Conformation of surface exposed N-terminus part of bacteriorhodopsin studied by transferred NOE technique

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Abstract Interaction of the monoclonal antibody A5 raised against native bacteriorhodopsin (BR) with the synthetic peptide pGlu[1]-Ala-Gln-Ile-Thr-Gly-Arg[7]-NH_2, corresponding to the amino acid sequence 1–7 was studied by transferred nuclear Overhauser effect (TRNOE) spectroscopy. The denaturing reagents and the specially designed pulse sequences which eliminate broad signals from the TRNOE spectra were used to favour evaluation of the TRNOE peaks. On the basis of the data obtained, the conformation of peptide bound with A5 was calculated. A model of the mutual arrangement of bacteriorhodopsin N-terminus and the first transmembrane α-helical segment 8–32 was proposed.

Key words: Bacteriorhodopsin; Monoclonal antibody; NMR; Spatial structure

1. Introduction

Bacteriorhopsin (BR) — the light driven proton pump of *Halobacterium halobium* is a single chain membrane protein of 248 amino acid residues and contains retinal chromophore bound to the ε-amino group of Lys^{216} via Schiff base [1]. Using electron microscopy in conjunction with a low dose electron diffraction the polypeptide chain of BR was shown to be arranged as seven transmembrane helices interconnected by relatively short loops on both sides of the membrane. Based on a wealth of biochemical and molecular biological data the three dimensional structure of the membrane embedded part of BR is now brought to 3.5 Å resolution [2]. However, there is no information available as to the conformation of outer membrane loops and N- and C-terminal parts of BR. It has been shown that BR retains its secondary and some elements of the native tertiary structure in membrane mimicking environments [3,4] stimulating the NMR studies of defined fragments of BR in methanol/chloroform mixture or detergent micelles [5,6]. These studies have resulted in determination of the spatial structure of the BR transmembrane segments with a high resolution, while the data on the conformation of the outer membrane segments required further refinement [5,6].

As a further development of NMR approaches to determination of the BR spatial structure we took the advantage of antibody–antigen complexes between monoclonal antibodies raised against the native BR membrane and respective synthetic epitopes. Due to a high affinity and stereospecificity of an antibody–antigen interaction, a short conformationally labile fragment of BR would undergo the conformation transformation upon binding with the antibody, so that the conformation of the antibody-bound fragment should be closely similar to the conformation of native antigen epitope had initiated the antibody production. Data supporting this view has been recently esserted by the results on NMR studies of interaction between the monoclonal antibody to recombinant interleukin-2 (IL-2) and its synthetic fragment [10,11], where trasferred NOE technique was used to reveal the conformation and orientation of the IL-2 segment in the native IL-2. At present antibodies against a variety of epitopes have been revealed (different types of bends [7], the extended structure [8], α-helical region [9]) suggesting that NMR studies toward this direction would be useful in the case of BR, as well.

In this paper we present data on the structure of the N-terminal fragment of BR as revealed by NMR studies of the antibody–antigen complex.

2. Experimental

Hybridoma A5 was prepared using the standard procedures [12–14] and cultured as ascites of BALB/c mice. Monoclonal antibody A5 was precipitated from the ascite fluid with 50% ammonium sulfate, pH 7.0, and purified as follows. The ammonium sulfate precipitate was solubilized in an appropriate buffer and incubated with an excess of purple membranes for 2 h at room temperature. This complex was washed several times with PBS buffer (0.14 M NaCl, 1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 2.7 mM KCl) by centrifugation at 100,000×*g* for 1 h. The complex was brought to pH 11.5 by adding 0.2 M CAPS and the purple membranes removed by centrifugation at 100,000×*g* and the eluted antibody was immediately brought to pH 7.0 by adding of 1 M imidazol-HCl. Finally, antibody was dialyzed against PBS. The epitope mapping was carried out by ELISA and immunoblotting using fragments of BR and synthetic peptides as antigens. The immunoglobulin isotype of the A5 was determined to be IgG_2. Affinity constant determined by the method [15] was equaled 3.65×10^8 M^{-1}.

The N-terminal peptide of bacteriorhodopsin pGlu[1]-Ala-Gln-Ile-Thr-Gly-Arg[7]-NH_2 (1–7)BR was synthesized by solid phase technique. A high purity of (1–7)BR was confirmed by ^1H NMR. The solvents used for preparing of NMR samples were ^2H_2O (99.96% ^2H, Stohler/KOR Stable Isotope) or a mixture of $^1H_2O/^2H_2O = 9:1$, containing PBS buffer and 0.015 M NaN_3. The concentrations of (1–7)BR and A5 were 3 and 0.14 mM, respectively. The pHs 7.2 and 4.7 were used for NMR samples dissolved in ^2H_2O and $^2H_2O/H_2O$, respectively. The last pH value was attained by adding 0.15 or 1.5 M HCl to the sample studied.

NMR spectra were obtained at 600 MHz (Varian UNITY 600

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Abbreviations: BR, bacteriorhodopsin; A5, the monoclonal antibody studied; (1–7)BR, the peptide pGlu[1]-Ala-Gln-Ile-Thr-Gly-Arg[7]-NH_2; pGlu, pyroglutamic acid; GnHCl, guanidinium hydrochloride; NOE, nuclear Overhauser effect; TRNOE, NOE which arised between the (1–7)BR protons in the (1–7)BR/A5 complex; RST, the values used to characterise non-selective NOE transfered to the peptide owing to the saturation of the A5 protons with radiofrequency field.

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Table 1
The parameters of the (1–7)BR proton signals observed in the presence and absence of A5: chemical shifts (δ, ppm); radiofrequeny saturation transfer (*RST*) observed after 0.7 s irradiation of A5 by radiofrequency field in the absence (0 M) and presence (0.6 M) of urea; transverse relaxation rate enhancements (ΔR_2; in s^{-1}) caused by A5 in the presence of 0.2 M GnHCl

Residue	Proton	δ (ppm)	*RST* 0 M urea	*RST* 0.6 M urea	ΔR_2 (s^{-1})
pGlu[1]	NH	7.89			9.6
	$C^{\beta}H$	2.58	1.8	8.5	
	$C^{\gamma}H_2$	2.46	1.8	6.9	5.6
Ala[2]	NH	8.42			7.8
	$C^{\beta}H_3$	1.43	1.1	3.7	9.9
Gln[3]	NH	8.50			10.8
	$C^{\beta}H$	2.03	1.7	6.0	
	$C^{\gamma}H_2$	2.40	1.4	5.3	8.6
Ile[4]	NH	8.29			8.2
	$C^{\gamma}H_3$	0.94	1.1	4.3	5.2
	$C^{\delta}H_3$	0.89	1.8	6.3	8.4
Thr[5]	NH	8.25			10.4
	$C^{\gamma}H_3$	1.25	1.0	3.7	3.0
Gly[6]	NH	8.45			5.9
	$C^{\alpha}H$	4.03	0.4	1.8	3.0
Arg[7]	NH	8.22			2.2
	$C^{\delta}H_2$	3.24		1	1.5

spectrometer) in the pure phase-sensitive mode by collecting hypercomplex data. DQF-COSY, TOCSY (mixing time 40 ms) were recorded using the standard VNMR 4.3 pulse sequences. The proton transverse relaxation rates (R_2) were measured using Carr-Purcell-Meiboom-Gill pulse sequence: $RD-90°-(-t-180°-t-)_{2n}-Acq$, where $RD = 5$–6 s, the time of restoration of the equilibrium state of the nuclear spin system; $t = 1$ ms; $(-t-180°-t-)_{2n}$=0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 s; $Acq = 0.512$ s, acquisition time. The radiofrequency saturation transfer (*RST*) from A5 to the signals of (1–7)BR and the nuclear Overhauser effects (TRNOE) arising between (1–7)BR protons in the (1–7)BR/A5 complex were determined from 1D and 2D spectra obtained with the following pulse sequences:(

$$[RD-d_2(F1,F2)-90°_{\varphi 1}-(-t_1-180°_{\varphi 2}-t_1)_{2n}-Acq(1,2)_{\varphi 3}] \quad (1a,b)$$

$$[RD-90°_{\varphi 4}-(t_2-180°_{\varphi 5}-t_2)_{2n}-d_4-90°_{\varphi 6}-\tau_m-90°_{\varphi 7}-Acq_{\varphi 8}] \quad (2)$$

where regularly repeated non-selective 180° radiofrequency pulses are intended to prevent phase distortion of proton signals; $RD = 3$ s and $RD = 1.2$ s for the pulse sequences 1 and 2, respectively; d_2 is the time for application of the weak continious radiofrequency field at frequency *F1* or *F2* (*F1* was applied on the aromatic proton signals of A5, and *F2* at an edge of the spectrum free of the proton signals); $t_1 = 1$ ms and $t_2 = 0.1$ ms; the durations of the $(t-180°-t)_{2n}$ cycles were about 0.032 ms and 0.01 ms (for the sequences 1 and 2, respectively); $Acq = 0.512$ and 0.341 s for the pulse sequences 1 and 2, respectively; d_4 and $\tau_m = 0.1$ s are evolution time and mixing time for NOESY pulse scheme. Phases $\varphi 1 = \varphi 3 = x,y,-x,-y$; $\varphi 2 = y,-x,y,-x$; $\varphi 4 = 8(x,-x),8(y,-y)$; $\varphi 5 = 4[2(y),2(-y)]$, $4[2(-x),2(x)]$; $\varphi 6 = (x),8(-x),8(y),8(-y)$; $\varphi 7 = 2[2(x),2(-x),2(y),2(-y)]$, $2[2(y),2(-y),2(-x),2(x)]$; $\varphi 8 = x,2(-x),x,y,2(-y),y,-x,2(x),-x,-y,2(y),-y,y,2(-y),y,-x,2(x),-x,-y,2(y),-y,x,2(-x),x$; hyperfine phase shift in ω1 direction was introduced by incrementing of phase of φ4 and φ5 by +90°. To decrease the water signal and the signal of admixture (at 2.9 ppm) in the 2D TRNOE spectrum (acquired using pulse sequence 2), these signals were continiously irradiated with weak radiofrequency fields during *RD* and τ_m. The number of complex data points acquired for 1D and 2D spectra were equal 8192 and 4096×(360−512), respectively. Chemical shifts were measured relative to the water resonance, which was arbitrarily taken as 4.72 ppm at 30°C.

The values of transverse relaxation rate enhancement (ΔR_2) of the (1–7)BR protons induced by A5 were determined in the presence of 0.2 M GnHCl, pH 4.7. The values RST were determined in the presence and absence of urea at pH 7.2 by the following equation: $RST = [(I_b - I_a)/I_b] \times 100$, where I_a and I_b are the intensities of (1–7)BR signal in the spectra obtained with the aid of the pulse sequences 1a and 1b, respectively.

The 2D spectra were analyzed using the program XEASY (ETH, Zurich, Switzerland). The cross-peaks volumes (*V*) measured in the TRNOE spectrum were converted to upper distance constraints (*b*) by using the relation $V = c/b^6$. The constant *c* was chosen such that the intraresidual distance between $C^{\alpha}H$ and NH protons were 2.5 Å or higher. The distance constrains were used by the program DIANA version 2.8 (ETH, Zurich, Switzerland) in order to calculate the conformation of (1–7)BR in the complex with A5 according to standard protocol. A model of the mutual arrangement of bacteriorhodopsin N-terminus and segment Pro^8-Met^{32} has been constructed using molecular modeling software SYBYL version 6.0 (TRIPOS Associates Inc., St. Louis, USA).

3. Results

Linewidths, spin–spin coupling constants and chemical shifts of the (1–7)BR protons were only slightly changed in response to the presence of A5 ([(1–7)BR]/[A5] = 20 : 1). Narrow signals of (1–7)BR are clearly visible against of the broad signals of A5 (Fig. 1a). The (1–7)BR signal assignments was

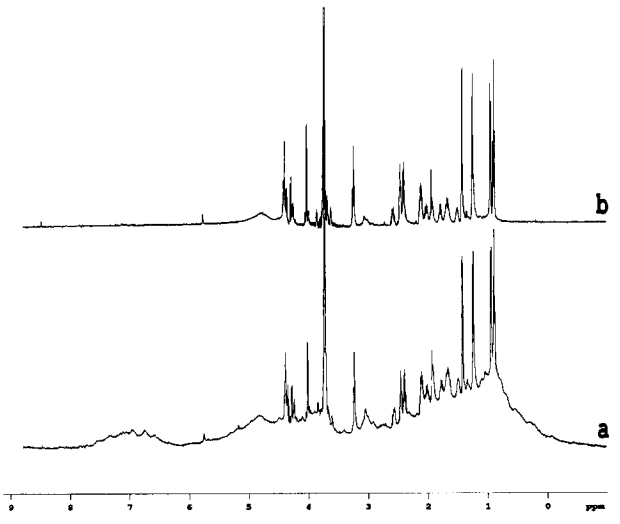

Fig. 1. ^1H NMR spectra of a mixture of [(1–7)BR]/[A5] = 20 : 1 obtained without (a) and with (b) introduction of a train of non-selective 180° radiofrequency pulses (see pulse sequence 1b, in section 2); ^2H_2O, PBS buffer, 0.015 M NaN_3, pH 7.2, 30°C.

based on a standard analysis [16] of DQF-COSY, TOCSY and 2D TRNOE spectra of (1–7)BR or the mixtures of (1–7)BR and A5. Chemical shifts of some (1–7)BR protons are given in Table 1.

In order to reveal the (1–7)BR-groups contacting with the A5 surface, a saturation transfer from the A5 protons on the (1–7)BR protons was studied using the pulse sequence 1a,b. The characteristic feature of this pulse sequence is the introduction of a delay which precedes the acquisition time (*Acq*). During this delay the broad signals disappear, while the narrow signals of (1–7)BR weaken only slightly (see Fig. 1b), so that the difference spectrum obtained by substracting the spectrum 1a from the spectrum 1b contains only the (1–7)BR signals which arise due to saturation transfer from A5. The saturation transfers were less than 8% even at $d_2 = 2$ s owing to slow exchange of (1–7)BR between the free and A5-bound states. It should be mentioned that long d_2 values diminish a selectivity of the saturation transfer [10]. Therefore we accelerated the exchange rate of (1–7)BR between the free and A5-bound states by adding of 0.6 M urea. In response of the urea presence the RST values increased 3–4 times (see Table 1) and became sufficiently well differentiated. This allowed to increase an accurracy of the RST value measurements and permitted to classify the (1–7)BR-groups according to the extent of contact with the A5 surface.

Nuclear Overhauser effect arising between the spatially proximated protons of macromolecule-bound ligand could be registered on the signals of the free ligand if its lifetime in the bound state is considerably shorter than the longitudinal relaxation times of the protons in the bound and free ligand, and the mixing time of the NOESY experiment. This method named TRNOE spectroscopy have been often used to determine the conformation of a ligand bounded to a macromolecule [17]. The narrow TRNOE cross-peaks between the protons of (1–7)BR were hardly detectable against the background of intensive broad signals in the standard NOESY spectra ($\tau_m = 0.2$–0.5 s) of the mixture [(1–7)BR]/[A5] = 20 : 1. This is accounted for unsufficiently fast exchange rate of (1–7)BR between the free and A5-bound states. To increase the exchange rate urea or GnHCl were added to the sample. Even with 0.2 M GnHCl the exchange rate of (1–7)BR between the free and bound states was not fast, so that the TRNOE cross-peaks were not large enough to neglect the presence of the rapidly relaxing broad signals of A5. The broad signals had been cleared out by introducing an additional delay into the standard NOESY pulse sequence after the first 90° pulse. A non-selective 180° radiofrequency pulse or continious radiofrequency field have been used during this delay to prevent a distortion of the cross-peak phases [18,19]. We tested the pulse sequence MLEV-16, continious radiofrequency field as well as a train of rapidly repeated non-selective 180° radiofrequency pulses to attain the same goal. The last method was preferred and used in the present study. In the 2D TRNOE spectrum of the mixture of (1–7)BR and A5 acquired using the pulse sequence 2 ($\tau_m = 0.1$ s) about hundred cross-peaks between the protons of (1–7)BR were detected. The facts, that these cross-peaks were in phase with the spectrum diagonal as well as absence of the cross-peaks in the analogous spectrum of (1–7)BR acquired in the absence of A5, clearly indicate that these cross-peaks are due to NOE arised between the spatially proximated protons of (1–7)BR in the (1–7)BR/A5 complex. In the end the 46 intraresidual and 21 interresidual TRNOE-contacts between the protons of (1–7)BR were revealed.

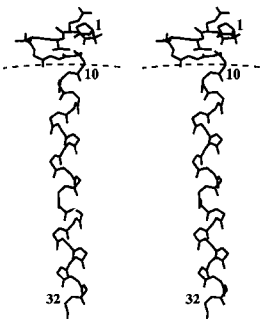

Fig. 2. Mutual arrangement of the N-terminus and α-helical transmembrane segment Pro^8-Met^{32} of BR. All heavy atoms are shown for the fragment $pGlu^1$-Gly^6, the rest of the polypeptide chain is represented by the backbone heavy atoms only. The positions of $pGlu^1$, Trp^{10} and Met^{32} are indicated by numerals. The dashed line represents the membrane surface.

The distance constraints estimated from the volumes of TRNOE cross-peaks were used to calculate the spatial structure of (1–7)BR in the (1–7)BR/A5 complex using DIANA program. The averaged values of torsion angles of the 10 best DIANA conformations of (1–7)BR indicate that our experimental data permit rather unambiguous determination of the (1–7)BR spatial structure from $pGlu^1$ to Thr^5: $pGlu^1$ $\psi = -152.1 \pm 0.1$; Ala^2 $\varphi = -117.3 \pm 0.2$, $\psi = -18.5 \pm 0.1$; Gln^3 $\varphi = -121.0 \pm 0.4$, $\psi = 44.2 \pm 0.7$, $\chi_1 = -35.8 \pm 0.1$, $\chi_2 = 102.4 \pm 0.2$; Ile^4 $\varphi = -40.5 \pm 0.6$, $\psi = 165.3 \pm 2.8$, $\chi_1 = -50.1 \pm 0.3$, $\chi_2 = -113.8 \pm 0.2$; Thr^5 $\varphi = -31.6 \pm 2.7$, $\psi = -34.1 \pm 1.3$, $\chi_1 = -75.4 \pm 0.9$. This clarity loses at Gly^6 ($\varphi = -139.6 \pm 25.2$, $\psi = -13.0 \pm 30.5$) and disappear at Arg^7. The torsion angles φ and ψ as well as the positioning of the side chain of Arg^7 are uncertain.

It is well known that the increasing of a size or conformational rigidity of a molecule (complex) is accompanied by raising of transverse relaxation rates of its nuclei. In fact the presence of A5 causes a differential raising of the transverse relaxation rates of the (1–7)BR protons (see ΔR_2, Table 1). Maximal ΔR_2 values were observed for the protons of $pGlu^1$-Thr^5 segment indicating its rigidity in the (1–7)BR/A5 complex. As moving from the protons of Gly^6 to the side chain protons of Arg^7 the values ΔR_2 become smaller. Besides the low ΔR_2 values, a high mobility of the Arg^7 side chain of (1–7)BR in the (1–7)BR/A5 complex is also apparent from the absence of the cross-peaks between the $C^\gamma H_2$ and $C^\delta H_2$ protons of Arg^7 in the TRNOE spectrum of the mixture of (1–7)BR and A5.

4. Discussion

The values of the spin–spin coupling constants of H-NC^α-H protons and the ROESY spectrum (data not shown) indicate that (1–7)BR takes a random coil spatial structure in solution. This is a typical feature for peptides of such size. In the absence of additivities (1–7)BR comparatively slow exchanges between the free and antibody-bound states. In the presence of 0.6 M urea or 0.2 M GnHCl the exchange rate was significantly accelerated, making possible NMR investigation of the (1–7)BR bound state conformation using TRNOE technique. As a result the spatial structure of (1–7)BR in the (1–

7)BR-A5 complex as well as the surface of (1–7)BR in contact with A5 were evaluated.

Increasing of the transverse relaxation rates of the (1–7)BR protons in response to the presence of A5 indicates that the amino acid residues $pGlu^1$-Thr^5 penetrate into the antigen-binding cleft of A5, residue Gly^6 is positioned on the surface of complex, while the side chain of Arg^7 is surrounded by solvent. In the complex the conformation of the $pGlu^1$-Thr^5 segment is unambiguously determined as a consequence of its interactions with the antigen binding site of A5. The values of the torsion angles φ and ψ indicate (see section 3) that the conformation of the Ile^4-Arg^7 residues is close to that of a type I β-turn [20]. The hydrogen bond between the carbonyl oxygen of Ile^4 and the backbone amide proton of Arg^7 was revealed in 7 out of 10 best DIANA conformations of (1–7)BR.

On binding to A5, (1–7)BR adopts the conformation which is close to the conformation of the antigen which had initiated A5 production. The same groups of (1–7)BR and BR form contacts with the A5 binding site. These groups of BR should be located on its surface and accessible for the interaction with the antibody binding site. Recognizing these facts we suppose a model of the mutual arrangement of the bacteriorhodopsin N-terminus and transmembrane α-helical segment Pro^8-Met^{32} (see Fig. 2). On building of this model the conformation of the $pGlu^1$-Gly^6 residues has been brought into line with the conformation of (1–7)BR in the (1–7)BR-A5 complex (see section 3), while those of Trp^{10}-Met^{32} had been aligned in accordance with the literature data [2,6]. Thereafter the relative position of these fragments was adjusted by changing the values of the torsion angles Arg^7, Pro^8 and Glu^9. In the course of the adjustment the protons of the N-terminus best contacting with A5 surface (large RST values) were maximally removed from the transmembrane segment Trp^{10}-Met^{32} while the protons of Arg^7 and Gly^6 (small *RST* values) oriented towards the transmembrane segment. The A5 binding site is situated on the top of the model (see Fig. 2).

We are planing to apply the method used in the present study for determination of the conformation and orientation of the other solvent exposed segments of BR. To date we already have obtained 15 clones of the hybridomas against different parts of native BR.

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