]Leu was obtained by a pyridoxal-catalyzed hydrogen exchange reaction (Le Master and Richards, 1982) in ^2H_2O (99.9% deuterium; Isotop, USSR).

C2 Fragment ((1–71)BR or [^2H]Leu-(1–71)BR) was prepared by α-chymotrypsin cleavage (en-

zyme/protein ratio was 1:10) of peptide bond Phe^{71}–Gly^{72} (Gerber et al., 1980) of the pre-bleached membrane, followed by separation of the products on a Sephadex LH-60 column (2.5 × 90 cm) in chloroform/methanol (1:1), 0.1 M LiCl. The corresponding fraction was evaporated and, after salt removal by centrifugation in H_2O, dried.

NMR samples were prepared by solubilizing of lyophilized fragment C2 in the chloroform/methanol (1:1), 0.1 M $LiClO_4$ mixture. Solvents were C^2H_3OH (98% deuterium; Isotop, USSR), $C^2H_3O^2H$ (99.5% deuterium; Stohler Isotope Chemicals), and C^2HCl_3 (100% deuterium; Stohler Isotope Chemicals).

^1H NMR spectra of the 1–2 mM solution of fragment C2 were obtained at 600 MHz (Varian Unity 600 spectrometer) at 20, 30 and 40°C. DQF-COSY (Rance et al., 1983), TOCSY (Bax and Davis, 1985) with mixing times 30, 50, and 80 ms, and NOESY (Jeener et al., 1979) with mixing time 200 ms, were recorded in the pure phase-absorption mode by collecting hypercomplex data (States et al., 1982). Relaxation delays of 1–1.2 s were used. Chemical shifts were measured relative to a residual signal of the methyl group of methanol, whose shift to tetramethylsilane was arbitrarily chosen at 3.50 ppm.

Processing of NMR data was performed with FELIX, Hare Research Inc. and VNMR, Varian software.

To measure deuterium exchange rates of amide protons, six TOCSY spectra at 20 C were obtained during 30 h (5 h per spectrum), starting at 30 min after C2 solubilization in $C^2H_3O^2H$/C^2HCl_3 (1:1), 0.1 M $LiClO_4$. Intensities of cross peaks were digitized using the program FELIX, and exponential fitting was performed by the 'Analyze' routine from VNMR.

RESULTS AND DISCUSSION

After solubilization of fragment C2 in chloroform/methanol (1:1), 0.1 M $LiClO_4$, TOCSY (Figs. 1A and 2A), NOESY and DQF-COSY spectra were recorded. To simplify the assignment procedure, spectra of fragment C2, wherein the leucines are deuterated at the β-position, were also obtained. A region of the TOCSY spectrum of deuterated C2 (Fig. 1B) demonstrates the absence of cross peaks from $C^\beta H_2$ protons of leucine residues (compare Fig. 1A and B). Taking into account that leucine deuterated in the α- and β-positions was used for biosynthesis, one can expect that cross peaks of $C^\alpha H$ protons should disappear as well. However, these cross peaks are present in spectra of deuterated C2 (Fig. 2). Thus, the deuterated C2 contains [β-$^2H_2$]Leu rather than [α,β-$^2H_3$]Leu. This is explained by the transamination during biosynthesis (Muchmore et al., 1989).

The sequence-specific resonance assignments were made as follows. First, DQF-COSY and TOCSY spectra were analyzed to delineate most spin systems with different types of residues. Because of significant overlap of resonances in the aliphatic region, those for $C^\gamma H$ and $C^\delta H_3$ protons were identified only for few leucine residues. Aromatic proton spin systems were linked to their NH-$C^\alpha H$-$C^\beta H_2$ proton spin systems using NOEs between aromatic and $C^\beta H_2$ protons (Billeter et al., 1982). Chemical shifts of $C^\varepsilon H$ and $C^\zeta H$ protons of phenylalanine residues were not assigned due to the neighborhood of the corresponding cross peaks with the diagonal. The spin system of Gln^3 is unique in the amino acid sequence of C2. It was identified on the basis of NOEs between $N^\varepsilon H_2$ and $C^\gamma H_2$ protons. So, we had a number of starting points for sequence-specific assignment: Gln^3, seven Ala, two Trp, four Tyr, two Asp, and a few other residues. Analyzing $d_{\alpha N}(i,i+1)$, $d_{\beta N}(i,i+1)$ and $d_{NN}(i,i+1)$ connectivities from the amide proton of residue $i+1$ to

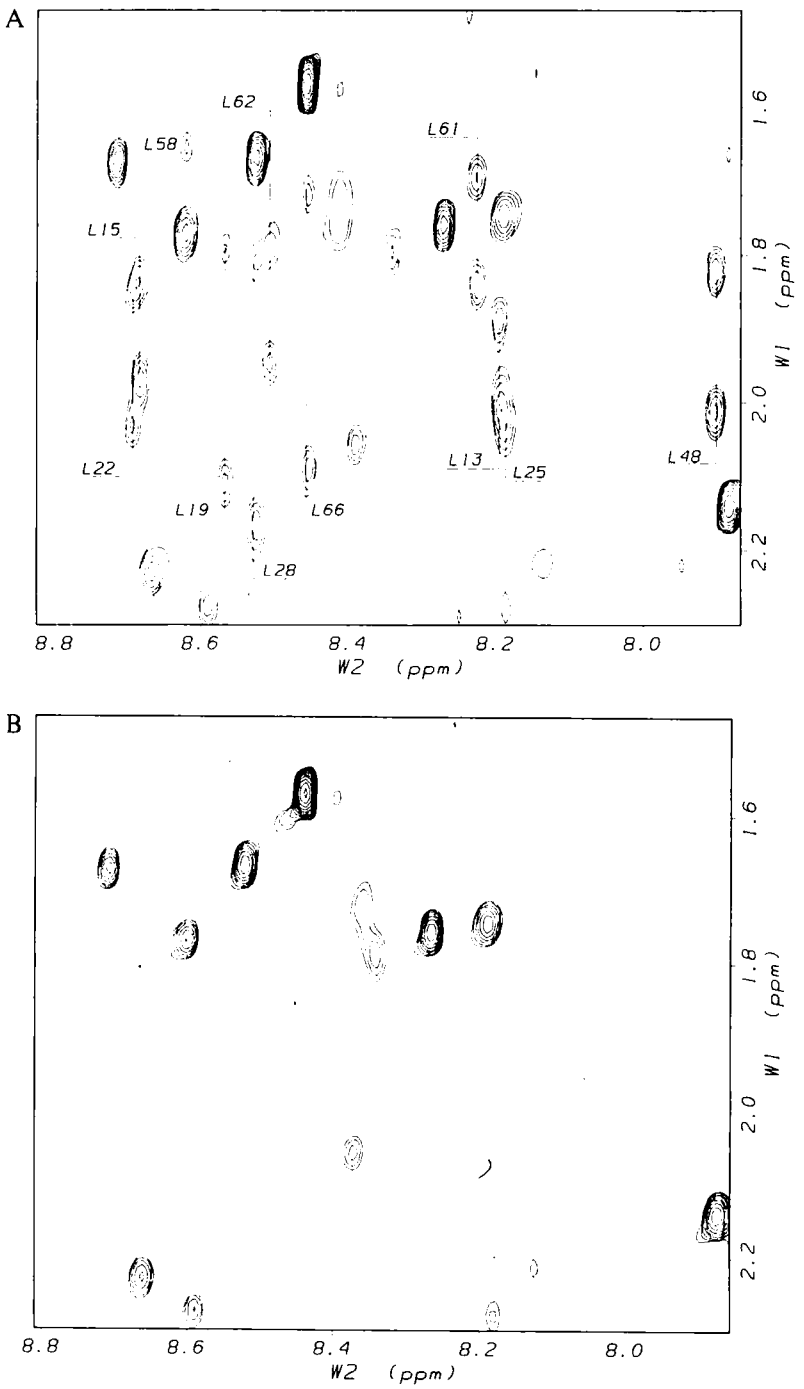

Fig. 1. NH/C$^{\beta}$H (ω_1 = 1.4–2.3 ppm, ω_2 = 7.8–8.8 ppm) region of TOCSY spectra of (A) (1–71)bacterioopsin and (B) the same peptide but β-protons of all leucines were replaced by deuterons. Sample concentration was 1 mM in chloroform/methanol (1:1), 0.1 M $LiClO_4$, at 30°C. Assignments of N$_i$H/C$_i^{\beta}$H cross peaks are indicated for leucine residues.

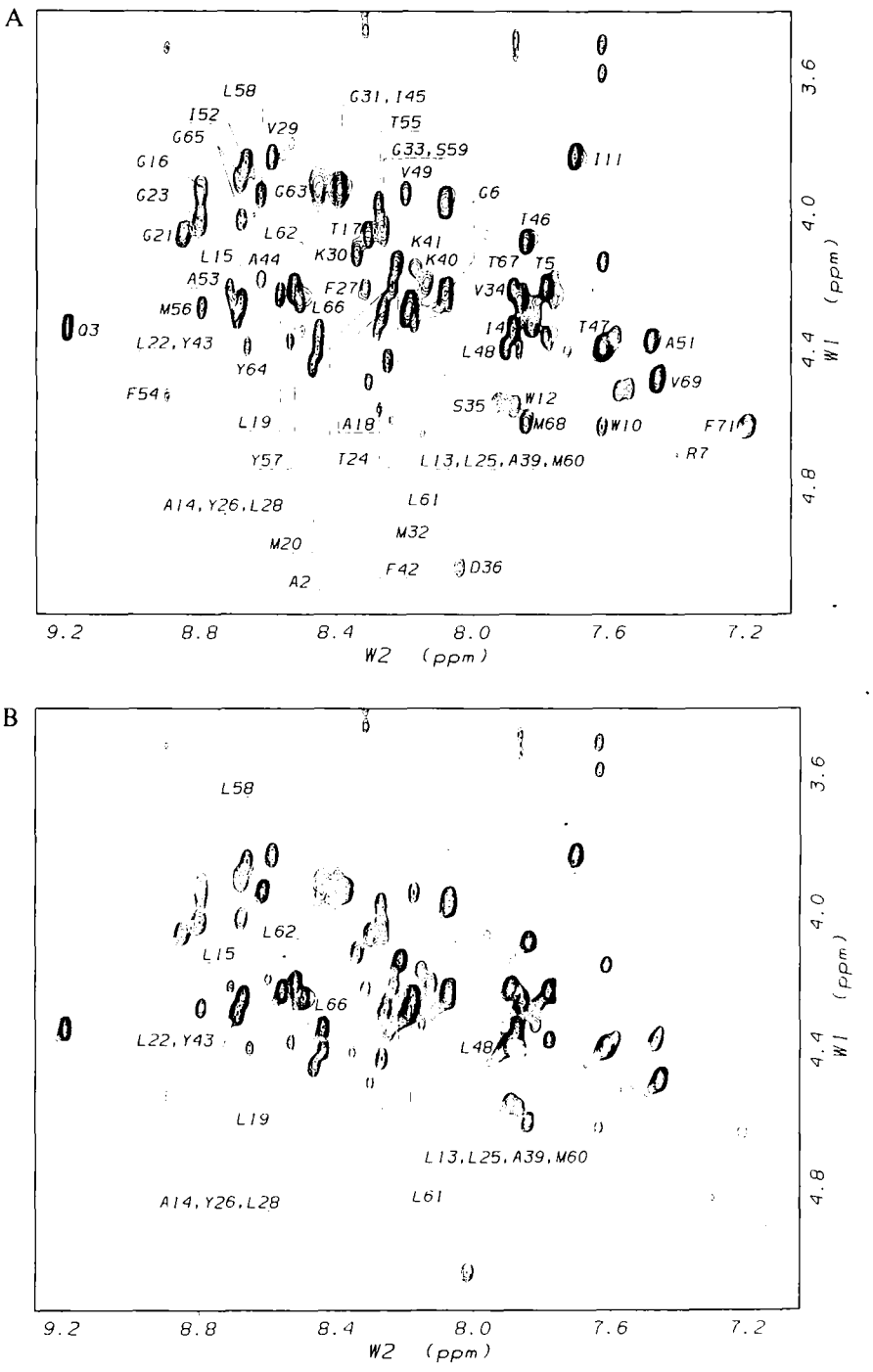

Fig. 2. NH/C$^{\alpha}$H (ω_1 = 3.4–5.2 ppm, ω_2 = 7.1–9.3 ppm) region of the same TOCSY spectra as in Fig. 1. Assignments of N,H/C$_i^{\alpha}$H cross peaks are indicated for (A) all residues and (B) leucine residues only. The one-letter code of amino acid residues is used.

the $C^{\alpha}H$, $C^{\beta}H$, and amide protons of the preceding residue i, respectively, we found that the major part of the sequence-specific assignment can be performed using only $d_{NN}(i,i+1)$ (Fig. 3) and $d_{\beta N}(i,i+1)$ connectivities. Strong overlap of NOE cross peaks made impossible the identification of d-connectivities for some residues. This was overcome on the basis of the spin system identification and the principle of self-consistency of the assignment.

A survey of NOE connectivities obtained is shown in Fig. 4, and chemical shifts of assigned proton resonances for fragment C2 are listed in Table 1.

The fragment C2 has 4 proline residues in positions 8, 37, 50, and 70. The X–Pro peptide bond in *cis* and *trans* configurations can be distinguished when using characteristic NOE connectivities (Arseniev et al., 1984). The presence of the intensive NOE cross peak between $C^{\alpha}H$ protons of preproline and proline residues is characteristic of the *cis*-configuration of the X–Pro peptide bond. On the other hand, a strong NOE cross peak between the $C^{\alpha}H$ proton of the i-th residue and the $C^{\delta}H$ proton of the $(i+1)$-th proline is the inherent feature of the *trans* X–Pro peptide bond. Keeping in mind these rules, the conclusion was made that peptide bonds Arg^{7}–Pro^{8}, Asp^{36}–

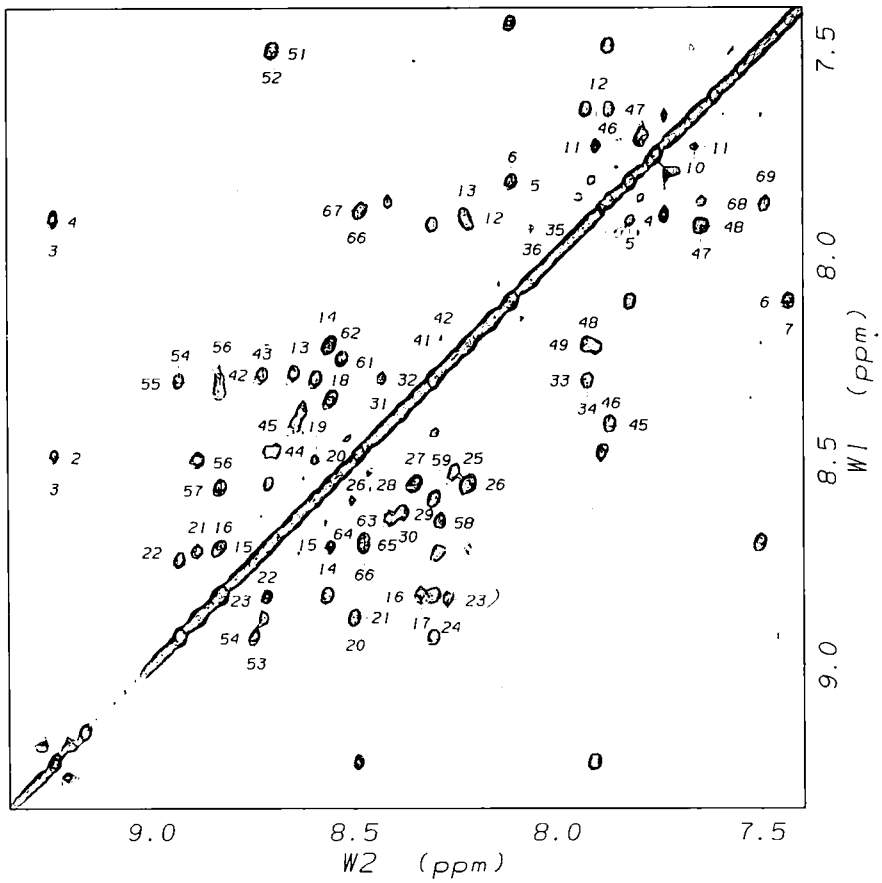

Fig. 3. NH/NH (ω_1,ω_2 = 7.4–9.3 ppm) region of NOESY (τ_m = 200 ms) spectrum of (1–71)bacterioopsin in chloroform/methanol (1:1), 0.1 M $LiClO_4$, at 30 C. Assignments of $N_iH/N_{i+1}H$ cross peaks are indicated.

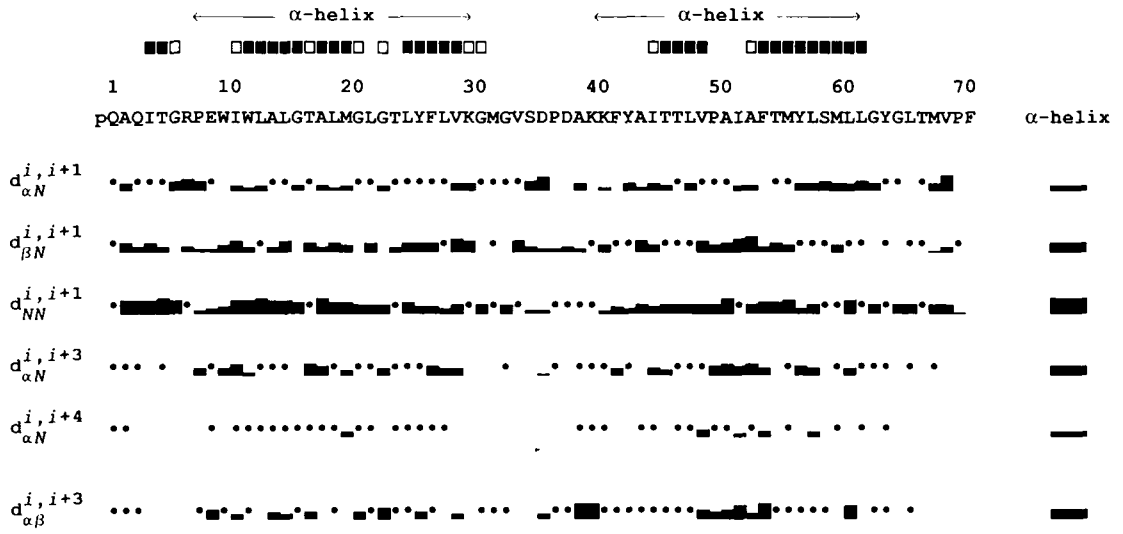

Fig. 4. Amino acid sequence of (1–71)bacterioopsin and survey of NOE connectivities involving NH ($C^{\delta}H_2$ protons of proline residues considered as NH protons), $C^{\alpha}H$ and $C^{\beta}H$ protons. The one-letter code of amino acid residues is used and pQ denotes a pyroglutamic acid residue. Thickness of lines corresponds to cross-peak intensity and the rightmost column shows the NOE pattern expected for a residue in the canonical right-handed α-helix. If the presence of a cross peak is somewhat doubtful, it is indicated by a black circle. Residues with slowly exchanging amide protons are indicated by black squares above the sequence, and residues with intermediate exchange rates by open squares (see Table 1). At the top the α-helical regions, as obtained from NOE connectivities and deuterium exchange rates, are indicated.

Pro^{37}, and Val^{69}–Pro^{70} have *trans* configurations. None of the cross peaks mentioned above was found in NOESY spectra for the Val^{49}–Pro^{50} peptide bond. This left the configuration of the corresponding peptide bond undetermined until the more detailed analysis of the fragment C2 structure.

The peptide secondary structure can be deduced from a comparison between theoretical and experimental $d_{\alpha N}$-, $d_{\beta N}$- and d_{NN}-connectivities (Wüthrich et al., 1982; Arseniev et al., 1984; Wagner et al., 1986). The canonical α-helix ($\varphi = -57°, \psi = -47°$) is characterized by a very specific connectivity pattern – the rightmost column in Fig. 4. NOEs observed for fragment C2 (Fig. 4) along with amide proton exchange measurements (Fig. 4 and Table 1) indicate 2 stable α-helical regions.

The first α-helix extends from Pro^{8} to Lys^{30}. The helix is destabilized in the middle which leads to a faster deuterium exchange of Leu^{22} and Thr^{24} (Fig. 4 and Table 1). Apparently, the destabilization has its origin in the absence of significant sterical hindering in the Gly-enriched region. The α-helix is terminated by Pro^{8} and Gly^{31}, which is consistent with typical roles of proline and glycine residues in α-helices (Richardson and Richardson, 1989). Usually, 4 amide groups at the N-terminus of the α-helix do not form intramolecular hydrogen bonds. Since we did not find any evidence (see Fig. 4) that Arg^{7} is integrated in the α-helical region, it is unclear how to account for the rather slow deuterium exchange of the amide proton of Ile^{11}. A possible explanation is the formation of a hydrogen bond between the NH proton of Ile^{11} and the side chain oxygen of Glu^{9}. However, this must await more detailed analysis of the NMR data.

The second α-helix extends from Lys^{41} to Leu^{62}. Pro^{50} is positioned in the middle of the helix, which clearly destabilizes the hydrogen bond pattern (Fig. 4). Analyzing the NMR data for Pro^{50}

TABLE I
PROTON RESONANCE ASSIGNMENT AND AMIDE PROTON DEUTERIUM EXCHANGE TIMES OF (1–71)BACTERIOOPSIN SOLUBILIZED IN CHLOROFORM/METHANOL (1:1), 0.1 M $LiClO_4$, AT 30 C

Residue	δ ± 0.01 ppm				
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others	$t_{1/2}$[b] (h)
pGln[1]	–[a]	–	–		–
Ala[2]	8.43	4.40	1.57		< 0.5
Gln[3]	9.19	4.33	2.23	$C^{\gamma}H_2$ 2.58	< 0.5
				$N^{\varepsilon}H_2$ 7.57;6.96	
Ile[4]	7.87	4.34	2.13	$C^{\gamma}H_2$ 1.67;1.41	10
				$C^{\gamma}H_3$ 1.08, $C^{\delta}H_3$ 1.05	
Thr[5]	7.78	4.22	4.36	$C^{\gamma}H_3$ 1.24	10
Gly[6]	8.07	4.23;3.97			2.5
Arg[7]	7.40	4.69	2.28	$C^{\gamma}H_2$ 1.77;1.73	< 0.5
				$C^{\delta}H_2$ 3.36;3.29	
				$N^{\varepsilon}H$ 8.36	
Pro[8]		4.38	2.37;1.93	$C^{\gamma}H_2$ 2.14, $C^{\delta}H_2$ 3.97	
Glu[9]	10.05	4.08	2.15;2.05	$C^{\gamma}H_2$ 2.59;2.42	< 0.5
Trp[10]	7.63	4.63	3.58;3.51	$C^{\delta}H$ 7.47, $N^{\varepsilon}H$ 10.20	< 0.5
				$C^{\zeta}H$ 7.72, $C^{\eta}H$ 7.52	
				$C^{\varepsilon 3}H$ 7.18, $C^{\eta}H$ 7.28	
Ile[11]	7.70	3.83	2.05	$C^{\gamma}H$ 1.69;1.27	2.5
				$C^{\gamma}H_3$ 0.93, $C^{\delta}H_3$ 1.00	
Trp[12]	7.86	4.57	3.54;3.48	$C^{\delta}H$ 7.25, $N^{\varepsilon}H$ 10.18	20
				$C^{\zeta}H$ 7.72, $C^{\eta}H$ 7.52	
				$C^{\varepsilon 3}H$ 7.18, $C^{\eta}H$ 7.28	
Leu[13]	8.19	4.29	2.04;1.92	$C^{\delta}H_3$ 1.10	20[c]
Ala[14]	8.52	4.21	1.66		20[c]
Leu[15]	8.63	4.24	2.00;1.86	–	20
Gly[16]	8.79	3.96;3.92			10
Thr[17]	8.30	4.06	4.49	$C^{\delta}H_3$ 1.32, $O^{\gamma}H$ 5.09	2.5
Ala[18]	8.26	4.29	1.75		100[c]
Leu[19]	8.56	4.22	2.12;1.80	$C^{\delta}H_3$ 1.10	15
Met[20]	8.46	4.44	2.46;2.32	$C^{\gamma}H_2$ 2.87;2.76	25
Gly[21]	8.85	4.06			2.5
Leu[22]	8.69	4.29	2.06;1.88	$C^{\delta}H_3$ 1.10	< 0.5
Gly[23]	8.80	4.03			2.5
Thr[24]	8.23	4.21	4.60	$C^{\gamma}H_3$ 1.48	< 0.5
Leu[25]	8.19	4.28	2.04;1.67	–	20[c]
Tyr[26]	8.52	4.21	3.30	$C^{\delta}H$ 6.98, $C^{\varepsilon}H$ 6.78	20[c]
Phe[27]	8.31	4.22	3.46;3.41	$C^{\delta}H$ 7.50, $C^{\varepsilon}H$ 7.41	5
Leu[28]	8.52	4.21	2.08;1.82	–	20[c]
Val[29]	8.59	3.83	2.27	$C^{\gamma}H_3$ 1.22;1.09	20
Lys[30]	8.34	4.12	1.78	$C^{\gamma}H_2$ 1.39, $C^{\delta}H_2$ 1.65	2.5
				$C^{\varepsilon}H_2$ 2.99;2.93	
Gly[31]	8.40	3.96;3.90			2.5
Met[32]	8.27	4.42	2.40;2.29	$C^{\gamma}H_2$ 2.90;2.71	< 0.5
Gly[33]	8.26	4.04			< 0.5
Val[34]	7.89	4.21	2.37	$C^{\gamma}H_3$ 1.16;1.13	< 0.5
Ser[35]	7.89	4.55	4.12;3.95		< 0.5
Asp[36]	8.02	5.04	3.11;2.94		< 0.5

TABLE 1 *(continued)*

Residue	δ ± 0.01 ppm				
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others	$t_{1/2}$[b] (h)
Pro[37]		4.49	2.53;2.15	$C^{\gamma}H_2$ 2.15 $C^{\delta}H_2$ 4.18;4.09	
Asp[38]	8.13	4.65	2.98;2.80		< 0.5
Ala[39]	8.19	4.29	1.74		< 0.5
Lys[40]	8.12	4.21	2.21;2.06	$C^{\gamma}H_2$ 1.67, $C^{\delta}H_2$ 1.89 $C^{\varepsilon}H_2$ 3.15	< 0.5
Lys[41]	8.15	4.17	2.17;2.05	$C^{\gamma}H_2$ 1.61 $C^{\delta}H_2$ 1.92;1.87 $C^{\varepsilon}H_2$ 3.09;3.06	< 0.5
Phe[42]	8.24	4.35	3.36	$C^{\delta}H$ 7.25	< 0.5
Tyr[43]	8.65	4.26	3.33	$C^{\delta}H$ 7.26, $C^{\varepsilon}H$ 6.91	< 0.5
Ala[44]	8.59	4.19	1.67		< 0.5
Ile[45]	8.37	3.93	2.05	$C^{\gamma}H_2$ 1.89;1.43 $C^{\gamma}H_3$ 1.09, $C^{\delta}H_3$ 1.02	2.5
Thr[46]	7.84	4.08	4.20	$C^{\gamma}H_3$ 1.12	20
Thr[47]	7.61	4.39	4.15	$C^{\gamma}H_3$ 1.27, $O^{\gamma}H$ 4.62	100
Leu[48]	7.89	4.38	2.03;1.84	$C^{\delta}H_3$ 1.14	100
Val[49]	8.17	3.94	2.55	$C^{\gamma}H_3$ 1.28;1.12	5
Pro[50]		4.38	2.02	$C^{\gamma}H_2$ 2.38 $C^{\delta}H_2$ 3.76;3.71	
Ala[51]	7.47	4.37	1.76		< 0.5
Ile[52]	8.66	3.97	2.22	$C^{\gamma}H_3$ 1.13	< 0.5
Ala[53]	8.71	4.21	1.66		2.5
Phe[54]	8.89	4.53	3.51;3.37	$C^{\delta}H$ 7.43	5
Thr[55]	8.27	3.97	4.57	$C^{\gamma}H_3$ 1.47, $O^{\gamma}H$ 5.31	20
Met[56]	8.79	4.27	2.47;2.31	$C^{\gamma}H_2$ 2.90;2.70	10
Tyr[57]	8.53	4.38	2.32	$C^{\delta}H$ 7.12, $C^{\varepsilon}H$ 6.84	100
Leu[58]	8.62	3.94	1.76;1.64	$C^{\delta}H_3$ 1.00	100
Ser[59]	8.26	4.08	4.26	$O^{\gamma}H$ 4.90	–
Met[60]	8.18	4.27	2.41;2.28	$C^{\gamma}H_2$ 2.90;2.64	20[c]
Leu[61]	8.22	4.13	1.84;1.71	$C^{\delta}H_3$ 0.97	100
Leu[62]	8.49	4.24	1.96;1.80	–	10
Gly[63]	8.44	3.95;3.90			< 0.5
Tyr[64]	8.65	4.39	3.29	$C^{\delta}H$ 7.20, $C^{\varepsilon}H$ 6.86	< 0.5
Gly[65]	8.68	4.02;3.91			< 0.5
Leu[66]	8.44	4.33	2.09;1.71	–	< 0.5
Thr[67]	7.86	4.26	4.40	$C^{\gamma}H_3$ 1.42, $O^{\gamma}H$ 5.11	< 0.5
Met[68]	7.84	4.61	2.15	$C^{\gamma}H_2$ 2.62;2.55	< 0.5
Val[69]	7.45	4.48	2.25	$C^{\gamma}H_3$ 1.11;1.04	< 0.5
Pro[70]		4.61	2.17;1.97	$C^{\gamma}H_2$ 2.07 $C^{\delta}H_2$ 3.98;3.68	
Phe[71]	7.22	4.64	3.35;3.22	–	< 0.5

[a] Dash (–) denotes protons unassigned.

[b] Deuterium exchange times were measured at 20 °C using a series of 2D TOCSY spectra. The value '< 0.5' means that the cross peak from the amide proton was absent in the first TOCSY spectrum, which was recorded for 5 h, starting at 30 min after C2 solubilization in $C^2H_3O^2H/C^2HCl_3$ (1:1), 0.1 M $LiClO_4$.

[c] The resonance is overlapped with others, hence the value corresponds to an averaged deuterium exchange time.

and its neighbors we found just the situation previously observed for (34–65)bacterioopsin (Arseniev et al., 1988). The observed NOE pattern indicates that the Val^{49}-Pro^{50} peptide bond has a *trans* configuration and that the Pro^{50} residue does not break the α-helix. An observation of $d_{\alpha\beta}(i,i+3)$ connectivities for residues Ala^{39} and Lys^{40} (Fig. 4) reflects a tendency of prolongation of the α-helix at its N-terminus. On the other hand, the C-terminus of the α-helix ends with the Gly^{63} residue.

It is necessary to point out a slow deuterium exchange of Ile^4, Thr^5, and Gly^6. Unfortunately, the d-connectivity pattern is not exhaustive for this part of the peptide due to overlap of informative cross peaks. Application of the modified CONFORNMR program (Lomize et al., 1990) to determine the local structure on the basis of NOE cross-peak volumes, leads us to the suggestion that residues Ala^2–Gly^6 form a turn of the right-handed helix. The hydrogen bond pattern for this part of the peptide is still unclear.

A loop region including the Met^{32}–Asp^{38} residues and the section from Tyr^{64} to Phe^{71} are not stabilized by hydrogen bonds. Analysis of their conformations will be performed later on the basis of quantitative NOE data.

Earlier hydrogen bonds between side-chain OH groups of threonines and serines and backbone carbonyls were observed in α-helical regions of fragment BP2 (residues 163–231) of bacteriorhodopsin (Barsukov et al., 1990). There are 5 threonine residues in positions 17, 24, 46, 47, and 55, and Ser^{59} in the α-helical regions of the fragment C2 (Fig. 4). Proton resonances of OH groups of Thr^{17}, Thr^{47}, Thr^{55}, Thr^{67}, and Ser^{59} were assigned in DQF-COSY and TOCSY spectra. For OH protons of other threonines and serines, no cross peaks were found in 2D spectra because of fast exchange of these OH protons with solvent, or masking of corresponding resonances by a strong solvent signal. Additional information was obtained from NOESY spectra. Quite strong NOEs were found between side-chain OH and backbone amide protons of Thr^{17} and Thr^{55} as well as between side-chain OH protons and $C^{\alpha}H$ protons of Ala^{14} and Ile^{52} ($(i-3)$-th residues), respectively. This indicates that orientation of corresponding threonine side chains favors the formation of a hydrogen bond between the side chain and backbone carbonyls of $(i-3)$-th and $(i-4)$-th residues (i.e. hydrogen bonds of carbonyl groups of Leu^{13}, Ala^{14} with the OH group of Thr^{17}, and carbonyls of Ala^{51} and Ile^{52} with the OH group of Thr^{55}). For side-chain OH groups of Thr^{47}, Thr^{67} and Ser^{59} residues, no interresidual NOE cross peaks were found. This leaves the side-chain orientation undetermined for the 3 residues until more rigorous analysis of the NMR data is carried out.

No evidence was obtained to confirm formation of the helical 'hairpin' in the C2 fragment of bacteriorhodopsin. It might be due to unfolding of the 'hairpin' in organic solvent or due to our inability to recognize corresponding NOEs in the heavily overlapped aliphatic region of NOESY spectra.

It is essential that the results on fragment C2 described above fit well with those obtained by electron cryo-microscopy for entire bacteriorhodopsin (Henderson et al., 1990). α-Helical regions revealed by electron cryo-microscopy (Henderson et al., 1990) are namely, Trp^{10}–Met^{32} and Asp^{38}–Leu^{62}, while NMR reveals Pro^8–Lys^{30} and Lys^{41}–Leu^{62}.

Being a part of our project on the BR spatial structure determination, the present work confirms the possibility of investigation of a membrane protein in organic solvent, at least as long as the secondary structure is considered.

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