Volume 231, number 1, 81–88 FEB 05737 April 1988

Two-dimensional ^{1}H-NMR study of bacterioopsin-(34–65)-polypeptide conformation

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Received 22 January 1988

Conformation of the synthetic 32-residue polypeptide, an analog of membrane spanning segment B (residues 34–65) of the *Halobacterium halobium* bacteriorhodopsin in the membrane mimetic system, methanol/chloroform (1:1), was investigated by ^{1}H-NMR spectroscopy. Previously it was shown by ^{19}F-NMR spectroscopy that this medium retains the native conformation of membrane bound BR and its fragments. The spectrum resonance was assigned by means of the sequential signal assignment porcedure using phase-sensitive DQF-COSY, MLEV17 HOHAHA and NOESY techniques. Interproton nuclear Overhauser effects, spin-spin coupling constant of vicinal H-NC$^\alpha$-H protons and deuterium exchange rates of individual NH groups were derived from two-dimensional NMR spectra. The data unequivocally define the peptide conformation as the right-handed α-helix, extremely rigid in the central region from Phe 42 to Nle 60 and flexible in the N- and C-terminal parts.

Bacteriorhodopsin; Synthetic peptide; Solution conformation; NMR

1. INTRODUCTION

Bacteriorhodopsin is a membrane protein found in *Halobacterium halobium* (reviews [1–3]). BR contains one polypeptide chain of 248 amino acid residues [4,5] and the retinal chromophore bound via the Schiff base to lysine 216 [6]. Light absorption by the chromophore triggers a photocycle that leads to proton translocation across the cell membrane.

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Abbreviations: BR, bacteriorhodopsin; sB, bacterioopsin-(34–65)-polypeptide analog which corresponds to the membrane spanning segment B of BR; Nle, norleucine residue; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; DQF-COSY, double-quantum filtered homonuclear correlated spectroscopy; MLEV17 HOHAHA, two-dimensional Hartmann-Hahn spectroscopy

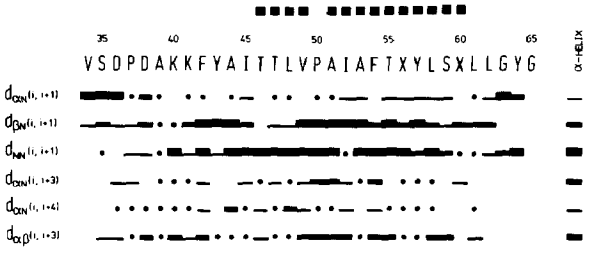

Fig.1. Amino acid sequence of the bacterioopsin synthetic segment B analog and survey of NOE connectivities involving NH, C$^\alpha$H and C$^\beta$H protons (C$^\delta$H$_2$ protons of proline residues are considered as honored NH protons). One-letter code of amino acid residues is used, X denotes norleucine residues. The residues with slowly exchanged peptide NH protons (i.e. protons with the NH/C$^\alpha$H cross-peaks observable in DQF-COSY spectrum recorded from 56 to 62 h after dissolving of the sample in CD_3OD-$CDCl_3$) are indicated by black squares above the sequence. The observed NOEs classified as strong, medium, and weak (based on counting the exponentially spaced contour levels) are shown by thick, medium and thin lines. If the corresponding cross-peak was somewhat doubtful, it is indicated by black circle. On the right-hand side of the diagram NOEs pattern expected for *i* residue in regular right-handed α-helix [20–23] is shown for comparison.

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00145793/88/$3.50

Spatial structure of BR has been a challenge for many years. The major breakthrough was achieved by electron microscopy; image reconstruction from low-dose micrographs revealed that the structure consists of seven cylindrical electron-dense regions, roughly perpendicular to the membrane plane [7]. However, the lack of well-ordered three-dimensional crystals limited the determination of the structure to a resolution of ~6 Å [8,9]. Additional information on BR backbone folding was obtained from labeling, immunochemical and proteolysis studies (review [1–3]). These data have been interpreted to provide approximate locations of seven (A–E) membrane spanning segments in the BR sequence [10]. Nevertheless, the precise folding and conformation of BR remain obscure.

An additional way of solving the problem is to make use of high resolution NMR spectroscopy,

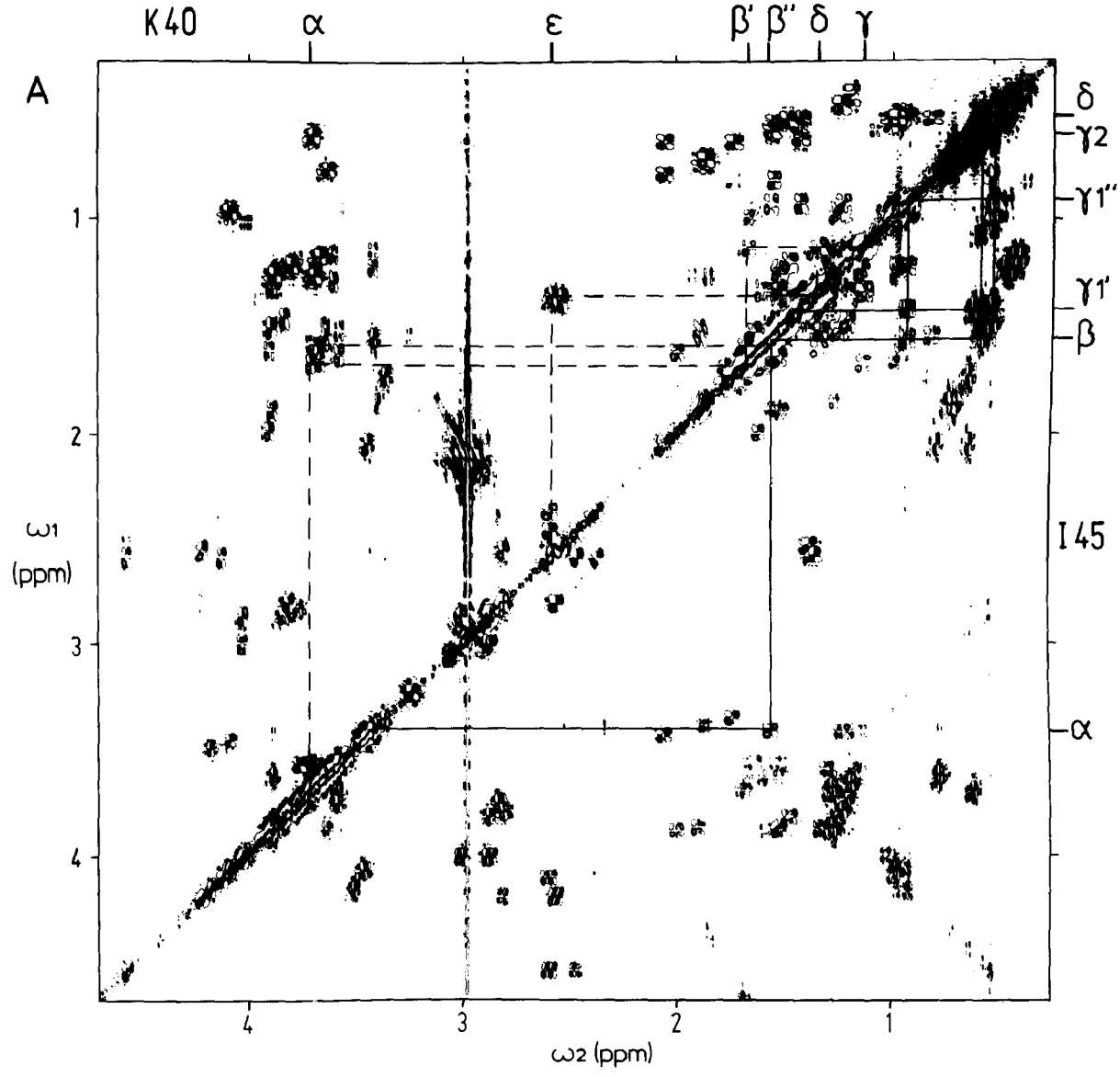
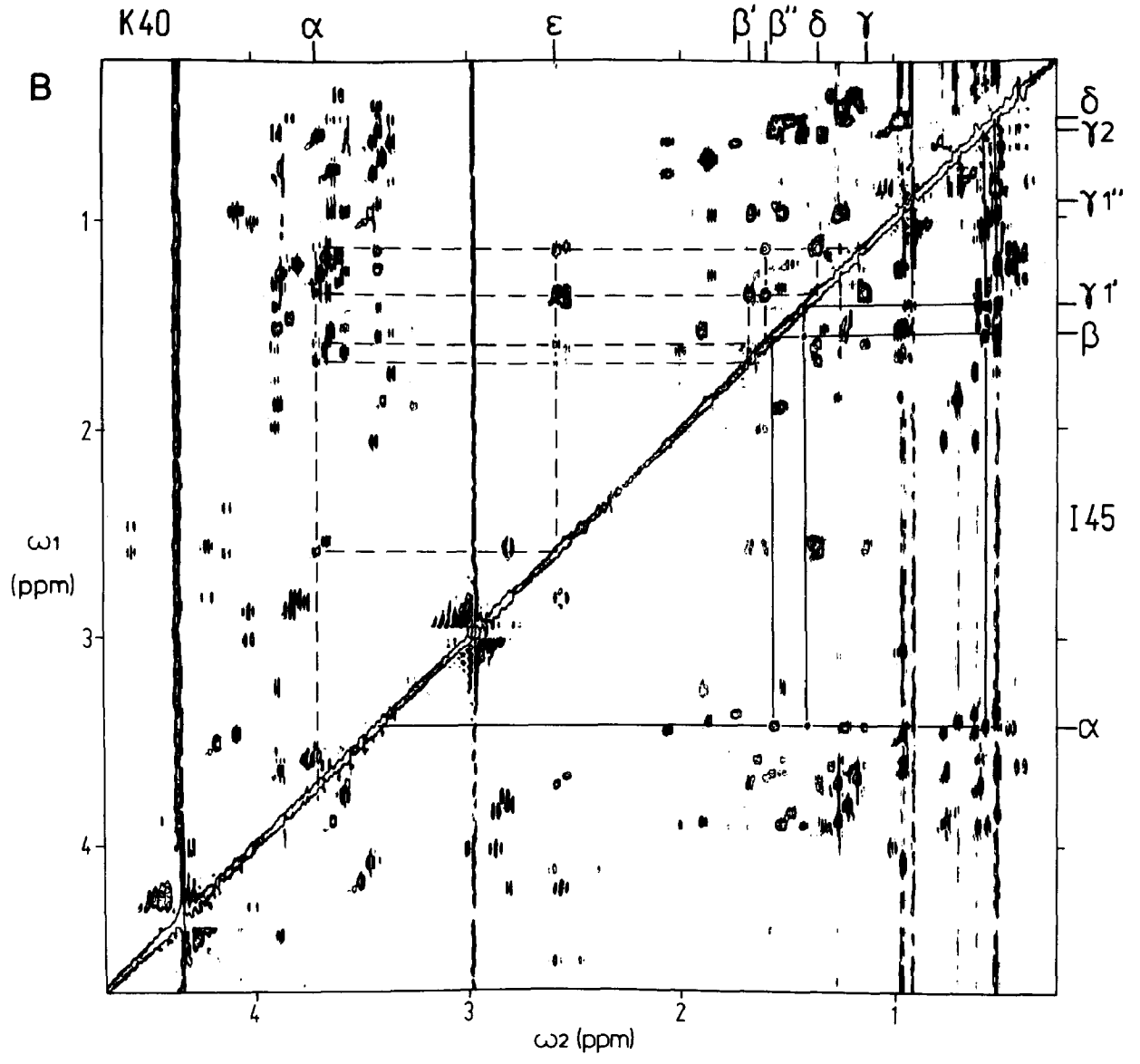


Fig.2. A, DQF-COSY and B, MLEV17 HOHAHA spectra of the aliphatic region of segment B in CD_3OD-$CDCl_3$ solution at 30°C. The cross-peaks in A arise from spin-spin interaction through two and three bonds; in addition B contains cross-peaks due to multiply

which becomes an efficient tool for spatial structure evaluation of relatively small proteins in solution [11]. Unfortunately, the membrane bound BR demonstrates very broad resonances; thus the proper media condition mimicking the native environment has to be found. Previously it was shown by ^{19}F-NMR spectroscopy [12] that BR solubilized in methanol/chloroform retains a secondary structure of the fully active chromoprotein in the purple membrane and possesses a specific tertiary structure. Moreover, individual fragments of BR isolated after polypeptide chain cleavage retain their 'native' conformations in the organic solvent [12].

In this paper we describe the first results of our conformational studies of membrane spanning segment B, bacterioopsin-(34–65)-polypeptide analog, solubilized in the membrane mimetic solu-



relayed through bond connectivities within spin-systems of amino acid residues. In order not to overcrowd the drawings only connectivities in the spin systems of Lys 40 and Ile 45 residues are identified.

tion. The ^{1}H-NMR spectrum is completely assigned and the secondary structure is delineated from qualitative analysis of the NOEs, spin-spin coupling constants of H-NC$^\alpha$-H protons and deuterium exchange rates of individual amide NH groups.

2. MATERIALS AND METHODS

The 32-residue peptide (fig.1) was synthesized by a conventional solution technique as described [13]. The peptide corresponds to the bacterioopsin-(34–65)-polypeptide fragment with methionine residues 56 and 60 substituted for norleucines for technical reasons. Such isosteric replacements usually do not affect the polypeptide conformation and function (see e.g. [14]), simplifying at the same time the synthesis. The peptide is further referred to as sB of BR.

CD_3OH (99% deuterium, Isotop, USSR), CD_3OD (99.5% deuterium, Stohler Isotope Chemicals) and $CDCl_3$ (100% deuterium, Stohler Isotope Chemicals) were used throughout.

^{1}H-NMR spectra (500 MHz) of 5–10 mM solutions of sB in methanol/chloroform (1:1) were obtained at 20, 30 and 40°C using a Bruker WM 500 NMR spectrometer. All two-dimensional spectra were recorded in pure phase absorption mode using the time proportional phase incrementation method [15]. The following spectra were recorded: DQF-COSY spectra [16], NOESY spectra [17] at mixing times of 100 and 200 ms, and MLEV17 HOHAHA spectra [18] at a mixing time of 35 ms. The OH resonance of methanol was suppressed by continuous irradiation except during the acquisition time period. Chemical shifts were measured relative to residual signal of $CHCl_3$ whose shift to tetramethylsilane was arbitrarily chosen at 7.27 ppm. Splittings related to coupling constants (^3J) of H-NC$^\alpha$-H vicinal protons were measured in DQF-COSY spectra along the ω_2 axis (digital resolution of 0.4 Hz/point).

3. RESULTS AND DISCUSSION

sB was dissolved in methanol/chloroform (1:1) and after overnight incubation at 50°C demonstrated NMR high-resolution spectra shown in figs 2 and 3. Incubation led to a single set of resonance lines observed for individual amino acid residues, indicating chemical purity and single (in the NMR time scale) conformation of the polypeptide.

Sequence specific resonance assignments were made as follows. Initially the DQF-COSY spectra (e.g. figs 2a and 3a) were analyzed to delineate the principal spin-systems of amino acid residues. Then the through-bond J-connectivities between geminal and vicinal protons observed in DQF-COSY spectra were complemented by multiple relayed J-connectivities in MLEV17 HOHAHA spectra (e.g. fig.2b). Thus the HN-C$^\alpha$-H-C$^\beta$H-...

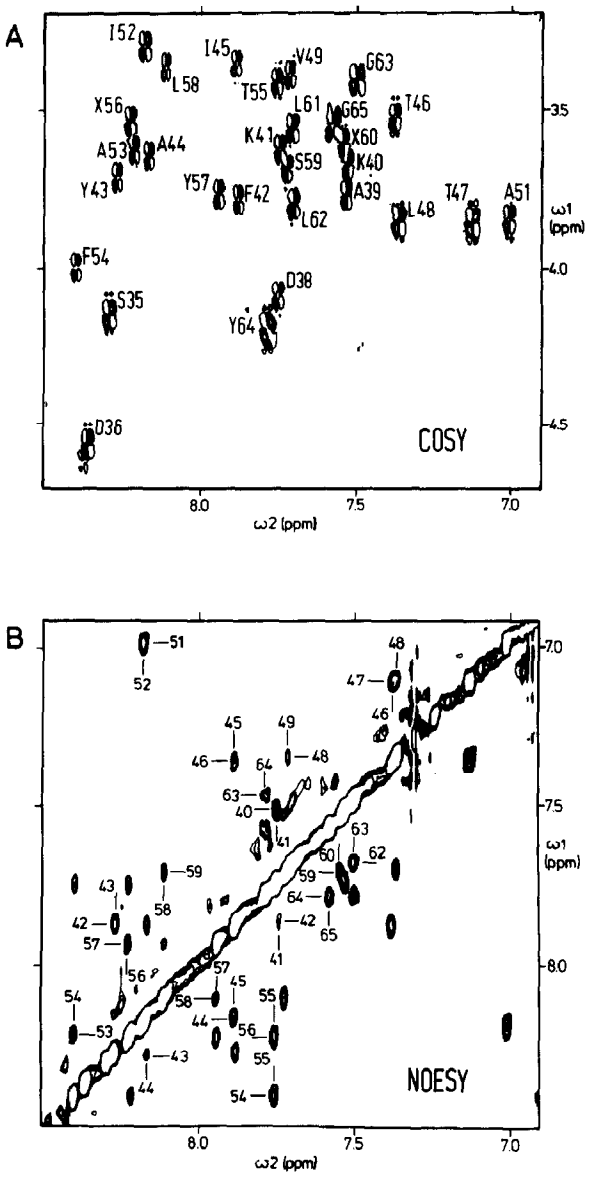


Fig.3. A, NH (ω_2 = 6.9–8.5 ppm)/C$^\alpha$H (ω_1 = 3.2–4.7 ppm) region of the DQF-COSY spectrum and B, NH/NH (ω_2,ω_1 = 6.9–8.5 ppm) region of the NOESY spectrum (mixing time 200 ms) of the segment B solution in CD_3OH-$CDCl_3$ at 20°C. Assignments of the N_iH/C_i^α cross-peaks in A and $N_iH/N_{i+1}H$ cross-peaks in B are indicated.

spin-systems of individual amino acid residues were evaluated. In the DQF-COSY spectrum, aromatic proton spin-systems of all three tyrosines (C$^\delta$H-C$^\delta$H) and one of two phenylalanine residues (C$^\delta$H-C$^\epsilon$H-C$^\zeta$H) were identified. Aromatic proton spin-system of second phenylalanine was not iden-

tified in DQF-COSY spectrum, apparently due to equivalence of chemical shifts of its $C^{\delta}H$-C^{ϵ}-$C^{\zeta}H$ protons. Therefore the intense signal at 6.9 ppm in the ^{1}H-NMR spectrum of sB is assigned to five aromatic proton spin-systems of the second phenylalanine. The aromatic proton spin-systems were linked to the NH-$C^{\alpha}H$-$C^{\beta}H_2$ proton spin-system of corresponding phenylalanine and tyrosine residues through NOE connectivities between $C^{\delta}H$ and $C^{\beta}H$ protons as proposed in [19].

Next, spin-systems of amino acid residues were assigned to specific positions in the amino acid sequence (fig.1) through $^1d_{\alpha N}$, $^1d_{\beta N}$ and $^1d_{NN}$ connectivities for the amide proton of residue $i+1$ with the $C^{\alpha}H$, the $C^{\beta}H$ and the amide protons of the preceding residue i, respectively. In the case of Pro 37 and Pro 50 residues $C^{\delta}H_2$ protons were considered as honored amide protons. A survey of the sequential connectivities obtained in the sB is presented in fig.1, and the chemical shifts for the assigned resonances are listed in table 1. The complete resonance assignments were obtained mainly by the sequential connectivities based on observation of $^1d_{NN}$ (fig.3b) and $^1d_{\beta N}$. The connectivities between Ala 39 and Lys 40 could not be established unambiguously due to interference of their amide proton signals at all the employed temperatures.

It is standard practice [20] to carry out the NMR conformation analysis of a polypeptide in three steps: investigation of (i) configuration of the X-Pro peptide bonds, (ii) secondary structure domains, and (iii) three-dimensional structure.

sB has two prolines in fragments Asp 36–Pro 37 and Val 49–Pro 50. It was shown [21] that *cis* and

Table 1

Proton resonance assignment (chemical shift δ, ppm) and apparent splittings, related to spin-spin coupling constants ($^3J_{\alpha N}$, Hz) of the H-NC^{α}-H protons of segment B in methanol-chloroform (1:1) at 20°C

Residue[a]	δ (± 0.01 ppm)				Splitting (± 0.4 Hz)[b]
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others	
Val 34	–	3.37	1.84	$C^{\gamma}H_3$ 0.72, 0.72	–
Ser 35	8.28	4.14	3.47, 3.47		7.8
Asp 36	8.35	4.56	2.63, 2.47		7.2
Pro 37	–	3.87	1.60, 1.98	$C^{\gamma}H$ 1.66, 1.76	–
				$C^{\delta}H$ 3.51, 3.56	
Asp 38	7.74	4.08	2.37, 2.59		7.6
Ala 39	7.53	3.77	1.20		6.8
Lys 40	7.52	3.69	1.58, 1.64	$C^{\gamma}H$ 1.13, 1.13	6.5
				$C^{\delta}H$ 1.33, 1.33	
				$C^{\epsilon}H$ 2.58, 2.58	
Lys 41	7.73	3.65	1.62, 1.62	$C^{\gamma}H$ 1.13, 1.13	6.5
				$C^{\delta}H$ 1.35, 1.35	
				$C^{\epsilon}H$ 2.53, 2.53	
Phe 42	7.87	3.79	2.87, 2.87	$C^{\delta}H$ 6.71; $C^{\epsilon}H$ 6.39	6.5
				$C^{\zeta}H$ ~6.9	
Tyr 43	8.26	3.72	2.83, 2.83	$C^{\delta}H$ 6.74; $C^{\epsilon}H$ 6.39	6.5
Ala 44	8.16	3.65	1.26		6.2
Ile 45	7.88	3.38	1.51	$C^{\gamma}H$ 0.91, 1.39	6.7
				$C^{\gamma}H_3$ 0.57; $C^{\delta}H_3$ 0.51	
Thr 46	7.37	3.53	3.68	$C^{\gamma}H_3$ 0.57	7.1
Thr 47	7.12	3.86	3.60	$C^{\gamma}H_3$ 0.74	8.2
Leu 48	7.36	3.86	1.31, 1.49	$C^{\gamma}H$ 1.39	7.0
				$C^{\delta}H_3$ 0.57, 0.61	
Val 49	7.70	3.41	2.82	CH_3 0.62, 0.77	6.2
Pro 50	–	3.87	1.49, 1.89	$C^{\gamma}H$ 1.51, 1.85	–
				$C^{\delta}H$ 3.18, 3.24	
Ala 51	7.01	3.85	1.25		6.4

Table continued on the following page.

Table 1 (contd)

Residue[a]	δ (± 0.01 ppm)				Splitting (± 0.4 Hz)[b]
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others	
Ile 52	8.17	3.34	1.70	$C^{\gamma}H$ 0.79, 1.52 $C^{\gamma}H_3$ 0.62; $C^{\delta}H_3$ 0.52	7.2
Ala 53	8.20	3.64	1.17		6.2
Phe 54	8.39	4.00	2.86, 2.98	$C^{\delta}H$, $C^{\epsilon}H$, $C^{\zeta}H$ ~6.9	6.5
Thr 55	7.75	3.44	4.07	$C^{\gamma}H_3$ 0.97	7.2
Nle 56	8.22	3.56	1.49, 1.61	$C^{\gamma}H$ 0.94, 1.25 $C^{\delta}H$ 1.24, 1.24 $C^{\epsilon}H_3$ 1.84	6.8
Tyr 57	7.93	3.76	2.80, 2.80	$C^{\delta}H$ 6.59; $C^{\epsilon}H$ 6.32	6.8
Leu 58	8.10	3.40	1.12, 1.21	$C^{\gamma}H$ 1.20 $C^{\delta}H_3$ 0.44, 0.47	6.2
Ser 59	7.71	3.70	3.57, 3.57		6.0
Nle 60	7.54	3.62	1.51, 1.51	$C^{\gamma}H$ 0.91, 1.21 $C^{\delta}H$ 0.96, 0.96 $C^{\epsilon}H_3$ 1.65	6.8
Leu 61	7.70	3.58	1.25, 1.36	$C^{\gamma}H$ 1.15 $C^{\delta}H_3$ 0.38, 0.42	7.2
Leu 62	7.69	3.81	1.44, 1.44	$C^{\gamma}H$ 0.51 $C^{\delta}H_3$ 0.96, 0.96	7.5
Gly 63	7.49	3.42, 3.42		–	
Tyr 64	7.78	4.18	2.55, 2.81	$C^{\delta}H$ 6.76; $C^{\epsilon}H$ 6.32	8.6
Gly 65	7.57	3.56, 3.56			–

[a] Nle denotes norleucine residues, numbers correspond to positions of residues in the amino acid sequence of BR [4,5]

[b] Splitting was measured in the phase-sensitive DQF-COSY spectrum using cross-sections along the ω_2 axis. The values were obtained as the distance between antiphase peak components and no corrections were made for the effect of the large line width of the components [24]. Based on the amide proton signal line widths of ~10 Hz, the splitting (distance between peak components) of 6–7 Hz corresponds [24] to $^3J_{\alpha N}$ coupling constant $\leqslant$5 Hz

trans X-Pro peptide bonds are distinguishable by their unique NOE patterns. An observation of intensive NOE cross-peaks between proline $C^{\delta}_{i+1}H$ protons and the $C^{\alpha}_{i}H$ proton of the preceding residue is an inherent feature of the *trans* X-Pro peptide bond. The intensive $C^{\alpha}_{i}H/C^{\alpha}_{i+1}H$ NOE cross-peak between the i residue and $i+1$ proline, respectively, is inherent to the *cis* X-Pro peptide bond. In the case of the Asp 36-Pro 37 fragment intensive cross-peaks between $C^{\delta}H_2$ of Pro 37 and $C^{\alpha}H$ of Asp 36 were observed; thus the *trans* Asp-Pro peptide bond is established and torsion angle ψ of Asp 36 is in the region around 120° (see fig.21 in [21]). Both types of the cross-peaks $C^{\alpha}_{i}H/C^{\delta}_{i+1}H$ and $C^{\alpha}_{i}H/C^{\alpha}_{i+1}H$ are definitely absent for the Val 49 and Pro 50 residues. This leaves the configuration of the Val 49–Pro 50 peptide bond undetermined, pointing at the same time to ~ – 60° torsion angle ψ of the Val 49 residue [21].

The secondary structure of peptide segments is usually deduced from comparison of $d_{\alpha N}$, $d_{\beta N}$ and d_{NN} distances for principal types of regular secondary structures [20–23] with experimental NOE data. The most useful for this purpose are inter-residue 'short range' NOEs between NH, $C^{\alpha}H$ and $C^{\beta}H$ protons of i and j residues where $|i-j| \leqslant 4$. A summary of NOEs observed for sB and expected for a regular right-handed α-helix is given in fig.1. The information is sufficient to delineate a right-handed α-helix which extends at least from the Lys 40 to the Ser 59 residue. Further refinement of the secondary structure is based on deuterium exchange rates of amide NH groups and spin-spin coupling constants of NH-$C^{\alpha}H$ protons. Both data

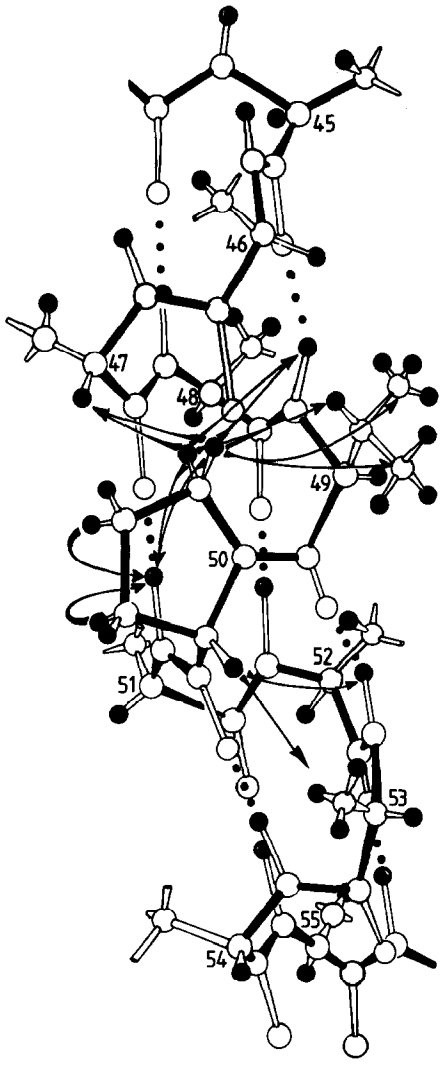


Fig.4. Schematic presentation of the right-handed α-helical conformation of segment B in the Val 49–Pro 50 region. Arrows indicate interproton NOE interactions observed between protons of Pro 50 and protons of other residues. Rows of three dots show the interresidue hydrogen bonds where slow deuterium exchange rates for amide NH groups were observed (see fig.1).

sets (fig.1 and table 1) strongly indicate that a stable right-handed α-helix starts from the Phe 42 carbonyl and terminates at the NH group of the Nle 60. This segment is characterized by relatively low $^3J_{\alpha N} < 5$ Hz values and slow deuterium exchange of peptide NH protons. The amino acid residues outside this region are more mobile even though they retain a tendency (rapidly lowering towards both N- and C-terminals) to form the α-helix, as can be judged by NOE and deuterium exchange data (fig.1).

With the above data at hand we were able to distinguish between the *trans* and *cis* Val 49–Pro 50 amide configurations in favor of the former. For that purpose we constructed wire models of sB in which the amide bond in question was positioned between the relevant helical segments. It turned out that only in the case of *trans* configuration the observed HN-C$^{\alpha}$H couplings and NOEs agreed with the values predicted from the model (fig.4).

4. CONCLUSION

The objective of the project is to provide a direct spectral insight into the spatial structure of BR. Previously it was shown [12] that isolated BR segments retain their intact bilayer membrane conformations in methanol/chloroform (1:1) solution. sB is the first membrane spanning segment of BR whose molecular conformation was determined by direct NMR methods. Currently, the secondary structure of other segments as well as the folding motive of the intact polypeptide chain of BR in methanol/chloroform solution are under investigation.

REFERENCES

[1] Ovchinnikov, Yu.A. (1982) FEBS Lett. 148, 179–191.
[2] Dencher, N.A. (1983) Photochem. Photobiol. 38, 753–767.
[3] Stoeckenius, W. (1985) Trends Biochem. Sci. 10, 483–486.
[4] Ovchinnikov, Yu.A., Abdulaev, N.G., Feigina, M.Yu., Kiselev, A.V. and Lobanov, N.A. (1979) FEBS Lett. 100, 219–244.
[5] Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K. and Biemann, K. (1979) Proc. Natl. Acad. Sci. USA 76, 5046–5050.
[6] Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2225–2229.
[7] Henderson, R. and Unwin, N. (1975) Nature 257, 28–32.
[8] Leifer, D. and Henderson, R. (1983) J. Mol. Biol. 163, 451–466.
[9] Tsygannik, I.N. and Baldwin, J.M. (1987) Eur. Biophys. J. 14, 263–272.
[10] Engelman, D.M., Henderson, R., McLashlan, A.D. and Wallace, B.A. (1980) Proc. Natl. Acad. Sci. USA 77, 2023–2027.
[11] Wuthrich, K. (1986) NMR of Proteins and Nucleic Acids, Wiley, New York.

[12] Arseniev, A.S., Kuryatov, A.B., Tsetlin, V.I., Bystrov, V.F., Ivanov, V.T. and Ovchinnikov, Yu.A. (1987) FEBS Lett. 213, 283–288.
[13] Kozhich, A.T., Vaskovsky, B.V., Mikhaleva, I.I. and Ivanov, V.T. (1984) in: Chemistry of Peptides and Proteins (Voelter, W., Bayer, E., Ovchinnikov, Yu.A. and Wunsch, E. eds) vol.2, pp.79–85, Walter de Gruyter, Berlin, New York.
[14] Wunsch, E., Moroder, L., Gillessen, D., Soerensen, U.B. and Bali, J.-P. (1982) Hoppe-Seylers Z. Physiol. Chem. 363, 665–669.
[15] Marion, D. and Wuthrich, K. (1983) Biochem. Biophys. Res. Commun. 113, 967–974.
[16] Rance, M., Sorensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R. and Wuthrich, K. (1983) Biochem. Biophys. Res. Commun. 117, 479–485.
[17] Jeener, J., Meier, G.H., Bachman, P. and Ernst, R.R. (1979) J. Chem. Phys. 71, 4546–4553.
[18] Bax, A. and Davis, D.G. (1985) J. Magn. Reson. 65, 355–366.
[19] Billeter, M., Braun, W. and Wuthrich, K. (1982) J. Mol. Biol. 155, 321–346.
[20] Arseniev, A.S., Kondakov, V.I., Maiorov, V.N. and Bystrov, V.F. (1984) FEBS Lett. 165, 57–62.
[21] Arseniev, A.S., Kondakov, V.I., Maiorov, V.N., Volkova, T.M., Grishin, E.V., Bystrov, V.F. and Ovchinnikov, Yu.A. (1983) Bioorgan. Khim. (USSR) 9, 768–793.
[22] Arseniev, A.S., Kondakov, V.I., Maiorov, V.N., Bystrov, V.F. and Ovchinnikov, Yu.A. (1983) Bioorgan. Khim. (USSR) 9, 1667–1689.
[23] Wagner, G., Neuhaus, D., Worgotter, E., Vasak, M., Kagi, J.H.R. and Wuthrich, K. (1986) J. Mol. Biol. 187, 131–135.
[24] Neuhaus, D., Wagner, G., Kagi, J.H.R. and Wuthrich, K. (1985) Eur. J. Biochem. 151, 257–273.