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Towards structural investigations on isotope labelled native bacteriorhodopsin in detergent micelles by solution-state NMR spectroscopy

Heiko Patzelt[a], Anne S. Ulrich[b], Hermann Egbringhoff[a], Petra Düx[b], Jennifer Ashurst[b], Bernd Simon[b], Hartmut Oschkinat[b,*] and Dieter Oesterhelt[a]

[a]*Max-Planck-Institut für Biochemie, Am Klopferspitz, D-82152 Martinsried, Germany*
[b]*European Molecular Biology Laboratory, P.O. Box 10 2209, D-69012 Heidelberg, Germany*

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Summary

^{1}H NMR signals of the retinal moiety in detergent-solubilized bacteriorhodopsin are assigned, enabling the interpretation of NOEs within the chromophore. To achieve this, a number of differently labelled samples were prepared to test the applicability of the various assignment and distance measurement strategies. In measurements with and without light, ^{1}H and ^{13}C chemical shifts of the retinal in the native protein were partially assigned for both the dark- and the light-adapted states. Additionally, samples with residue-specific ^{1}H amino acids and/or retinal in an otherwise deuterated protein were prepared to measure the distances between either two kinds of amino acids or between individual amino acids and the retinal moiety. With the observation of NOE within the bound retinal and between retinal and its neighbouring amino acids, an important step towards the elucidation of distance constraints in the binding pocket of the proton pump is made.

Introduction

Many cellular processes are based on the function of integral membrane proteins, of which the receptors for extracellular stimuli and the components of bioenergetic systems are prominent examples. However, the study of structure–function relationships in membrane proteins is, in most cases, hampered by a lack of suitable structural data, since procedures for obtaining sufficiently large and well-ordered crystals are not straightforward. To date, only a few membrane protein structures have been solved by X-ray crystallography (Deisenhofer et al., 1985; Allen et al., 1988; Weiss and Schulz, 1993; Abrahams et al., 1994; Iwata et al., 1995) or by electron microscopy of 2D crystalline arrays (Henderson et al., 1990; Kühlbrandt et al., 1994).

As a complement to diffraction methods, solid-state NMR spectroscopy has recently been applied to the structural analysis of membrane proteins (Cross and Opella, 1994). Membrane proteins that are embedded in the lipid bilayer can be studied by magic angle spinning techniques (Smith and Griffin, 1988; Smith and Peersen, 1992) or by using static oriented samples (Ketchem et al., 1993). The application of such methods leads to the measurement of either individual distances or orientational constraints, and specific isotope labelling at the site of interest is required.

In order to obtain a larger number of distance constraints simultaneously from a particular sample, it is desirable to employ some of the solution-state NMR methodology. This involves usually multidimensional NMR spectroscopy which, for small proteins of up to 30 kDa, yields large sets of NOEs, from which high-resolution structures can be calculated. However, the solution-state NMR methods are limited to systems which tumble rapidly in solution and whose NMR signals therefore

*To whom correspondence should be addressed.
Abbreviations: BR, bacteriorhodopsin; DM, dodecyl maltoside; δ, chemical shift; HMQC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; OG, octyl glycoside; PM, purple membrane; ppm, parts per million; Ret, retinal.

show relatively long relaxation times. To investigate membrane proteins by this approach, they need to be solubilized in suitable detergent micelles which maintain a stable, homogeneous sample and show sufficiently short correlation times.

Here, we test the applicability of the solution-state NMR to the integral membrane protein bacteriorhodopsin, solubilized in detergent micelles. The intention is to assign a large number of proton resonances in the core of the protein and to measure NOEs between the retinal moiety and its environment. The protein–detergent micelles are still of considerable size (50–100 kDa (Seigneuret et al., 1991b)), and not all of the high-resolution methodology will be applicable. Therefore, special methods for labelling needed to be developed, as well as spectroscopic and instrumental arrangements required for sample illumination.

The light-driven proton pump bacteriorhodopsin (BR) has a molecular weight of 26 kDa and occurs naturally in 2D arrays of the purple membrane (PM) of *Halobacterium salinarum* (Tittor, 1991; Oesterhelt et al., 1992; Khorana, 1993). The chromoprotein absorbs at 570 nm and contains retinal as a prosthetic group which is covalently bound to the side chain of Lys^{216} via a protonated Schiff base linkage (Bayley et al., 1981; Lemke and Oesterhelt, 1981; Mullen et al., 1981). Electron crystallographic refinement has yielded the structure of BR with a resolution of 3.5 Å (Grigorieff et al., 1996). Seven transmembrane α-helices are grouped around the retinal. With the aid of computer modelling, the positions of most side chains could be located (Nonella et al., 1991; Zhou et al., 1993; Grigorieff et al., 1996).

The function of BR as a proton pump is based on a thermoreversible all-*trans* to 13-*cis* photoisomerization of the retinal moiety to which the transient deprotonation of the protonated Schiff base is connected. The photocycle of the molecule is comprised of a number of intermediates and has been analysed in detail by spectroscopy and many other biophysical techniques (reviewed by Lanyi and Váró (1995)). Of special interest is the so-called M-intermediate, which contains the protonated Schiff base in a deprotonated 13-*cis*,15-*anti* configuration (Farrar et al., 1993). Variants of BR have been produced, for example D96N, where M is the dominant intermediate accumulating under constant illumination (Mogi et al., 1988; Butt et al., 1989). The initial (light-adapted) state BR_{568} (all-*trans*,15-*anti* configuration) equilibrates in the dark with a second species (BR_{548}) of 13-*cis*,15-*syn* geometry (Smith et al., 1989) which is not taking part in the photocycle. In the so-called dark-adapted state of BR these two forms occur in a mixture, of which ratios of approximately 1:1 or 1:2 (all-*trans*:13-*cis*) have been reported (Oesterhelt et al., 1973; Scherrer et al., 1989).

The local structure of the chromophore, its environment, and the structural changes during the photocycle were the focus of many solid-state NMR applications to BR (reviewed by Engelhard and Bechinger (1995)). The mobilities and protonation states of ^{2}H-, ^{13}C-, ^{15}N- or ^{19}F-labelled amino acid side chains were investigated (Bowers et al., 1986; Engelhard et al., 1990; Metz et al., 1992; Zheng and Herzfeld, 1992; Tuzi et al., 1996), as well as the chromophore conformation and orientation in the initial and the M-states (Smith et al., 1989; Creuzet et al., 1991; Zheng and Herzfeld, 1992; Farrar et al., 1993; Ulrich et al., 1994,1995; Sakurai et al., 1996).

A small number of 1D solution-state NMR studies on BR in SDS micelles have been carried out with protein containing a selectively ^{13}C-labelled chromophore (Yamaguchi et al., 1981). The differential mobilities of aromatic residues (Mayo et al., 1988) and methionine side chains (Seigneuret et al., 1991a,b) were also analysed. In previous 2D NMR investigations of separated BR fragments in SDS micelles (Lomize et al., 1992) or of the entire polypeptide in organic solvents (Orekhov et al., 1992), the lack of native tertiary protein structure precluded the description of the active site around retinal.

In the 2D NMR study presented here, we focus on the assignment and analysis of the retinal moiety in the intact and functional protein (as monitored by UV/vis spectroscopy), which shows nearly no degradation over weeks if kept in the dark. Labelling concepts are explored to help overcome the intrinsic problems of line broadening and assignment difficulties in this exceptionally large system (Wagner, 1993). In general, there are two different solution-state NMR approaches: one applies extensive ^{13}C and ^{15}N labelling together with heteronuclear 2D, 3D and 4D NMR spectroscopy (Kay et al., 1990; Ikura et al., 1992; Oschkinat et al., 1994), while the other is based on a uniform deuteration of the protein and selective protonation of individual units in the system (Brodin et al., 1989; LeMaster, 1994). Our studies were designed to allow an assessment of the potential of both concepts for solubilized BR. We have thus established methods for the preparation of uniformly labelled ^{2}H-, $^{2}H,^{15}N$- and $^{13}C,^{15}N$-bacterioopsins (Bop's), that were reconstituted with ^{1}H-retinal or with specifically labelled ^{13}C-retinal analogues, either biosynthetically (Oesterhelt and Krippahl, 1983) or chemically (Gärtner et al., 1983). This work forms the basis for further assignments and for the collection of distance constraints between retinal and surrounding amino acids, with the final aim of obtaining further insight into the mechanism of proton translocation on a molecular level.

Materials and Methods

Preparation of labelled BR in detergent micelles

Samples with ^{2}H or ^{13}C labels in the chromophore and/or the protein were prepared using a combination of the following techniques:

(1) ^{13}C-labelled retinal was incorporated into BR (or protonated retinal into the uniformly deuterated protein) chemically, or

(2) by using a retinal-negative strain such as JW5;

(3) ^{2}H-labelled protein, possibly containing selected ^{1}H-amino acids, was prepared by growing *H. salinarum* in D_2O on a ^{2}H- or ^{2}H,^{15}N-labelled medium prepared from *Scenedesmus obliquus* algae, and

(4) ^{13}C,^{15}N-labelled BR on a medium from *Chenopodium rubrum* cell cultures.

(i) Preparation of ^{13}C,^{15}N-labelled peptone Cell suspension cultures of *Chenopodium rubrum* were grown photoautotrophically in sealed 50 ml two-tier culture flasks or 1 l Fernbach flasks (Hüsemann and Barz, 1977) under an atmosphere of $^{13}CO_2$ and constant illumination at 20 °C, in a medium described by Murashige and Skoog (1962). KNO_3 and NH_4NO_3 were substituted by the corresponding ^{15}N-labelled salts. After 3–4 weeks, the cells (50 g wet weight/l) were centrifuged and the cell walls were digested with cellulases and pectinases (J. Ashurst et al., in preparation). The residual protoplasts (12.5 g) were lyophilized and repeatedly extracted with acetone (2 × 1000 ml per 400 g wet weight), and hydrolyzed with 4 N HCl in H_2O for 3 days at 70 °C. This material consisted of 50% amino acids and oligopeptides as estimated by NMR spectroscopy.

(ii) Preparation of ^{2}H- or ^{2}H,^{15}N-labelled peptone Cultures of the green alga *Scenedesmus obliquus* were grown in D_2O (with unlabelled CO_2) at 30 °C as described above. After 3–4 weeks, the cells were harvested by centrifugation and lyophilized, followed by consecutive extractions with hexane, acetone, chloroform, and 20% methanol/80% chloroform in a Soxhlet apparatus. The residue was hydrolyzed (1 N DCl in D_2O, 48 h) and the resulting slurry was filtered over a pad of activated charcoal. Evaporation of the solvent and lyophilization yielded a white solid peptone/saccharide mixture (80% w/w from the dry cells), with a typical amino acid content of 45–55%. A degree of ^{2}H and ^{15}N labelling above 99% was obtained as shown by GC-MS analysis of the derivatized amino acids. Table 1 gives a typical amino acid analysis of the extract.

(iii) Preparation of purple membranes from Halobacterium salinarum For the production of ^{13}C,^{15}N-labelled [or ^{2}H,^{15}N-labelled] BR, the following medium was prepared per litre of H_2O [D_2O]: isotopically labelled peptone (4.6 g), NaCl (250 g), $MgSO_4 \cdot 7H_2O$ (20 g) [$MgSO_4$ anhyd (6.8 g)], KCl (2.0 g), KH_2PO_4 (150 mg), $K_2HPO_4 \cdot 3H_2O$ (150 mg), KNO_3 (100 mg), $CaCl_2 \cdot 2H_2O$ (0.7 mg), $CuSO_4 \cdot 5H_2O$ (0.05 mg), $FeCl_2 \cdot 4H_2O$ (2.3 mg), ascorbic acid (0.02 mg), $MnSO_4 \cdot H_2O$ (0.3 mg), $ZnSO_4 \cdot 7H_2O$ (0.4 mg), thiamine (2.0 mg), folic acid (2.0 mg) and biotin (0.2 mg). After the addition of supplementary buffer (Tris-HCl [Tris-DCl], 1.2 g), the solution was filtered over a Buchner funnel and sterilized (filter 0.45 μm). The pH was adjusted to 7.2 [meter reading 6.8 in the case of D_2O]. The cells of a 35 ml culture of the strain S9 [adapted to D_2O] were used as inoculum and grown in the described medium (700 ml medium in 2 l Erlenmeyer flasks) for 24–28 days, at 37 °C, on a rotary shaker at 100 rpm. BR production was monitored in 1 ml samples of the culture broth after centrifugation and resuspension in 1 ml H_2O to which also a crystal of DNase I was added. The absorbance at 570 nm, based on a molar extinction coefficient of 63 000 $M^{-1}\,cm^{-1}$, gave the BR content of the culture.

After centrifugation (14 500 × g, 20 min, 4 °C) the cells were resuspended in 10 ml basal salt solution per litre of cell culture, containing ca. 1 mg DNase I, and dialyzed against water (12 h, 4 °C). The lysate was centrifuged (48 000 × g, 30 min, 4 °C), and the pellet was resuspended and homogenized (Potter, Elvejhem). This procedure was repeated 3 times before the PM was purified on a sucrose gradient (30–60% w/w [40–60% w/w for deuterated samples], 80 000 × g, 10 °C, 14 h). Dialysis against water (6 h, 10 °C) and centrifugation (48 000 × g, 4 °C, 1 h) yielded the labelled PM.

(iv) Chemical reconstitution of BR with ^{13}C-labelled retinal Bleaching of the PM and reconstitution with (10,11-^{13}C)- or (14,15-^{13}C)-retinal was performed according to the procedure of Oesterhelt et al. (1974), adjusted to a preparative scale. BR (1.5–2.0 μmol) was reacted with 0.2 M aqueous hydroxylamine (pH 7.0) for 8 h and washed with water. Reconstitution was achieved with an ethanolic solution of ^{13}C-labelled retinal (1.6–2.2 μmol) (Oesterhelt and Schuhmann, 1974).

TABLE 1

TYPICAL CONTENT OF ^{2}H,^{15}N-LABELLED AMINO ACIDS OF AN EXTRACT FROM *S. OBLIQUUS* AFTER (COLUMN 2) AND BEFORE (COLUMN 3) ACID HYDROLYSIS (6 N HCl, 12 h)

Amino acid	Relative content after hydrolysis (%)	Relative content without hydrolysis (%)
Asx	10.7	10.2
Thr	5.5	5.1
Ser	4.8	4.6
Glx	12.9	12.4
Pro	6.0	5.8
Gly	6.6	6.3
Ala	9.3	8.9
Val	6.1	5.8
Met	2.4	2.2
Ile	4.1	4.0
Leu	3.8	8.7
Tyr	4.2	3.9
Phe	5.8	5.6
His	2.1	2.0
Lys	7.2	6.8
Arg	8.2	7.5
Cys	0.4	0.3

The hydrolyzed extract contained a total of 50.5% amino acids, while, without hydrolysis, 35.1% free amino acids were detected.

(v) In vivo labelling of ^{2}H- or $^{2}H,^{15}N$-labelled PM with protonated retinal Cultures of the retinal-negative strain JW5 (Oesterhelt and Krippahl, 1983) were grown in D_2O as described above. A total of 14 μmol all-*trans*-retinal (Fluka) in isopropanol (1.4 ml) per 700 ml culture was administered in four aliquots every second day, beginning 24 h after the onset of growth (3.–5. days). When the BR production had reached a plateau, the labelled PM (10–12 mg per culture) was isolated and purified as described above.

(vi) Preparation of ^{2}H-labelled PM, containing ^{1}H-tryptophan and/or ^{1}H-threonine Depending on whether labelled PM with ^{2}H- or ^{1}H-retinal was produced, the protocols described at (iii) or (v), respectively, were followed. However, ^{1}H-Trp (0.5 mM) and/or ^{1}H-Thr (5.0 mM) were added to the fermentation medium prior to sterilization.

(vii) Solubilization of PM and preparation of NMR samples PM was solubilized in dodecyl maltopyranoside (DM) or octyl glucopyranoside (OG) according to Schertler et al. (1991): pelleted PM (16–50 mg) was resuspended in H_2O [D_2O for deuterated samples] (2–4 ml) and stirred intensively in a solution of Triton X-100 (1%) in low salt buffer (10 mM NaH_2PO_4 [in D_2O], pH 5.6, 15 ml) for 18 h, at 34 °C. After centrifugation (86 000 × g, 30 min, 15 °C), the supernatant was applied to an anion exchange resin (DEAE-Sephacel). The column was washed with 1% DM or OG in low salt buffer until the eluent was free of absorptions by aromatics (280 nm, ca. 50 ml). BR was eluted with 1% DM (or OG) in 500 mM NaH_2PO_4 [D_2O] (pH 5.6) and concentrated (Centricon x-50, Amicon), then dialyzed against the low salt buffer (10 °C, 12 h) and concentrated to a volume of 1.4 ml. With final OD_{552} values between 23 and 80, the NMR samples typically contained 12–41 mg BR.

NMR experiments

(i) NMR spectroscopy The NOESY experiments were carried out on Bruker AMX600 and DRX750 spectrometers and the 2D HMQC spectrum of the uniformly $^{13}C,^{15}N$-labelled sample was recorded on a Bruker AMX500 spectrometer. A probe head for 8 mm sample tube diameter was used for the measurements on AMX600 and DRX750 except in the case of the spectra shown in Figs. 2 and 4a. The kind of samples measured, the sample conditions, and some NMR parameters are summarized in Table 2. The sample buffer was 20 mM KPi, pH 6.5. The detergent concentrations varied between 2% and 4%, due to enrichment during sample concentration. The spectra were recorded with standard pulse sequences. To avoid problems with quadrature images of the very intense detergent signals, the transmitter frequencies were usually positioned in the range of 9–12 ppm, using spectral widths of 17–28 ppm. The lengths of the proton pulses were around 15 μs, while the carbon pulses were 19–22 μs long. Relaxation delays of 1.5–3 s were employed. Mild presaturation of the water was applied to avoid antidiagonals.

The spectra were calculated with Bruker UXNMR software, using exponential multiplications of −20 Hz and Gaussian broadenings of 0.01 in F2. In F1, a slightly shifted squared cosine function was applied. The calculated spectra had a size of 2K × 2K points in F2/F1, respectively.

(ii) Illumination set-up For the observation of the light-adapted state, the sample needs to be illuminated inside the spectrometer magnet during the NMR measurements. The experimental set-up (Düx, 1993) consists of a mercury lamp equipped with suitable UV (cutoff at 515 nm) and IR filters to obtain an appropriate excitation spectrum centred around the 560 nm absorbance of BR. The excitation of M412 is avoided. The light is passed into a fibre optical quartz cable (3 mm diameter) which is immersed into the NMR sample tube. A light intensity of around 5–10 mW was registered around the tip of the conically shaped light guide. The modification of the light spectrum by the filter characteristics is shown in Fig. 1.

Results

Sample preparation

(i) Medium preparation Halobacteria are sensitive towards a variety of chemical impurities in the medium. It is known, for example, that bile acids lead to a suppression of BR biosynthesis or even cell lysis (Kamekura et al., 1988). Therefore, special attention had to be paid to the removal of lipids and chlorophylls during the preparation of the labelled peptones. In the case of the ^{13}C, ^{15}N-labelled peptone from *Chenopodium rubrum* (Hüsemann and Barz, 1977), simple acetone extractions were sufficient to produce a nontoxic medium.

The deuterated medium was derived from the green alga *Scenedesmus obliquus*, which is known to be cultivatable in D_2O under photoautotrophic conditions to serve as a source of labelled microbial nutrients (Crespi and Katz, 1972; Markley, 1972). In the course of this study, it turned out that considerable attention to toxic components was required. In our hands, the described extraction procedures (Crespi, 1982; Brodin et al., 1989) did not yield sufficiently pure amino acid mixtures for the growth of halobacteria unless the harvested cells were lyophilized intact, followed by repeated extractions with hexane, acetone, chloroform and 20% methanol in chloroform, and hydrolysis in 1 M DCl. With this procedure, typical yields of more than 80% (based on the lyophilized cells) were obtained, which are considerably higher than those described before (Brodin et al., 1989).

(ii) Cultivation of labelled H. salinarum A number of chemically defined medium compositions for the growth of *H. salinarum* have been described in the literature (DasSarma et al., 1995). For our purposes, an adaptation

TABLE 2
SPECTRAL PARAMETERS AND SAMPLE CONDITIONS

Sample	Concentration (OD × ml)	Detergent	Method of retinal incorporation	Spectrum	MHz	Temperature (°C)	Mixing times/coupling evolution time (ms)	Spectral width in F1/F2 (ppm)	No. of scans/experiments
U-(^{13}C,^{15}N)-BR	44	DM		1D ^{13}C	600	40	2.2	221	12K/1
				2D HMQC	500			221/14	32/512
Ret-(14,15-^{13}C)-BR	82	OG	Chemically	HMQC (± hv)	600	22/27	2.2	300/17	128/256
Unlabelled	68	OG	Chemically	NOESY	600	35	5,10,20	21/21	32/643
^{2}H-BR, ^{1}H-Ret	55	DM	Chemically	NOESY	600	50	10,20	24/24	64/300
^{2}H-BR, ^{1}H-Ret	22	^{2}H-OG	In vivo	NOESY	600	23	10	14/14	192/169
^{2}H-BR, ^{1}H-Trp, ^{1}H-Ret	21	DM	In vivo	NOESY	750 (600)	35	7.5,15,30 (20)	17/27 (31/31)	64/1024 (96/780)
^{2}H-BR, ^{1}H-Trp, ^{1}H-Thr	30	DM	In vivo	NOESY	750	35	10,30	27/27	64/800

of the minimal medium of Weber et al. (1982) was found to be most successful. The metabolically active citrate was omitted and Tris-HCl was added to compensate for the loss of buffer capacity. NH_4Cl proved unnecessary for halobacterial growth, a catalytic amount of ascorbic acid was added as antioxidant for Fe^{2+}, and the amount of the essential vitamins thiamine, folic acid and biotin was doubled. The quantity of the algal or plant cell extracts necessary for optimum growth and BR production was found to be 4.6 g/l. When *H. salinarum* strain S9 was grown on ^{13}C,^{15}N-labelled peptone, approximately 40 mg of uniformly ^{13}C,^{15}N-labelled PM was obtained per litre of culture broth.

A stepwise adaptation of the same strain to D_2O (20%, 50%, 100%) was possible in a standard medium based on (protonated) Oxoid peptone (Oesterhelt and Krippahl, 1983). Cultures from the final stage were suspended in a basal salt solution in 100% D_2O and served as an inoculum for the preparation of ^{2}H- or ^{2}H,^{15}N-labelled PM. Approximately 30 mg of uniformly labelled PM was isolated from 1 l of culture broth.

Protonated retinal was incorporated by the addition of a solution of ^{1}H-retinal in isopropanol to cultures of JW5, growing in D_2O on a peptone made from ^{2}H- or ^{2}H,^{15}N-labelled *S. obliquus* cells. When the amount of administered retinal was raised to almost toxic concentrations and the schedule of feeding was carefully optimized, the yield of fully ^{2}H-labelled BR, containing ^{1}H-retinal, could be augmented to about 18 mg per litre of culture broth. ^{1}H-Trp and/or ^{1}H-Thr were efficiently incorporated upon supplementation of the growth medium with excessive amounts of the unlabelled amino acids.

(iii) Sample preparation and stability The stability of the detergent-solubilized NMR samples, as monitored by UV/vis spectroscopy, was found to vary considerably with the method of preparation. While most DM samples remained intact for several weeks even using temperatures of 50 °C for the NMR measurements, OG gave less reproducible results. Several samples with chemically exchanged chromophore deteriorated already within 12–24 h, presumably due to minute impurities entrained by the chromophore exchange procedure. The biosynthetic incorporation of ^{1}H-retinal and the solubilization in DM were therefore preferred.

NMR measurements

The feasibility of structural studies on large proteins generally depends on the possibility of assigning the ^{1}H resonances and on the measurement of a sufficient number of NOEs (Wüthrich, 1986). Both tasks are usually

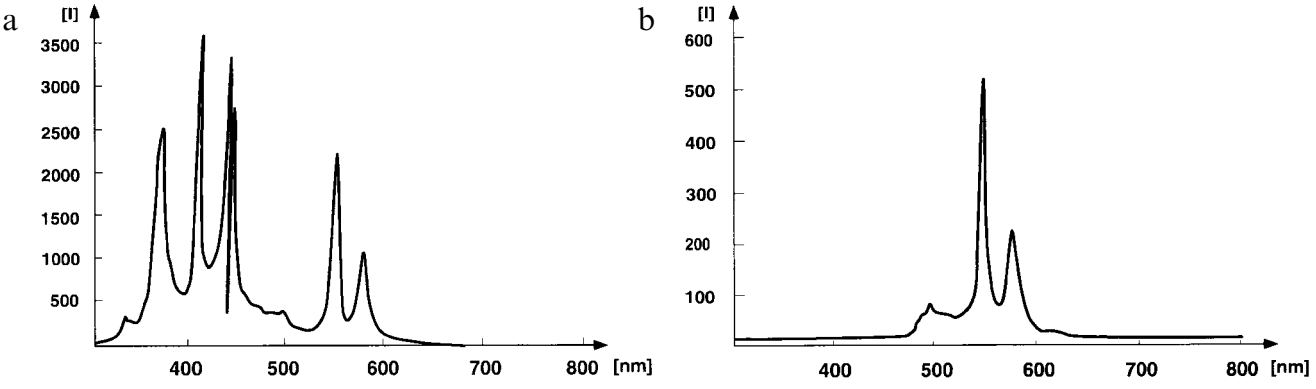

Fig. 1. (a) Light spectrum of the mercury lamp. (b) The same spectrum after filtering and passing through the fibre optic cable as experienced by the sample. The intensity [I] is given in arbitrary units. The same scaling, however, is used for both scales.

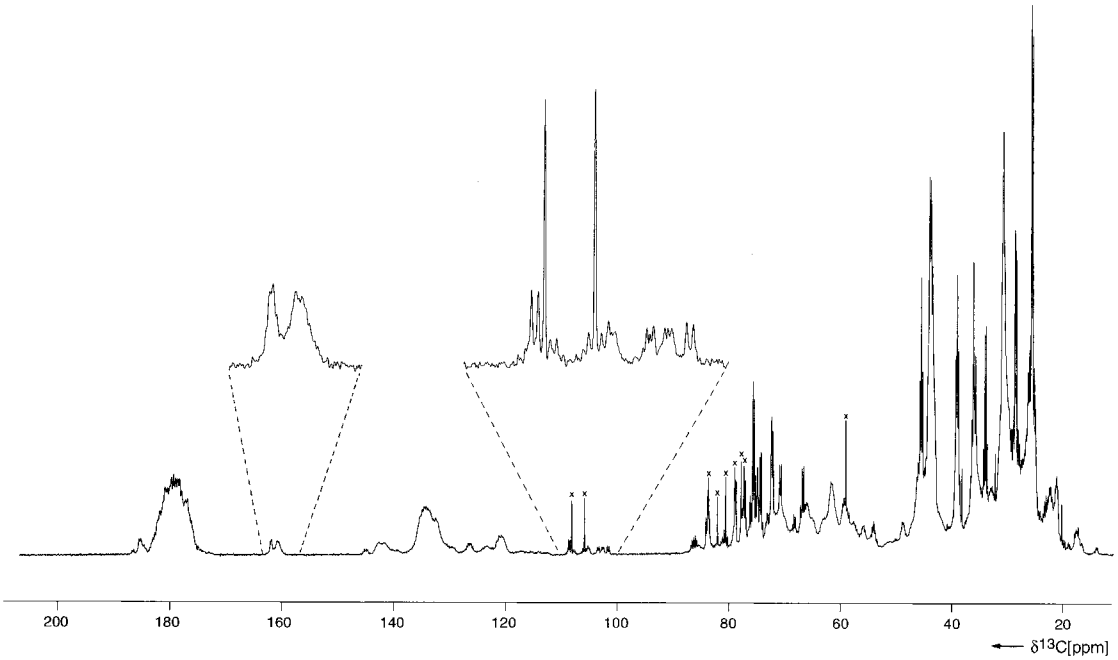

Fig. 2. 1D ^{13}C NMR spectrum of uniformly ^{13}C,^{15}N-labelled BR in DM micelles at 40 °C. The regions with the signals of the protonated Schiff base carbons (around 160 ppm) and with the signals of the anomeric carbons of sugar moieties (100–110 ppm) are shown above the spectrum at larger scale. Note the different line widths of sugar and protein signals. The signals marked with an x are due to DM.

accomplished by ^{15}N and ^{13}C chemical shifts. For protein–detergent micelles of the size expected for BR in OG or DM (50–100 kDa), however, only NMR techniques with few free precession periods can be applied considering the short transverse relaxation times (Wagner, 1993). The NOESY technique is known to work for larger molecules, and the basic heteronuclear experiments, such as the HMQC or HSQC techniques, are also sufficiently sensitive. The latter heteronuclear techniques may then be used to assign individual resonances of uniformly or selectively ^{13}C-labelled samples. As a first demonstration of detailed structural studies on solubilized membrane proteins, and of BR in particular, we describe the assignments of retinal protons in BR using ^{13}C HMQC spectroscopy and of NOEs between a protonated chromophore and protonated amino acids and otherwise uniformly deuterated protein.

(i) ^{13}C NMR measurements of labelled BR The 1D ^{13}C NMR spectrum of uniformly ^{13}C,^{15}N-labelled BR recorded in the dark shows the usual protein resonances (see Fig. 2) and, as a special feature, the C15 and C13 signals of the retinal bound to the protein at around 160 ppm (Smith et al., 1989). The chemical shift of the C15 signals is characteristic of a protonated Schiff base linkage. The signal of the aldehyde carbon of free retinal occurs at around 190 ppm (Pattaroni and Lauterwein, 1981). Apart from resonances directly belonging to BR, strong and narrow singlets in the range of 40–70 ppm and around 105–110 ppm occur due to the mobile DM molecules. Interestingly, between 100 and 105 ppm there are a number of small, relatively sharp doublets which show ^{1}J carbon–carbon couplings. Because of their doublet nature, these signals must be caused by components from the ^{13}C-labelled cells. Their chemical shift values indicate that they are due to the anomeric carbons of sugar moieties.

Further analysis of the constituents in the sample is achieved by ^{13}C-^{1}H HMQC spectroscopy. An overview plot of the HMQC spectrum of uniformly ^{13}C,^{15}N-labelled BR solubilized in DM micelles (Fig. 3) shows unresolved peaks of the aromatic amino acids (around 7 ppm proton chemical shifts), much sharper and intense signals in the area around 60–85 ppm/3.0–4.5 ppm in the carbon/proton dimensions, respectively, and signals due to aliphatic amino acid side chains in the upper right quadrant. The sharp signals in the centre ($\delta^{13}C = 60$–85 ppm) and around 100 ppm confirm the hypothesis of the presence of sugar moieties, presumably of glycolipids that have not been replaced by the detergent.

The part of the HMQC spectrum deserving special attention is the region around 160 ppm carbon chemical shift and 7–9 ppm proton chemical shift, where the signals of the C15-H15 group of the retinal occur. Two signals are expected due to the two forms present in dark-adapted samples. In Fig. 3 they are not visible at the chosen plot level. To assign them unambiguously through their response to light, a selectively labelled sample was prepared with ^{13}C in the C14 and C15 positions of the retinal chain, using the chemical replacement method. HMQC spectra of this BR preparation in OG micelles

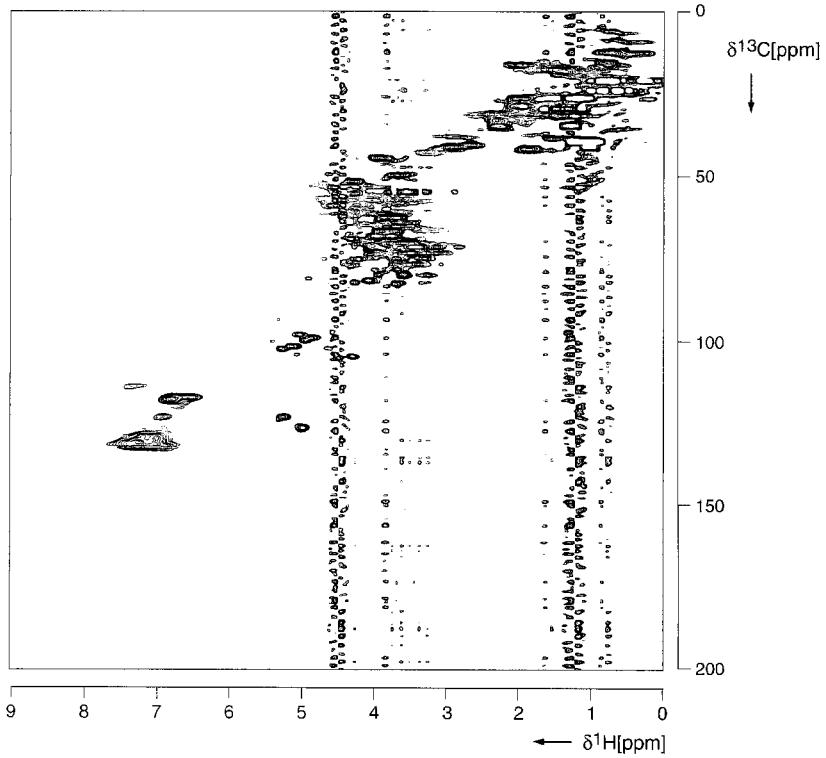

Fig. 3. 2D $^{1}H,^{13}C$ HMQC spectrum of BR in DM micelles at 40 °C. Apart from the usual features of a protein 2D $^{1}H,^{13}C$ HMQC spectrum, various signals arising from sugar moieties of archebacterial lipids tightly bound to the protein occur in the range of 60–80 ppm/3.8–5.0 ppm and around 100 ppm/5.0 ppm carbon/proton chemical shifts, respectively. Their amplitudes are considerably stronger than those of most of the protein signals due to the inherent flexibility of the sugar head groups.

were acquired both in the dark and with continuous illumination. The relevant spectral region is shown in Fig. 4 as a dark–light–dark–dark series. The two expected cross peaks are observed at 163 ppm/8.7 ppm and at 160 ppm/7.2 ppm in the carbon/proton dimensions, respectively. One of these peaks should result from the all-*trans*,15-*anti*-retinylidene protonated Schiff base, and the other from the 13-*cis*,15-*syn* form (Smith et al., 1989). The spectrum in Fig. 4a was measured in the dark at a temperature of 22 °C, using a 5 mm sample tube and a volume of 0.6 ml, containing 22 mg BR. To measure the light-adapted protein, the sample was increased in volume to 1.2 ml and transferred into an 8 mm NMR tube to accommodate the light guide. A spectrum using the same spectral parameters was acquired under continuous illumination and is shown in Fig. 4b. Compared to the dark-

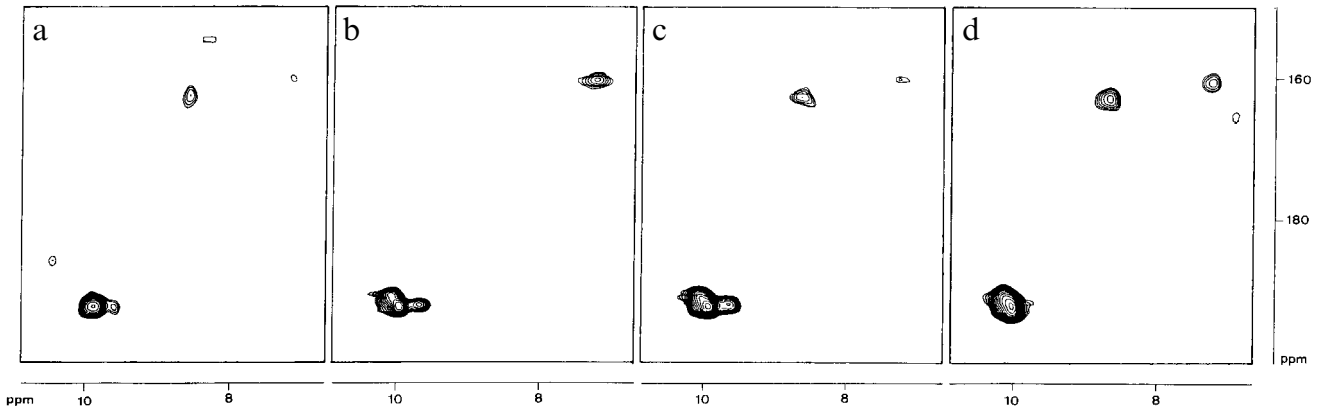

Fig. 4. Regions of 2D HMQC spectra of BR in OG micelles containing selectively (14,15-^{13}C)-labelled retinal, showing the signals due to the C15-H15 fragment. The four spectra were recorded (a) in the dark, (b) with light, (c) in the dark, all at 22 °C, and (d) again in the dark but at 27 °C. The peak which vanishes when the sample is illuminated (b) was assigned to the 13-*cis*,15-*syn* form of BR; hence, the peak which is left in panel b at 160 ppm carbon chemical shift is assigned to the all-*trans* form. The intensities of the two signals around 160 ppm in panel d suggest a 1:2 distribution of the all-*trans* and 13-*cis*,15-*syn* forms, respectively.

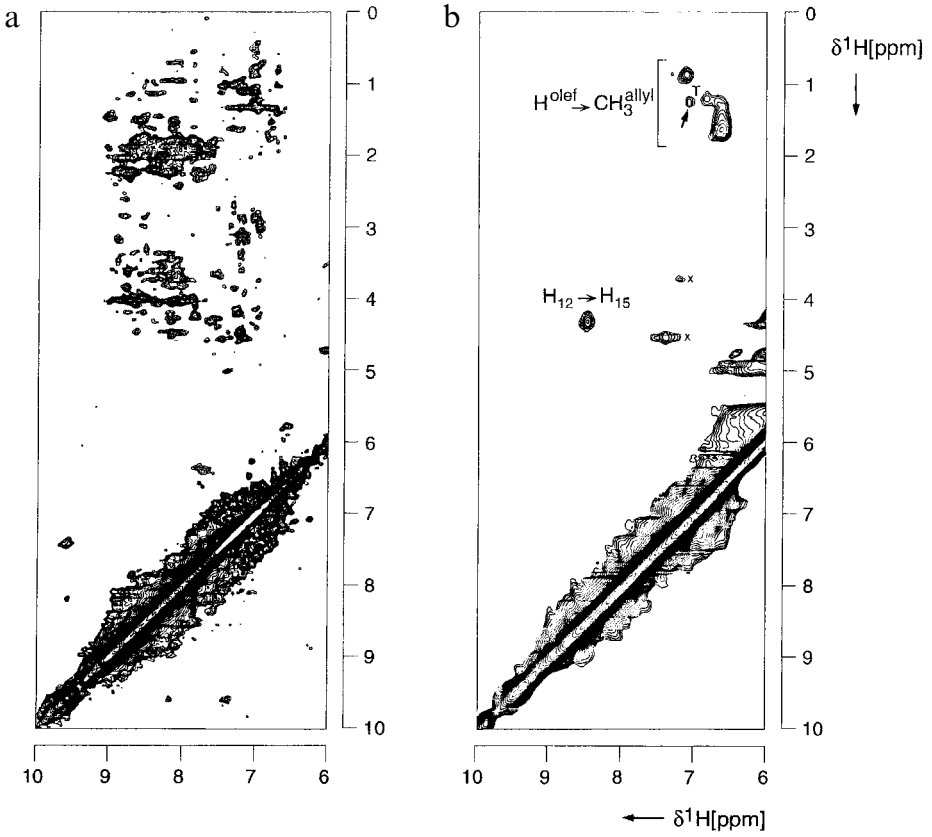

Fig. 5. (a) Amide region (6–10 ppm in F2; 0–10 ppm in F1) of the NOESY spectrum of unlabelled BR in detergent solution, using D_2O as a solvent. The spectrum is dominated by cross peaks involving aromatic protons in the F2 area between 6.0 and 7.8 ppm and aliphatic protons of hydrophobic residues in F1; and by cross peaks between nonexchanged amide protons (7.4–9.0 ppm in F2) and aliphatic protons, primarily showing NOEs to their own α-protons and to side-chain resonances of hydrophilic residues. (b) NOESY spectrum of deuterated BR containing protonated retinal. The resonances indicated by an x are baseline distortions due to the proximity of the detergent diagonal signals. The residual cross peaks are due to NOEs within the retinal moiety. The signal indicated by an arrow is due to residual Triton X-100 which was used for solubilization.

adapted state, the intensity of the signal at 8.7 ppm (in F2) decreases considerably, and the signal at 7.2 ppm becomes much stronger. Hence, the latter peak can be assigned to the all-*trans*,15-*anti* form of retinal in light-adapted BR. Reversibility of light adaptation was shown by repeating exactly the same measurement in the dark, 2 h after the light had been switched off (Fig. 4c). Again, a stronger signal from the 13-*cis*,15-*syn* form (8.7 ppm) than from all-*trans*-retinal (7.2 ppm) is seen. To exclude temperature effects, the same measurement was repeated in the dark at 27 °C instead of 22 °C (Fig. 4d). In this case, the overall intensities of both peaks increase but their ratio remains the same. The intensity increase is a result of the expected temperature-dependent decrease in the viscosity of the solution, which should result in shorter proton correlation times and hence longer transverse relaxation times.

The intense signals seen in the lower left corner of all four plots around 190 ppm carbon chemical shifts are due to C15 carbons of free retinal isomers which occur in small amounts in the sample when it is illuminated. Since the free retinal is more mobile than when bound to the protein, it shows longer transverse correlation times and thus causes a relatively strong signal. The free retinal concentration was monitored by recording UV spectra before and after each experiment. At the beginning, approximately 4% of the total content was free retinal, which increased to 8% at the end of the NMR measurements 4 days later.

The HMQC spectra of the same sample also contain the signals from the ^{13}C label in position C14. Two peaks were identified in the spectra of a dark-adapted sample at 122 and 129 ppm carbon chemical shift and around 6 ppm proton chemical shift (not shown). Since C14 of free all-*trans*-retinal is known to give a signal at around 129 ppm (Pattaroni and Lauterwein, 1981), the signal at 122 ppm was assigned to the 13-*cis*,15-*syn* isomer of the chromophore. This value is in good agreement with the chemical shift data from solid-state MAS and a previous solution-state study (Yamaguchi et al., 1981; Smith et al., 1989). A sample labelled in the C10 and C11 positions was used to assign H10 of the 13-*cis*,15-*syn* isomer on the basis of a cross peak observed at 133 ppm carbon and 6.9 ppm proton chemical shifts in an HMQC spectrum in the dark. Other signals arising from C10 and C11 were not identified on the basis of this spectrum.

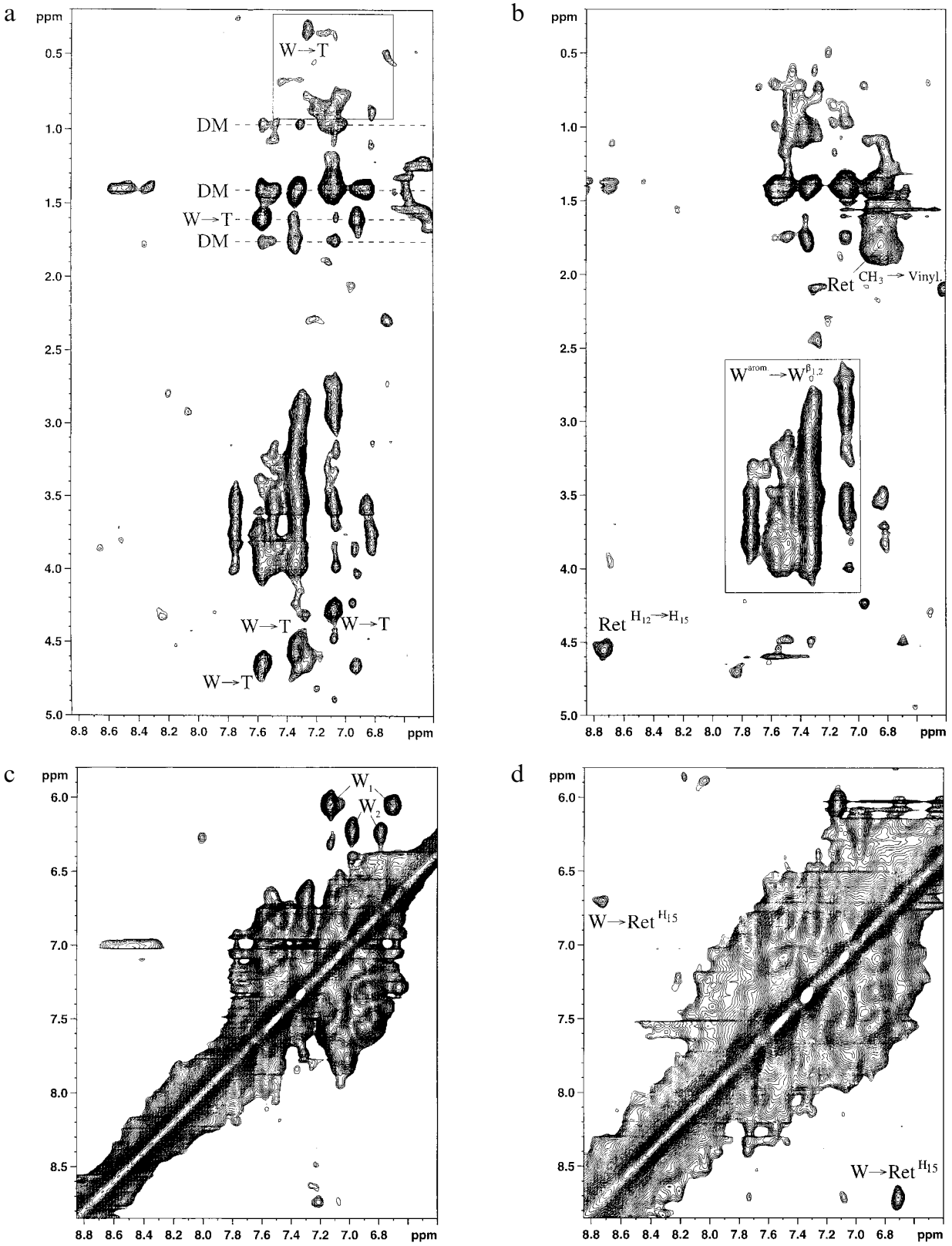

Fig. 6. Amide regions of the NOESY spectra of samples of deuterated BR containing either protonated tryptophan and threonine (Figs. 6a and c) or protonated tryptophan and retinal (Figs. 6b and d). Cross peaks occurring in both spectra should be due to intraresidue NOEs of the tryptophans, cross peaks occurring in Figs. 6a and c only should be due to intraresidue NOEs within the threonines or due to NOEs between tryptophan and threonine, and cross peaks occurring in Figs. 6b and d only are due to intra-retinal interactions or due to NOEs between tryptophan and the retinal moiety. W stands for tryptophan, T for threonine, Ret for retinal, and DM indicates cross peaks involving dodecyl maltoside. The signal-to-noise ratio of the spectrum shown in Figs. 6b and d is approximately twofold higher compared to the other one.

(ii) NOESY spectroscopy of BR NOESY spectra of large molecular systems such as BR should yield, at shorter mixing times, cross peaks of comparable intensity as small proteins, not considering line width effects (Wagner, 1993). To analyse more specifically NOEs from the interior of the protein, samples containing (i) BR with deuterated amino acids and protonated retinal, (ii) ^{2}H-BR with ^{1}H-Trp and ^{1}H-Ret, and (iii) ^{2}H-BR with ^{1}H-Trp and ^{1}H-Thr were prepared.

A first sample with only protonated retinal was prepared by chemical exchange of the retinal and solubilization in DM micelles, and a second one by in vivo labelling and solubilization in OG. The in vivo approach turned out to be necessary because an apparent instability of NMR samples prepared by chemical reconstitution had been observed. For comparison, a sample of unlabelled BR in D_2O solution using OG as detergent was also prepared. A region of the NOESY spectrum of the latter sample containing plain, protonated BR recorded with 10 ms mixing time is shown in Fig. 5a. It contains clusters of cross peaks between aromatic protons and signals from aliphatic protons, especially in the region between 6 and 8 ppm in F2 and 1 and 5 ppm in F1. Some of the amide protons may not have been exchanged for deuterons in this sample, leading to resonances further downfield (8–10 ppm).

The same region of the NOESY spectrum of the deuterated ^{1}H-retinal sample is shown in Fig. 5b, documenting the dramatic simplification achieved by uniform deuteration and selective protonation. The remaining signals in the range between 1.0 and 2.0 ppm in F1, and between 6.5 and 7.5 ppm in F2 are mostly due to NOEs between allylic methyl groups and olefinic protons of the retinal. A pronounced cross peak is observed at the chemical shifts of 4.4 ppm/8.7 ppm in F1/F2 . The F2 coordinate corresponds to the chemical shift of the proton on C15 in the 13-*cis*,15-*syn* form of dark-adapted BR (see Fig. 4). This cross peak must therefore be due to an NOE between the protons at C15 and C12, which are spatially very close to one another in the 13-*cis* geometry. Hence, a chemical shift of 4.4 ppm is assigned to the proton at C12.

Since the methyl signals on the one hand and the olefinic signals on the other show relatively similar chemical shifts, a complete assignment of the retinal signals on the basis of this spectrum alone is difficult. A similar spectrum was recorded using a more dilute sample containing OG micelles (data not shown) and the same cross peak pattern was obtained. A comparison of the two spectra shows that the signals at 1.2 and 1.6 ppm in F1 and 7.0 ppm in F2 are due to residual Triton X-100 left in the sample after detergent exchange.

The NOESY spectrum from the ^{1}H-Trp/^{1}H-Thr sample (Figs. 6a and c) demonstrates, when compared to the spectrum from the ^{1}H-Trp/^{1}H-Ret sample (Figs. 6b and d), the presence of NOEs between tryptophan and threonine. They are most apparent in the region between 0.2 and 1.0 ppm/6.8 and 7.6 ppm in F1/F2, respectively. In fact, the 1D spectrum of this sample shows four nicely separated threonine methyl groups in this aliphatic region. The corresponding region of the 2D NOESY then shows the full spin system of these amino acids.

The spectrum shown in Fig. 6a exhibits also three relatively strong yet unassigned cross peaks between threonine H^{α}/H^{β} signals and tryptophan aromatics, as indicated in the area between 4 and 5 ppm in F1. A comparison of the two spectra in Figs. 6a and b shows also the presence of cross peaks between detergent and the aromatic protons of the tryptophan, as indicated in Fig. 6a. The aromatic region of this spectrum shows two tryptophan patterns with distinct chemical shifts (W_1 and W_2, Fig. 6c).

The spectrum of the ^{1}H-Trp/^{1}H-Ret sample (Figs. 6b and d) confirms the position of the retinal signals (see also Fig. 5b) and shows, apart from intra-tryptophan signals, a clearly identifiable cross peak between the retinal H15 signal of the dark-adapted form and one of the tryptophans (Fig. 6d). Weaker signals, supposedly due to other protons of this tryptophan spin system, are visible at 8.73 ppm F1 chemical shift. Some weaker cross peaks which are probably due to tryptophan–retinal NOEs are also present in Fig. 6a at various F1 positions and at 8.73 ppm in F2.

Discussion and Conclusions

NMR investigations of integral membrane proteins in a solubilized form by solution-state NMR seem to be a formidable task, but it is demonstrated here that the basic requirements for accomplishing it can be fulfilled. In the case of BR, it has been shown that ^{13}C labelling may be applied to assign individual proton resonances and to resolve at least the 1D proton NMR spectrum by means of heteronuclear chemical shifts. Furthermore, samples which employ selective protonation on a deuterated background may be used to measure individual NOEs, as shown with the examples of NOESY spectra obtained on the samples containing ^{1}H-retinal only or ^{1}H-Ret and ^{1}H-Trp or ^{1}H-Trp and ^{1}H-Thr in an otherwise deuterated protein. These NOEs may serve as a basis for assignments in further partially protonated samples or as restraints in structure calculations.

The availability of structural information from the interior of the protein depends strongly on the possibility to measure NOEs which are still to some extent proportional to the distances. To check on this a series of NOESY spectra with different mixing times were recorded on the ^{1}H-Trp/^{1}H-Ret sample (7.5, 15.0 and 30.0 ms) and on the ^{1}H-Trp/^{1}H-Thr sample (10 and 30 ms). In the first set of spectra, the build-up of the two cross peaks involving H15 of the dark-adapted form (Figs. 6b and d) proceeds

TABLE 3
APPARENT PROTON LINE WIDTHS OBSERVED IN ^{13}C-LABELLED SAMPLES OR DEUTERATED SAMPLES (SEE ALSO TEXT)

Sample	In OG		In DM	
	22 °C	27 °C	40 °C	50 °C
(14,15-^{13}C)-retinal in BR	200, 250[a]	160, 160		
U-(^{13}C,^{15}N)-BR			200, 160	180, 130
U-(^{2}H,^{15}N)-BR, ^{1}H-retinal			75, –	75, –

The first value in each column shows the line width of the signal of the 13-*cis*,15-*syn* form and the second value shows the signal of the all-*trans* form in Hz (in analogy to Fig. 4).
[a] With illumination.

within 10 ms. The cross peaks within the isolated tryptophan signal sets (W_1 and W_2, Fig. 6c) and the intra-residue ones of those threonines whose methyl groups are shifted upfield show considerable proportionality up to 30 ms. Using appropriate choices of protonated/deuterated amino acids and prosthetic group, it is therefore expected that a large number of distances in the environment of the retinal can be determined from NOE data.

With regard to the practicability of the individual labelling concepts, the proton line widths observed in the spectra of either the carbon-labelled samples or the deuterated samples deserve some closer inspection. In Table 3, the 'apparent multiplet widths' at half height are displayed for the signals of the protons at C15 as observed in the 2D HMQC or 2D NOESY spectra. To improve the signal-to-noise ratio, an exponential multiplication of 20 Hz was applied which, however, did not result in a noticeable line broadening at half height. In the case of the carbon-labelled samples, the width of the multiplet contains proton–proton couplings, while all proton–carbon couplings were removed by decoupling. The deuterated sample used for the NOESY spectrum also contained ^{15}N nuclei; therefore, a $^{2}J_{NH}$ contributes to the apparent line width. Nevertheless, the line width from the deuterated sample (75 Hz) is much narrower than that from the sample containing ^{13}C (130–180 Hz). The increased proton line width in the latter sample can be primarily attributed to the large dipolar interactions introduced with the ^{13}C spin.

The effect of the kind of detergent (DM or OG) on the core structure of BR is very small, as only very minor chemical shift changes are observed in the spectra of samples with different detergents and no changes in the NOE pattern appeared. The DM solutions, however, appeared to be more stable. At the concentrations necessary for NMR measurements, relatively viscous solutions are obtained containing 2–4% of either detergent. As the line width observed reflects also the viscosity, it is necessary to measure at temperatures as high as possible. Using OG, a reasonable line width can already be obtained at 295 K, and temperatures up to 308 K are possible without causing a fast deterioration of the sample. More elevated temperatures (313–318 K) are required for measurements with DM to achieve a similar line width. However, the samples are still more stable when containing this detergent and when measured at this temperature than those containing OG at lower temperatures.

The apparent population of the two forms present in dark-adapted BR as observed as signal intensities in the HMQC spectra (Figs. 4a, c and d) is very close to a ratio of 2:1. Although different transverse relaxation times of the respective protons might to some degree obscure the signal intensities, the similarity of the observed line widths suggests an uneven population of the two forms.

In summary, if suitably labelled samples are available, NMR spectroscopic investigations on solubilized BR may yield important structural information such as chemical shifts and distance constraints derived from NOEs. The ^{1}H and ^{13}C chemical shifts of the protonated Schiff base carbon in the binding pocket of the native protein were partially assigned, and the population of different forms, as occurring in the dark- and light-adapted states, could be observed. Furthermore, it proved possible to measure an NOE which indicated the presence of the *cis* orientation of the C13/C14 double bond in the dark-adapted state and an NOE between the H15 signal of this form and one of the two tryptophans in the environment. Hence, it is conceivable to obtain distances around sites of interest in order to determine local structures. However, the possibility to determine a full structure of a membrane protein in solubilized form depends crucially on the ease of labelled sample preparation and sample stability.

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