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ARTICLE

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Two processes lead to a stable all-*trans* and 13-*cis* isomer equilibrium in dark-adapted bacteriorhodopsin; effect of high pressure on bacteriorhodopsin, bacteriorhodopsin mutant D96N and fluoro-bacteriorhodopsin analogues

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Abstract The combination of absorption spectroscopy and extraction techniques was applied to study the effect of high pressure on the dark-adapted state of bacteriorhodopsin, 14-(12-, 10-)fluoro-bacteriorhodopsin, a D96N bacteriorhodopsin mutant, and 14-(12-,10-)fluoro-D96N. Evidence is presented that, at high pressure, the isomers' equilibrium is shifted from all-*trans* isomers towards the 13-*cis* isomers. Two groups of values for calculated molar volume changes indicate that there are at least two different processes leading to a stable all-*trans* and 13-*cis* isomers' equilibrium called the dark-adapted bacteriorhodopsin. The first process may be attributed to changes in the distances and rearrangement of functionally important residues and a retinal Schiff base. It is suggested that the moved residues (probably Asp-212 with the contribution of Tyr-185 and/or Asp-85) closer to the chromophore could catalyse its *trans-cis* isomerization. These changes require smaller pressure changes and induce larger volume changes (large-volume-change process). The second process may be attributed to the formation of the three hydrogen bonds that additionally decrease the volume and strengthen further stabilization of the 13-*cis* isomer. To induce these changes, larger changes of pressure are required and the final molar volume changes are smaller (small-volume-change process). The total molar volume change between all-*trans* bacteriorhodopsin and 13-*cis* bacteriorhodopsin in the dark-adapted state of native bacteriorhodopsin was found to be about –28 mL/mol, which is much higher than the value of about –7 mL/mol obtained previously (Tsuda and Ebrey 1980, Schulte and Bradley 1995). The data provide a novel insight into factors leading to stable isomer equilibrium in dark-adapted bacteriorhodopsin.

Keywords Bacteriorhodopsin analogues · Dark-adapted bacteriorhodopsin · Molar volume changes · High pressure

Abbreviations *bO*: bacterioopsin · *bR*: bacteriorhodopsin · bR^{DA}: dark-adapted bacteriorhodopsin · bR^{LA}: light-adapted bacteriorhodopsin · *14-(12-,10-)F-bR*: bacteriorhodopsin with retinal fluorinated at position 14(12, 10) · *14-(12-,10-)F-D96N*: bacteriorhodopsin mutant with retinal fluorinated at position 14(12, 10) · *PM*: purple membrane

Introduction

Bacteriorhodopsin (bR) is a retinal protein found in the purple membrane (PM) of the bacterium *Halobacterium salinarum*. bR exists in two distinct forms: light-adapted and dark-adapted. Light-adapted bR (bR^{LA}) is the starting state for the physiological activity, the light-driven proton pump. It contains an all-*trans* chromophore and has an absorption spectrum with $\lambda_{max} = 568$ nm. If left in the dark for prolonged periods of time, light-adapted bR slowly converts to a less active form, dark-adapted bR (bR^{DA}). bR^{DA} is a mixture of all-*trans* and 13-*cis* ($\lambda_{max} = 548$ nm) isomers ($bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$) with a ratio close to 1 and a resultant absorption spectrum with $\lambda_{max} = 558$ nm. This contrasts with the behaviour of retinal in solution in which a *trans* configuration is more stable (Sheves and Baasov 1984). Obviously, the retinal binding site of bR stabilizes the 13-*cis* geometry of $bR^{13\text{-}cis}$.

The spectral (NMR, vibrational, electronic) differences between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ have been mainly ascribed to a twist of the C_{14}-C_{15} bond in the 13-*cis* isomer (Smith et al. 1987, 1989; Noguchi et al. 1990) and changes the vibrational degree of freedom of retinal (Ujj et al. 1994). However, these suggestions were not

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confirmed by other studies (Logunov and Schulten 1996; Helmle et al. 2000). A stronger steric interaction between C_{14}-H and C_{ϵ}-H_2 of Lys-216 in $bR^{13\text{-}cis}$ has also been postulated (Albeck et al. 1992). The changes may not only involve the nearest neighbours of the chromophore but also extend over the whole binding site by changing, for example, the hydrogen-bonding network (directly or through water molecules) (Logunov et al. 1995).

It was suggested that differences between $bR^{\text{all-}trans}$ and $bR^{13\text{-}cis}$ are the result of stronger interaction between the Schiff base and the associated counterions (Smith et al. 1989; Humphrey et al. 1994). For example, the steric relaxation which positions the carboxylate moieties of Asp-85 and Asp-212 closer to the Schiff base may be the main factor for forming optimal conditions for catalyzing *trans-cis* retinal isomerization. It may be done directly, because aspartate-212's carboxylate group can catalyze *cis-trans* isomerization of a positively charged retinal Schiff base (Seltzer 1992) or indirectly via the change of ionization state of Asp-85 (Balashov et al. 1993, 1996). However, spectroscopic studies (Becher and Cassim 1976; Cassim 1992 and literature therein) have not found any clear difference between bacterioopsin in $bR^{\text{all-}trans}$ and $bR^{13\text{-}cis}$. All mentioned difficulties can be related to the fact that dark adaptation may involve very subtle effects, structural changes within less than 1 Å, like a 0.3 Å distance change between the C_{14} position of the retinal and [*indole*-^{15}N]Trp86 (Helmle et al. 2000).

It occurred to us that two approaches might be useful in studies of subtle effects connected with dark-adapted bacteriorhodopsin: (1) a molar volume change as a diagnostic tool for changes and (2) chemical and/or genetic modifications as a method for amplification of the changes which take place in the native system.

The movement of as little as one or two residues of a protein during conformational changes, ionization changes and creation of hydrogen bonds can be expected to result in a measurable molar volume change, ΔV (Gross and Jaenicke 1994). The magnitude of the molar volume changes can be determined indirectly through the effect of pressure on the rate constants of the reaction or equilibrium constants.

Pressure dependencies have been determined for light-dark adaptation of bR (Kovacs et al. 1993; Schulte and Bradley 1995) and isomeric composition of the dark-adapted state (Tsuda and Ebrey 1980; Schulte and Bradley 1995; Schulte et al. 1995). We had expected that a closer examination of the pressure dependencies of the dark-adapted state of bR would reveal a significant molar volume change between all-*trans* and 13-*cis* that corresponds to the postulated steric rearrangement in bR. This was indeed observed, as we report here.

It is generally believed that the ratio of 13-*cis* to the all-*trans* isomer in bR^{DA} equals about one (Sperling et al. 1977; Fahr and Bamberg 1982; Dencher et al. 1983; Varo and Bryl 1988), although values up to 2:1 have been postulated (Scherrer et al. 1989). This ratio is altered in bR mutants (Soppa et al. 1989) and in bR analogues (Steinberg at al. 1991). Therefore, experiments with bR mutants and modified retinal chromophores have been proven to be powerful tools for obtaining information on $bR^{\text{all-}trans}$ and $bR^{13\text{-}cis}$ (Crouch 1986; literature cited in Logunov et al. 1995).

The incorporation of retinal analogues with a changed shape or altered electronic properties into the binding site of bR has been used to strengthen (or to diminish) the influence of the surrounding protein (Kollbach et al. 1998). The variations of the substituents at positions 13 and/or 14 have a major impact on the dark-adapted bacteriorhodopsin, which on changing them into fluoro groups, for example, change the electronic properties in close proximity to the Schiff base (Kollbach et al. 1998), steric interactions (Tierno et al. 1990) and may also influence the rearrangement of functional groups and the hydrogen bond network (Bryl and Yoshihara 2001).

In the present study, the pressure effects on absorption spectra and the isomeric composition of the retinal chromophores of bR, 14-(12-,10-)F-bR, D96N, and 14-(12-,10-)F-D96N in the dark-adapted state were studied. The two groups of values for the molar volume change were obtained for all bacteriorhodopsins studied. However, only the 14-F atom strengthened the effects of high pressure on the native bR. Since the strengthening effect appears to be restricted only to 14-F analogues, the regiospecific interactions with groups in the vicinity of the protonated Schiff base should be responsible for the observed effects. The results will be discussed in terms of steric rearrangement and isomerization changes.

Materials and methods

The synthesis of the halogenated retinal analogues was performed according to previously described methods (Asato et al. 1978; Asato and Liu 1986). The PM suspension of *Halobacterium halobium*, strain S9, and the PM suspension of the mutant D96N (Wacker, Germany) were used.

The regeneration of bacteriorhodopsin with halogenated retinal analogues was performed according to the previously described method (Tierno et al. 1990) with one modification. Instead of white membranes, apomembranes were used. The irradiation conditions and the protocol of verification of the light-adapted state of fluorinated bRs were described earlier (Chang et al. 1991). All samples were buffered at pH 7.2 with 0.01 M imidazole-HCl buffer. The absorbance of the suspension was about 1 OD at 570 nm in a 1.5 mm cuvette.

The absorbance changes (Shimadzu UV-Vis spectrophotometer) at varying pressures were determined in a thermostatted pressure cell (Frauenfelder at al. 1990) with three sapphire windows, two for the entry and exit of the measuring light and the third for the actinic light for light adaptation. High pressure was generated with an air-driven compressor using helium gas as the pressure-transmitting medium. The pressure was monitored with a Bourdon gauge accurate to 4 MPa.

The chromophore extractions were performed according to the previously described method (Tierno et al. 1990) with a slight modification for high-pressure experiments (Tsuda and Ebrey 1980). Briefly, 200 µL of the sample from the pressure cell (cooled to 2 °C) were denaturated by the addition of 4 mL of CH_2Cl_2-CH_3OH with sonication for 30 s at 0 °C and partitioned by the addition of 4 mL of hexane followed by centrifugation at 3000 rpm to enhance the phase separation. The upper organic layer was dried

by filtration through a small column of $MgSO_4$ and evaporated with a gentle stream of nitrogen. The residue was dissolved in 200 µL of CH_2Cl_2. One-fourth of the extract was injected into a Cosmosil Si-60 column, eluted at 6 mL/min with 10% diethyl ether in hexane, and monitored at 360 nm. The peaks were identified and quantitated with proper all-*trans*- and 13-*cis*-retinals for calibration.

Results and discussion

Retinal isomer ratio in dark-adapted bacteriorhodopsin and its analogues

All chromophore extractions were performed as quickly as possible (i.e., not longer than 4 min) in the dark to minimize the isomerization of the retinal, and particularly the 14-fluorinated retinal (Tierno et al. 1990). In the extracts from dark-adapted bR and D96N at 30 °C, and under atmospheric pressure, the results were $47 \pm 1\%$ all-*trans*-retinal and $53 \pm 1\%$ 13-*cis* retinal. The extraction from 14-F-bR and 14-F-D96N yielded $46 \pm 1\%$ all-*trans*-retinal and $54 \pm 1\%$ 13-*cis* retinal. Similar values were obtained for the 12-F and 10-F analogues: 46% all-*trans*-retinal and 54% 13-*cis*-retinal with an accuracy of 2.8%. The all-*trans* isomer content in light-adapted samples was 98–99% in all samples investigated. Obtained values of $bR^{\text{all-}trans}/bR^{13\text{-}cis}$ in the dark-adapted state are close to the previously published ratio of 50/50 (Kalisky et al. 1977; Sperling et al. 1977; Fahr and Bamberg 1982; Trissl and Gartner 1987). However, ratios between 50/50 and 34/66 can be also found in the literature (Scherrer et al. 1989; Helmele et al. 2000).

The data for D96N do not match the isomeric ratio obtained by Soppa et al. (1989) for the same mutant (all-*trans* to 13-*cis*, 54:46 in the DA state and 96:4 in the LA state). The differences might be related to the extraction technique, mostly extraction time, that influences the accuracy of the isomer content determination (Maeda et al. 1977; Pettei et al. 1977).

The dark equilibrium between $bR^{\text{all-}trans}$ and $bR^{13\text{-}cis}$ can be expressed by the equilibrium constant:

$$K = \left[bR^{\text{all}-trans}\right] / \left[bR^{13-cis}\right] \quad (1)$$

The extraction experiments yielded $K = 0.89$ for bR and D96N, and $K = 0.85$ for the fluorinated analogues under atmospheric pressure. Knowing the ratio of the two bacteriorhodopsin isomers in the dark- and light-adapted states, it is possible to calculate the absorption spectrum of $bR^{13\text{-}cis}$ (Sperling et al. 1977; Scherrer et al. 1989). Figure 1 shows examples of the absorption spectra of light- and dark-adapted bacteriorhodopsin and the spectra of the 13-*cis* isomers calculated on the basis of the equilibrium constants from the extraction experiments. The λ_{max} and the mutual positions of the light- and dark-adapted and $bR^{13\text{-}cis}$ spectra for bR and D96N agree with those obtained by others (Sperling et al. 1977; Soppa et al. 1989).

These absorption spectra can be applied to identification of changes induced by high pressure in a dark-adapted state. The absorption spectrum of light-adapted 14-F-bR is shifted about 12 nm toward shorter wavelengths compared with that obtained by Gat et al. (1997). This difference may be caused by the contamination of the spectra of the light-adapted state (obtained by Gat et al. 1997) by long-lived red-shifted species generated in 14-F-bacteriorhodopsin (Tierno et al. 1990). This problem will be described in detail elsewhere.

The effect of high pressure on the absorption spectra of bacteriorhodopsins

Figures 2 and 3 show the spectral changes of dark-adapted 14-F-bR and 14-F-D96N after being pressed to

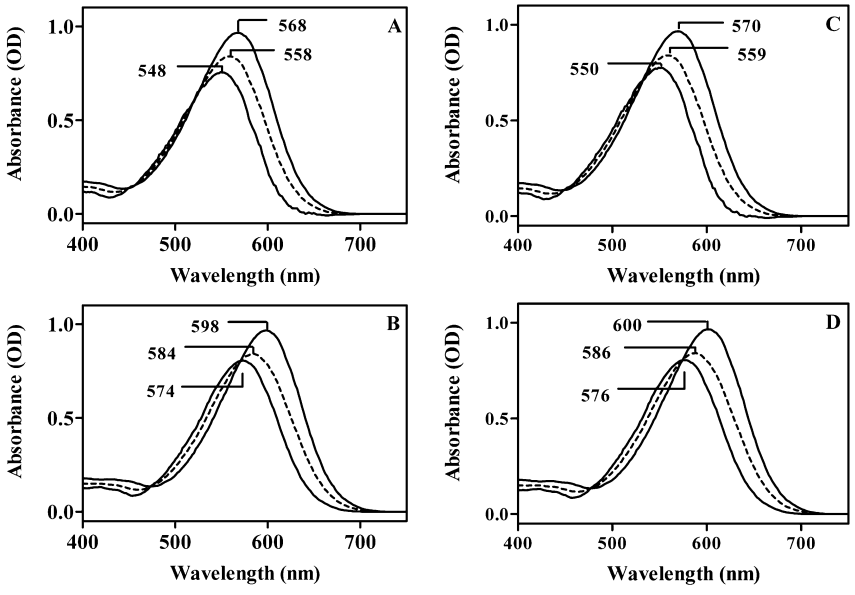

Fig. 1 Absorption spectra of $bR^{\text{all-}trans}$, $bR^{13\text{-}cis}$, and the mixture of both obtained after dark adaptation, measured at 30 °C for bR (**A**), 14-F-bR (**B**), D96N (**C**), and 14-F-D96N (**D**). *Upper* and *lower solid lines* represent absorption spectra of all-*trans* and 13-*cis* isomers, respectively. *Middle broken lines* were measured after dark adaptation. Spectra of $bR^{13\text{-}cis}$ calculated on the basis of $K = 0.89$ for bR and D96N, and $K = 0.85$ for 14-F-bR and 14-F-D96N

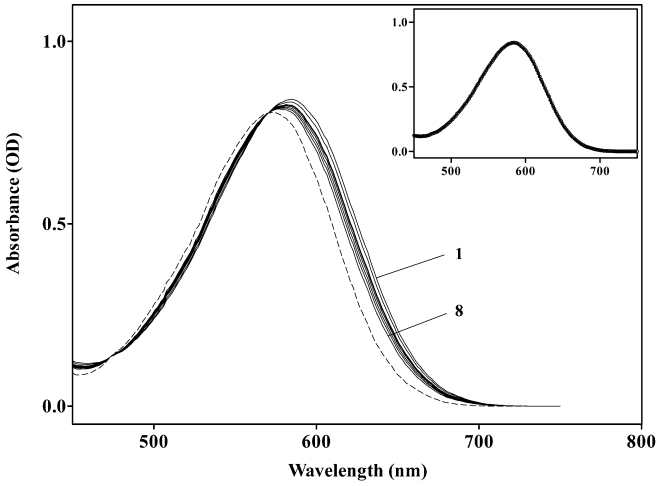

Fig. 2 Spectral changes of dark-adapted 14-F-bR under different pressure. *Curves 1–8* were recorded under 0.1, 25, 50, 75, 100, 150, 200, and 250 MPa pressure, respectively. The *broken line* represents the calculated spectrum of 14-F-bR$^{13\text{-}cis}$. *Inset*: absorption spectra recorded under atmospheric pressure of 0.1 MPa (*solid line*) and recorded after a releasing pressure of 200 MPa (*circles*)

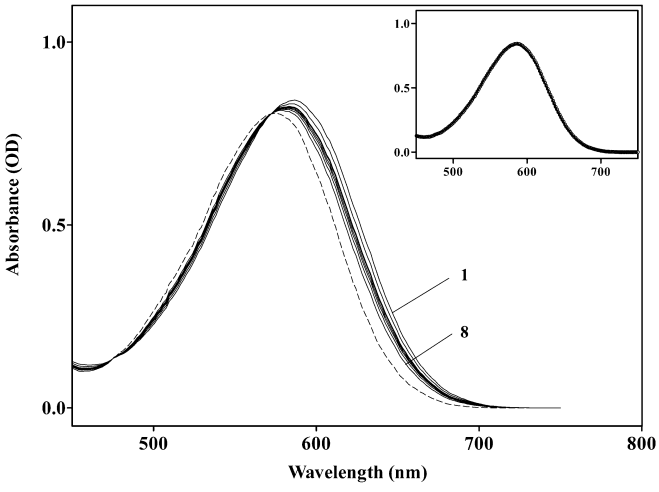

Fig. 3 Spectral changes of dark-adapted 14-F-D96N under different pressure. *Curves 1–8* were recorded under 0.1, 25, 50, 75, 100, 150, 200, and 300 MPa pressure, respectively. The *broken line* represents the calculated spectrum of 14-F-D96N$^{13\text{-}cis}$. *Inset*: absorption spectra recorded under atmospheric pressure of 0.1 MPa (*solid line*) and recorded after a releasing pressure of 200 MPa (*circles*)

different pressures at 30 °C. In these experiments, the sample was first allowed to completely dark-adapt at atmospheric pressure (0.1 MPa) and a constant temperature. Absorption spectra were taken at various pressures, ranging from 0.1 MPa to 300 MPa. Similar experiments were performed with dark-adapted bR and D96N (spectra not shown).

The spectral behaviour of all dark-adapted samples is very similar. The absorption spectrum of a dark-adapted sample, immediately after raising the pressure, increases in intensity and shifts to longer wavelengths. The increase in intensity and red-shift depends on the pressure applied. If the pressure is maintained, the absorption band decreases in intensity and slowly shifts to shorter wavelengths. This change saturates within hours. Figures 2 and 3 demonstrate the spectra after reaching a stable position. It is easily observed that the higher the pressure, the greater the spectral shift. The insets in Figs. 2 and 3 show the spectral changes of pressed bR samples after the pressure is reduced to atmospheric pressure. The absorption bands returned to those of the original spectra. However, this holds for pressures below 250–300 MPa. The effects of high pressure upon the absorption spectra of dark-adapted bRs were always reversible up to this pressure. A pressure higher than 300 MPa could cause irreversible changes in the samples (pressure denaturation), at least in our experiments. Hence, we limit our further interpretations to the pressure-induced reversible effects.

The higher pressure causes the absorption spectra of dark-adapted bRs to be closer to the spectra of pure 13-*cis* isomers (Figs. 1, 2, 3). It means that the increased pressure shifts the equilibrium between the all-*trans* and 13-*cis* isomers in dark-adapted bRs towards the 13-*cis* isomer (Tsuda and Ebrey 1980; Schulte and Bradley 1995). This result suggests that the equilibrium constant, K, is pressure dependent.

The pressure dependence of the 13-*cis* to all-*trans* equilibrium in dark-adapted bRs

The pressure dependence of the equilibrium constant K implies that there is a molar volume change between bR$^{\text{all-}trans}$ and bR$^{13\text{-}cis}$. The effect of pressure P on the equilibrium constant K at temperature T is related to the molar volume change by (Schulte and Bradley 1995):

$$K(T,P) = K(T,P_0)\exp(-P\Delta V/RT) \quad (2)$$

where ΔV is the molar volume change, R is the gas constant, and P_0 denotes the atmospheric pressure.

The molar volume change is calculated from the pressure dependence at a constant temperature from the following relationship:

$$\ln K = \text{constant} - P\Delta V/RT \quad (3)$$

The equilibrium constant, K, at each pressure was calculated from the retinal isomeric composition of the pressed bRs using deconvoluted absorption spectra (PeakFit, AISN Software). The deconvolution is based on the parameters of the all-*trans* component from the light-adapted state together with the parameters of the all-*trans* and 13-*cis* components of the dark-adapted state at atmospheric pressure. Figure 4A shows the plots of the logarithm of K versus pressure at 30 °C. The molar volume change is determined from the slope of these lines (Eq. 3). The results of a least-squares regression on the data are presented in Table 1 (absorption measurements).

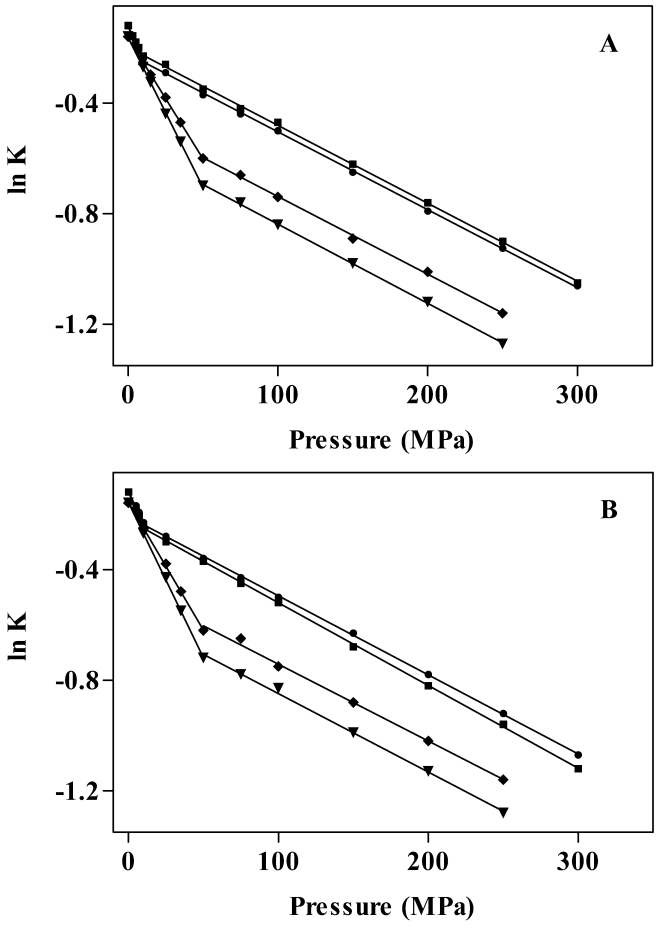

Fig. 4A, B Pressure dependence of the equilibrium constant, *K*, of all-*trans* and 13-*cis* isomers in dark-adapted bacteriorhodopsins at 30 °C. **A** The equilibrium constant calculated using absorption spectra. **B** The equilibrium constant determined from extraction experiments. *Squares*: bR; *circles*: D96N; *diamonds*: 14-F-bR; *triangles*: 14-F-D96N

Table 1 Molar volume change from absorption measurements and extraction experiments

Sample	Molar volume change (mL/mol)	
Absorption measurements		
bR	–6.96 ± 0.11	–21.09 ± 0.33
14-F-bR	–7.06 ± 0.24	–22.23 ± 0.12
12-F-bR	–7.14 ± 0.22	–19.48 ± 0.18
10-F-bR	–7.04 ± 0.19	–20.73 ± 0.24
D96N	–7.16 ± 0.32	–20.30 ± 0.29
14-F-D96N	–7.26 ± 0.22	–27.31 ± 0.18
12-F-D96N	–6.93 ± 0.21	–19.78 ± 0.13
10-F-D96N	–7.14 ± 0.28	–21.13 ± 0.23
Extraction experiments		
bR	–7.47 ± 0.32	–20.51 ± 0.33
14-F-bR	–6.98 ± 0.23	–23.20 ± 0.14
12-F-bR	–7.88 ± 0.17	–19.24 ± 0.12
10-F-bR	–7.32 ± 0.22	–21.13 ± 0.18
D96N	–7.08 ± 0.39	–21.99 ± 0.28
14-F-D96N	–7.14 ± 0.29	–28.84 ± 0.23
12-F-D96N	–7.28 ± 0.18	–20.42 ± 0.14
10-F-D96N	–6.99 ± 0.21	–20.73 ± 0.18
Data from literature		
bR[a,b]	–7.8 ± 3.2	–
bR[c,d]	–6.6 ± 3.0	–
bR[d,e]	–6.5 ± 0.2	–

[a]Tsuda and Ebrey (1980)
[b]Extraction experiment
[c]Schulte et al. (1995)
[d]Raman spectroscopy
[e]Schulte and Bradley (1995)

The lower value of the molar volume change for bR matches the values obtained by Tsuda and Ebrey (–7.8 mL/mol) and Schulte's group (about –6.6 mL/mol). The minus sign implies that the 13-*cis* isomer has a smaller volume than the all-*trans* isomer. The small-volume change (about –7 mL/mol) was attributed to the apoprotein and was interpreted as the formation of three hydrogen bonds rather than the ionization of, at most, two residues during the transformation of $bR^{all\text{-}trans}$ to $bR^{13\text{-}cis}$ (Tsuda and Ebrey 1980). The result for D96N can be interpreted in a similar way.

The most surprising results were obtained for the first points of the pressure changes. The values for the molar volume changes calculated for these points were about three times higher than those calculated for the remaining points (Table 1). For the first time, a large-volume change was found for native bR (and D96N).

In view of the fact that one can expect very small differences between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$, the introduction of the terms large- and small-volume change for obtained ΔV values seems to be justified.

Our results for low pressures (large-volume changes) differ from those obtained by Tsuda and Ebrey (1980), Schulte and Bradly (1995) and Schulte et al. (1995). They obtained only one value of the molar volume change, which is similar to the small-volume change found in our work. One reason for this difference may be connected with the number of experimental points: both groups used only one experimental point between 0.1 and 50 MPa. Therefore, the real process described by low-pressure dependence might be masked (hidden). The Schulte and Bradley (1995) data (their fig. 4) at high pressures fall on a line that is parallel to the high-pressure slopes (our Fig. 4A) but offset vertically from the native chromophore data. It is therefore possible that there might be systematic differences based mostly on experimental protocol (compare Scherrer et al. 1989). Hence, the possibility that the low-pressure process observed here might be sensitive to procedural differences should be taken into account.

The changes (in the diagram of ln*K* as a function of pressure) used for calculating the large-volume change are clear but rather small. Hence, the problem arises whether it is possible to magnify these changes and to provide more information required for the interpretation of the new value of ΔV. One possibility is to use bR (and/or bR mutant) analogues with a chemically modified chromophore to strengthen the expected effect as a result of the changed chromophore-opsin interaction (Crouch 1986; Kollbach et al. 1998). Figure 4A

demonstrates that the data for bRs with 14-F-retinal can be more easily analysed. The changes of the equilibrium constants obtained for low (up to 50 MPa) pressure are clear and the molar volume changes calculated for these constants are clearly different from those calculated for higher pressures. On the other hand, the diagrams of ln$K$ as a function of pressure for the 12-F and 10-F analogues were very similar to diagrams obtained for bR and D96N (Fig. 5A). This suggests that the strengthening effect is limited to the 14-F position of the chromophore.

Again, two groups of values for the molar volume change were obtained for fluorinated analogues (Table 1). The values for the molar volume changes are similar to those obtained for bR and D96N with division into large-volume changes (over –21 mL/mol) and small-volume changes (about –7 mL/mol). This result suggests that the initial pressing is more effective because smaller changes of pressure induce larger changes of K, i.e. larger changes in the isomeric ratio. After reaching a certain value of K, further changes in the isomeric ratio require larger pressures. The process of shifting bR$^{all\text{-}trans}$ to bR13-*cis* is less favourable.

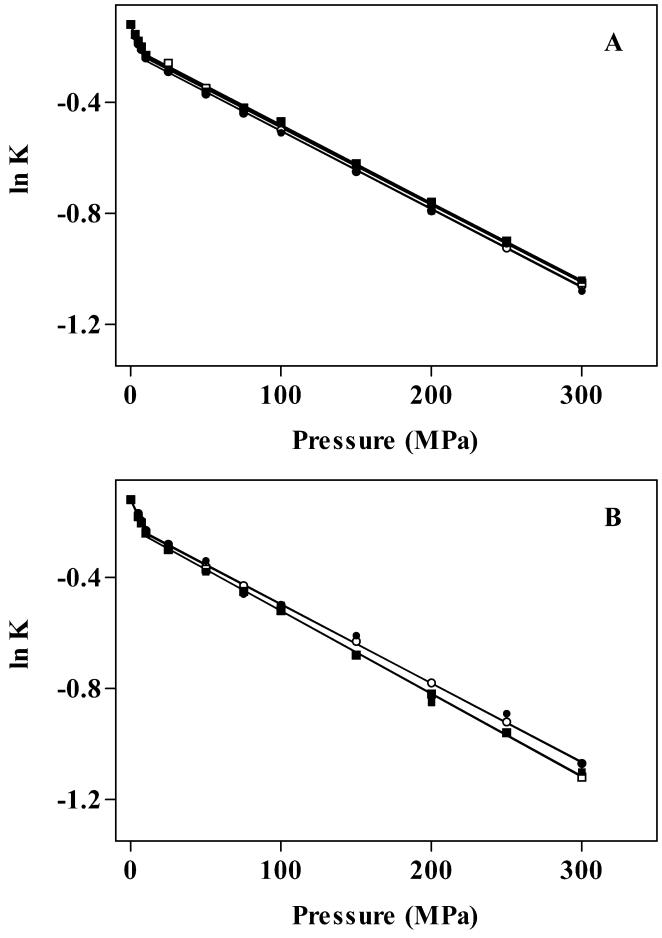

Fig. 5A, B Pressure dependence of the equilibrium constant, K, of all-*trans* and 13-*cis* isomers in dark-adapted bacteriorhodopsins at 30 °C. **A** The equilibrium constant calculated using absorption spectra. **B** The equilibrium constant determined from extraction experiments. *Filled squares*: 12-F-bR; *open squares*: 12-F-D96N; *filled circles*: 10-F-bR; *open circles*: 10-F-D96N

Figure 4A reveals another intriguing effect of pressure on fluorinated bRs. Lower values of ΔV for both 14-F-bR and 14-F-D96N are very similar (–7.06 and –7.26 mL/mol, respectively), but higher values of ΔV differ slightly (–22.23 and –27.31 mL/mol, respectively). This demonstrates that the initial pressure effect is slightly stronger for 14-F-D96N than for 14-F-bR.

The absorption measurements cannot directly determine the amount of all-*trans* and 13-*cis* pigments. Hence, to verify the results from the spectroscopic study, we tried to determine the proper amount of isomers in the pressed samples using the extraction method. We followed the 14-halogenated retinal extraction method (Tierno et al. 1990), with slight modification for extraction of the chromophore from the samples after pressing (Tsuda and Ebrey 1980). The data were nicely reproducible and the pressure effects were fully reversible up to 250 MPa. The log$K$ (obtained from extraction experiments) is plotted versus the pressure P in Figs. 4B and 5B. The molar volume changes are shown in Table 1 (extraction experiments). The extraction experiments confirmed the data from the absorption spectra: very similar values of molar volume change in addition to similar characteristics for equilibrium constant dependence on pressure.

One point should be emphasized at this point. Although "...the rather featureless absorption bands prohibit a direct determination of the chromophore structure" (Schulte and Bradley 1995), the absorption spectra can be usefully applied for the equilibrium constant calculation. Our results also support the earlier suggestion (Tsuda and Ebrey 1980) that the extraction technique might be a very useful method for chromophore identification from high-pressure experiments.

Two clearly different ΔV values suggest at least two different processes that might be involved in the pressure-induced shift of all-*trans* isomers into 13-*cis* ones: a large-volume-change process and a small-volume-change process. The small-volume-change process might be common for all bRs investigated: the final formation of hydrogen bonds. The easily reproducible value of ΔV [–6.6 mL/mol (Schulte and Bradley 1995; Schulte et al. 1995), –7.8 mL/mol (Tsuda and Ebrey 1980), and around –7 mL/mol (our results)] suggests that the earlier assumption concerning the formation of about three hydrogen bonds is justified. This hypothesis can be supported by calculations which demonstrate that the volume effect for the formation of one hydrogen bond in a solution is –2.2 mL/mol (Kharakoz 1992). The minus sign indicates that the formation of the hydrogen bond leads to a smaller molar volume. Thus, increasing pressure favours the 13-*cis* isomer, which has a smaller volume than the all-*trans* isomer in the dark-adapted state.

However, a large-volume-change process may indicate different changes in bR structure. The molar

volume changes, ΔV, in the range of –20 to –30 mL/mol favour steric rearrangements with eventual contribution from the ionization state of bR residues. The volume effect on ionization changes of weak acids is in the range of –10 to 20 mL/mol (Neuman et al. 1973). Hence, the changes in the molar volume change induced by the initial pressure may indicate the geometrical rearrangement to facilitate contact between functionally important residues and chromophore, and influence their ionization state, if required. The proximity and proper structure of these groups may induce retinal isomerization to a partly stable configuration with the retinal in the 13-*cis* form. Moreover, in view of the presented interpretation, the shape of the pressure dependence of ln$K$ (Figs. 4 and 5) may indicate that the isomerization involves both steric rearrangement and hydrogen bond formation, and that only hydrogen bond formation persists to high pressure. Hence, from the surprising sharpness of the break in the pressure dependence of ln$K$ it might be hypothesized that steric rearrangement could be saturated for the kinds of all-*trans* to 13-*cis* ratios at which the slope of ln$K$ versus P is observed to change (e.g., where the transition from the high-volume to low-volume process occurs).

The incorporation of retinal analogues with a changed shape or altered electronic properties into the binding site of bR has been used to strengthen the influence of the surrounding protein (Kollbach et al. 1998). We have already demonstrated that the variation of the substitute at position 14, by changing it into a halogen, leads to a specific protein-substrate interaction (Tierno et al. 1990).

Of the three retinal analogues (10-, 12-, 14-F), only the replacement of 14-H by a fluorine atom resulted in alterations of the bR properties (Bryl and Yoshihara 2001). The 14-F atom (but not 10- or 12-F) also strengthened the effects of high pressure on native bR. 14-Fluorination allowed the larger-volume process to persist to higher pressure and smaller values of K. In effect, fluorination at this site stabilizes the low-pressure behaviour with respect to the high-pressure behaviour. Since the strengthening effect appears to be restricted only to14-F analogues, the regiospecific interactions (rather than ionization state of bR residues) with groups in the vicinity of the protonated Schiff base should be responsible for the observed effects. Therefore, a candidate for the interaction should be positioned within "electrostatic distance" of the 14-F atom and should play an important role in stabilizing the structure of bR.

According to current models of bR based on crystallographic studies (Henderson et al. 1990; Grigorieff et al. 1996; Pebay-Peyroula et al. 1997; Essen et al. 1998; Luecke et al. 1998, 1999), several residues can fulfill the above-mentioned criteria.

Different biophysical studies have established that, in the light-adapted state of bR, the protonated Schiff base is stabilized by a diffuse counterion, presumably involving a H-bonded complex of water with the positively charged Arg-82 side chain and the deprotonated side chains of Asp-212 and Asp-85. The distance (in Å) of Asp212 to 14-C retinal is reported to be 4.4, 4.29, and 4.4 by Griffiths et al. (2000), Grigorieff et al. (1996), and Luecke et al. (1998), respectively. The same sources report Asp-85 to 14-C-retinal distances of >6.0, 5.13, and <6.0, respectively. In all cases, the side chain of Asp-212 is held in position by interactions with the side chains of Tyr-57 and Tyr-185, with a distance to the 14-C-retinal of <4.8 Å, and <4.3 Å, respectively. These data suggest that Asp-212 and/or Tyr-185 can be regarded as potential candidates for strong interaction with the 14-F atom.

However, other groups may interact directly or indirectly with the 14-F atom. Thr-89 is located one turn above the Asp-85 counterion on the C-helix. In a model of bacteriorhodopsin based on electron diffraction (Grigorieff et al. 1996), the hydroxyl oxygen of Thr-89 was found to be located about 3 Å from the Asp-85 and 3.8 Å from the nitrogen of the Shiff base. Thus, Thr-89 is in a good position to participate directly in the active site of bR and to interact with other (via hydrogen bond with Asp-85, for example) residues in the retinal binding pocket (Rothschild et al. 1993; Russel et al. 1997). The indole N-H of Trp-86 is near the carboxylate of Asp-85 (Hatanaka et al. 1997) and with a distance to the 14-C-retinal of 3.9 Å (Helmle et al. 2000). Thus, Trp-86 may be involved in binding the water molecules forming network of hydrogen bonds and participate in interaction with 14-F-retinal. Arg-82 points toward the extracellular side and the distance between the guanidinium group of Arg-82 and the Schiff base nitrogen is 11.5 Å. However, a molecular dynamics simulation of bR demonstrated that Arg-82 might be located at a distance of 4.5 Å from the protonated Schiff base, forming the complex counterion of the base (Nagel et al. 1997). Trp-86 is hydrogen bonded to Asp-85 and participates in the complex counterion of the Schiff base with Asp-85, Arg-82, and Asp-212 (Oesterhelt 1998).

All of the mentioned amino acids fulfill conditions to be good partners for interaction with the 14-F atom. However, if we accept that Asp-212 is located about 4.4 Å from C-14-retinal, that 1.35 Å is the van der Waals radius for fluorine, and that the F-C bond distance is 1.41 Å, these values give 2.99 Å as the distance between Asp-212 and the fluorine atom, the proper distance for strong electrostatic interaction. Hence, Asp-212 (together with Tyr-185) seems to be a very probable candidate for strong interaction with the 14-F atom. This interaction may lead to a small downward shift of helix G (which carries Asp-212) or shift of helix F (which carries Tyr-185). The shift of one helix (G or F) may induce the shift of another one owing to the strong cooperation between them (Oesterhelt et al. 2000).

The interaction of 14-F with opsin was strengthened by substituting aspartic acid 96 with asparagine. This puzzling result suggests the involvement of Asp-96 in the changes in the opsin region close to the proximal end of the chromophore. However, Asp-96 is far (more than 11 Å) from the place of investigation (Grigorieff et al.

1996). Therefore, it might be difficult to explain the direct interaction between 14-F and Asp-96. Surprisingly, this group is functionally linked with groups in the close vicinity of the Schiff base and influences their properties (Heberle et al. 1993; Rothschild et al. 1993; Riesle et al. 1996; Kandori et al. 2000). Particularly, the mutation of D96 introduces a local electrostatic change, which is the cause of the large-scale protein conformation shift in bacteriorhodopsin (Brown et al. 1997). It seems very probable that these structural changes may facilitate the electrostatic interaction between the 14-F atom and certain groups like Asp-212 (and/or Tyr-185), leading to their slightly larger shift and, as a result, to a slightly larger shift of helix G (and/or helix F) in D96N than that in native bR.

The incorporation of the 14-fluororetinal caused an enlargement of the hydrophobic part of the protein, which was manifested by an increase of hydrophobic mismatch between modified bR and lipids. From the extent of the hydrophobic mismatch and the distance of the long-range protein-lipid interaction, the increase of the hydrophobic part of the protein was estimated to be about 3 Å for 14-F-bR and about 7 Å for 14-F-D96N, compared with bR (Bryl and Yoshihara 2001). Hence, if one expects that rearrangement of Asp-212 (high-pressure induced, pushing this residue closer to the chromophore), together with linked groups, should lead to all-*trans*/13-*cis* isomerization, stronger effects on the large-volume change should be observed for 14-F-bR, but the strongest effects should be for 14-F-D96N, as compared with bR. This was indeed observed: –28.84 mL/mol for 14-F-D96N, –23.20 mL/mol for 14-F-bR, compared with 20.51 mL/mol for bR (data from extraction experiments). Hence, the large-volume change process is characteristic of native bR. The magnification of the effect by using retinal analogues revealed only a much larger rearrangement (molecular volume change) that led to a much larger 13-*cis* to all-*trans* ratio.

The steric rearrangement, which positions carboxylate moieties of Asp-212 (together with linked groups) closer to the Schiff base and the chromophore C_{13}-C_{14} bond, may be the main factor for forming optimal conditions for catalysing *trans-cis* chromophore isomerization. It may be directly, because aspartate-212's carboxylate group can catalyse *trans-cis* isomerization of a positively charged retinal Schiff base (Seltzer 1992), or indirectly, via the change of ionization state of functionally important residues, like Asp-85 (Balashov et al. 1995, 1996). The saturation of the steric rearrangement observed for all samples investigated may indicate that there is indeed an optimal arrangement of functionally important groups for the *trans-cis* equilibrium in dark-adapted bR.

The high-pressure experiments indicate that there are at least two processes involved in the shift of all-*trans* isomer into 13-*cis* isomer in the dark-adapted state of bR: (1) a large-volume-change process (with ΔV about –21 mL/mol for native bR), which may be attributed to the steric rearrangement of functionally important reside(s), most probably Asp-212 (and/or Tyr-185 with linked Asp-85), and (2) a small-volume-change process (with ΔV about –7 mL/mol for native bR), which may be attributed to the formation of about three hydrogen bonds. These two processes lead $bR^{all\text{-}trans}$ to accommodate the $bR^{13\text{-}cis}$ state, which is more favourable due to its smaller molar volume (ΔV about –28 mL/mol for total molar volume change between native $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$).

This interpretation of two reactions induced by high pressure can be strengthened by their reversibility. Figure 6 shows the pressure dependence of the equilib-

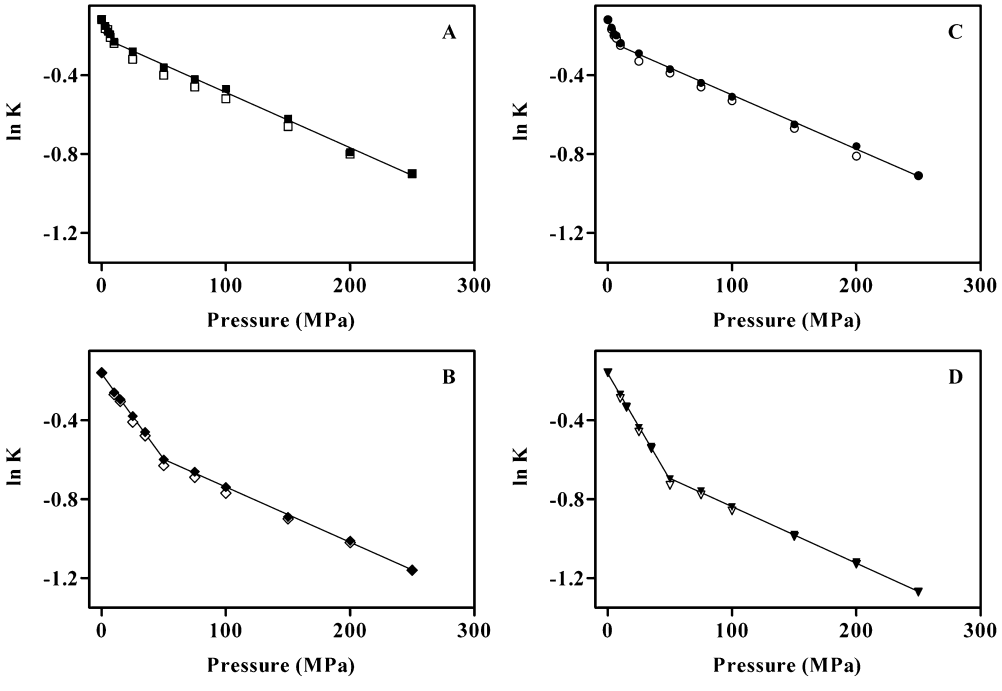

Fig. 6A–D Pressure dependence of the equilibrium constant, *K*, of all-*trans* and 13-*cis* isomers in dark-adapted bacteriorhodopsins at 30 °C. The equilibrium constant calculated using absorption spectra for bR (**A**), 14-F-bR (**B**), D96N (**C**), and 14-F-D96N (**D**). *Filled* and *open symbols* represent pressing and releasing pressure, respectively

rium constant, K, of all-*trans* to 13-*cis* isomers in dark-adapted bacteriorhodopsins at 30 °C. The equilibrium constants were calculated using the absorption spectra for bR (A), 14-F-bR (B), D96N (C), and 14-F-D96N (D). The samples were consecutively pressed up to a pressure of 250 MPa and the absorption spectra were registered as described earlier. The last spectrum in the first part of the experiments was considered as a first spectrum in the second part of the experiments in which the high pressure was released. The data demonstrate that pressing the samples up to 250 MPa and a subsequent release of the pressure led to fully reversible changes.

According to our interpretation, releasing the pressure causes a partial breaking of the hydrogen bonds and rearrangement of the retinal moiety to force part of the 13-*cis* retinals to accommodate the all-*trans* configuration. The whole system returns to the equilibrium called the dark-adapted state (under atmospheric pressure). The transformation from a dark-adapted state to a light-adapted state requires energy for the complex processes in bR that may be similar to those induced by high pressure. Hence, light energy used for light adaptation should be utilized for breaking hydrogen bonds and rearrangement of several residues to increase the opsin volume to be large enough for effective photocyclic reactions of bR^{LA} (small-volume-change process followed by large-volume-change process).

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