Pressure effects on the dark-adaptation of bacteriorhodopsin

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ABSTRACT The influence of hydrostatic pressure (340 MPa) on the dark-adaptation kinetics and the relaxation of dark-adapted bacteriorhodopsin following a pressure jump (0.1 MPa → 340 MPa) have been studied. We have also measured the temperature dependence of the equilibrium isomeric ratio of all-*trans* and 13-*cis* retinal in dark-adapted bacteriorhodopsin at 340 MPa. The results show that hydrostatic pressure affects both the dark-adaptation rate and the dark equilibrium isomeric ratio. With increasing pressure, the fraction of all-*trans* isomers decreases. The kinetics have been analyzed with a two-state model. The description of the pressure dependence using transition state theory is inappropriate for two reasons; (*a*) pressure changes the viscosity of the protein and its environment, and (*b*) pressure changes the population of conformational substates within either isomeric form of bacteriorhodopsin. The temperature independent ratio of all-*trans* and 13-*cis* isomers indicates that the all-*trans* and 13-*cis* conformations have the same conformational volume.

INTRODUCTION

Bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium halobium,* where it forms trimers arranged in a two-dimensional, hexagonal lattice (1). bR is a retinal-based protein complex that functions as a light-driven proton pump (2–4). The three-dimensional structure of bR has been determined to 3.5 Å resolution in the plane of the membrane and 10 Å perpendicular to the membrane (5). The polypeptide chain of the bR molecule forms seven trans-membrane helices in the center of which the chromophore, retinal, is bound to Lys-216 via a protonated Schiff-base. The loop regions missing from the structure have been added by molecular dynamics simulations (6).

Purple membrane exists in two spectroscopically distinct states: the dark-adapted (bR^{DA}) and the light-adapted form (bR^{LA}) with absorption maxima at 558 and 568 nm, respectively. After prolonged exposure to light, the light-adapted metastable state bR^{LA} is formed. Keeping the sample for an extended period in the dark, the sample converts back to the stable, dark-adapted state bR^{DA}. Thus the transition between bR^{DA} and bR^{LA} is completely reversible. bR^{LA} contains mainly (>98%) bR molecules with all-*trans* retinal, whereas bR^{DA} contains bR molecules with both all-*trans* and 13-*cis* retinals. We shall refer to these two major conformations of bR as bR_T and bR_C. It is generally believed that the ratio of bR_C to bR_T conformers in equilibrium bR^{DA} equals 1 (7–10), although a recent study by Scherrer et al. (11) yielded a value of 2 at atmospheric pressure.

After photoexcitation, both bR_T and bR_C run through cyclic processes that can be followed spectroscopically. The photocycle of bR_T is accompanied by the translocation of protons across the membrane in which the bR molecules are embedded. bR_C undergoes a different photocycle without proton translocation (8, 12–16), where part of the bR_C conformers are converted to bR_T conformers (17, 18). At high pH, a different bR_C photocycle has been observed with proton translocation (19).

Hydrostatic pressure can be used for generating small conformational changes in proteins (20). High pressure can change the population of conformational substates of the proteins according to their different molar volumes and also the transition rates among the substates. The overall reaction rate of proteins usually cannot be explained as a pure molar volume effect because it might involve substates with different molar volumes and different reaction rates (21).

In bR, pressure affects both the isomeric composition and the kinetics of the photocycle. Tsuda and Ebrey (10) found a shift of the isomeric composition towards the 13-*cis* isomer with increasing pressure and explained it by a difference of 7.8 ml/mol in the molar volumes of the 13-*cis* isomer and the all-*trans* isomer. Pressure studies of the kinetics of the photocycle indicate that the pressure effect cannot be explained by a difference in the molar volume only. While Tsuda et al. suggest a pressure-induced phase transition in the lipids of the purple membrane (22), Marque and Eisenstein explain their results with pressure-induced viscosity changes (23). The divergent interpretations indicate that the influence of pressure on purple membranes is not yet well understood. Here we study the influence of hydrostatic pressure on transitions between bR_T and bR_C with equilibrium measurements and with kinetic experiments monitoring the relaxation after light-adaptation and after a pressure jump.

MATERIALS AND METHODS

Purple membranes were isolated from the *Halobacterium halobium* strain R1M1 using the standard procedure (24). The purple mem-

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brane samples were buffered to pH 7 with 100 mM potassium phosphate buffer. The absorbance of the suspension was 1.2 OD at 570 nm in a cuvette of 1.5 mm path length.

High pressure in the sample cell was generated with helium gas pressurized with an Aminco compressor (model 46-14060). The pressure could be raised from atmospheric pressure (0.1 MPa) to 340 MPa in less than one minute. The pressure was monitored with a Bourdon gauge accurate to 4 MPa. The helium gas entered the pressure cell through a coiled stainless steel tubing so that the cell could be positioned without releasing the pressure. The bR sample was contained inside the pressure cell between two sapphire windows within a doughnut-shaped teflon spacer of 1.5 mm thickness. The details of the high pressure equipment are described elsewhere (21). The pressure cell was loaded into the sample chamber of a storage cryostat (model 8-DT; Janis Research Company, Inc., Wilmington, MA). The sample temperature was measured with a silicon diode temperature sensor and adjusted to within 0.1 K by a digital temperature controller (model DRC 93C; Lake Shore Cryotronics Inc., Westerville, OH). The cryogenic set-up was mounted on a Cary-14 spectrophotometer interfaced to an IBM PC/AT (On-Line Instrument Systems Inc., Jefferson, GA). The wavelength accuracy and reproducibility are 0.4 and 0.05 nm, respectively. To measure the kinetics of the spectral relaxation, successive spectra were taken between 350 and 750 nm in 2 nm intervals. The time for a single scan was 200 s.

The pressure-jump experiment was performed on bR^{DA} at 290 K. The dark-adaptation of the sample was assured by overnight incubation at 300 K followed by one hour incubation at 290 K. The pressure was raised from 0.1 MPa to 340 MPa in less than a minute. Then the relaxation was monitored by taking successive absorbance spectra. After the measurement was completed, the bR was light-adapted while being kept at the same pressure and temperature. Light-adaptation was accomplished by illumination with a 250-W tungsten lamp (model 66181; Oriel Corp., Stratford, CT) with heat filter and yellow glass filter for 5 min; then the light was switched off, and the kinetics of the relaxation into the dark-adapted state were monitored.

In the data analysis, the baseline of each spectrum was fitted with a linear baseline and subtracted from the spectrum. Because of the inhomogeneous nature of the bR absorption band it is inappropriate to characterize the position of the band by the wavelength of maximal absorbance, λ_{max}. Therefore, we calculated the band position, $\bar{\lambda}$, as the first moment of the absorption band in wavelength space.[1]

RESULTS

The dark- and light-adapted bR spectra measured at 0.1 MPa and 340 MPa at 290 K are shown in Fig 1. Pressure affects both the dark-adapted and the light-adapted form. Under high pressure, the peak absorbance of bR^{LA} increases by a factor of 1.26. The band position $\bar{\lambda}$ shifts from 562 to 570 nm, whereas the peak position λ_{max} shifts from 568 to 574 nm. The peak absorbance of the bR^{DA} spectrum increases by a factor of 1.16. The band position $\bar{\lambda}$ shifts much less, from 557 to 559 nm, and the peak position λ_{max} even shifts in the opposite direction, from 559 to 558 nm. When performing a pressure jump from 0.1 to 340 MPa, the band position shifts from 557 to 566 nm, and subsequently, conformational relaxation shifts the band to the new equilibrium position at 559 nm.

The red-shift of the bR^{LA} spectrum can be explained as an elastic effect of the hydrostatic pressure which compresses the proteins and decreases the distances between the atoms while leaving the arrangement of the atoms, i.e., the topology, unchanged. The elastic effect of pressure on spectral lines has been observed in crystals and proteins including bacteriorhodopsin (10), and the theory has been discussed by Slichter and Drickamer (25). bR^{DA} is a mixture of bR_T and bR_C, and the spectra of both components are expected to shift to the red as a result of the elastic pressure effect. In bR^{DA}, the band position shifts to the red while the peak position shifts to the blue. This observation indicates that the ratio of the two components in bR^{DA} changes with pressure. This conformational effect is characteristic of proteins. Pressure changes the arrangement of the atoms. Consequently, the protein moves from one conformational substrate to another (21). The gradual shift of the band position $\bar{\lambda}^{DA}$ from 566 to 559 nm following the pressure jump clearly indicates conformational relaxation. Similar effects of pressure on the spectral line of bR and on the dark equilibrium isomeric ratio have already been observed by Tsuda and Ebrey (10). They determined the equilibrium isomeric ratio by extracting the retinal and found that high pressure shifts the equilibrium between bR_T and bR_C isomers towards bR_C.

The kinetics of this process have been studied by pressure jump measurements. The pressure was raised from 0.1 to 340 MPa in less than a minute. Successive scans were taken to follow the relaxation until the new equilibrium was reached. The band position, $\bar{\lambda}(t)$, was determined for each scan. A relaxation function was calculated as

$$\Phi(t) = \frac{\bar{\lambda}(t) - \bar{\lambda}(\infty)}{\bar{\lambda}(0) - \bar{\lambda}(\infty)}, \qquad (1)$$

to parameterize the relaxation. For comparison, we also measured the kinetics of the dark-adaptation at high pressure. The relaxation functions for both the relaxation of bR^{DA} after pressure jump and the relaxation from the light-adapted to the dark-adapted state at 340 MPa are identical, as shown in Fig. 2 for T = 290 K.

We measured the kinetics of the dark-adaptation between 275 and 300 K at a pressure of 340 MPa. For comparison, we also measured at atmospheric pressure (0.1 MPa) between 285 and 305 K on the same sample. Experiments at atmospheric pressure have already been reported by other groups (8, 11, 13, 26) with slightly varying results. The relaxation functions, i.e., the normalized shifts as defined by Eq. 1, are shown in Fig. 3. The kinetics of the dark-adaptation are well described by an exponential process. The rate coefficients, listed in Table 1, show that high pressure not only shifts the spectra, but also increases the rate of dark-adaptation.

KINETIC MODEL AND DATA ANALYSIS

Fig. 2 shows that both the dark-adaptation and the pressure jump kinetics describe the same relaxation, namely

[1] The first moment $\bar{\lambda}$ is defined as $\int \mathcal{A}(\lambda)\lambda \, d\lambda / \int \mathcal{A}(\lambda) \, d\lambda$, where $\mathcal{A}(\lambda)$ represents the (baseline-corrected) absorption spectrum.

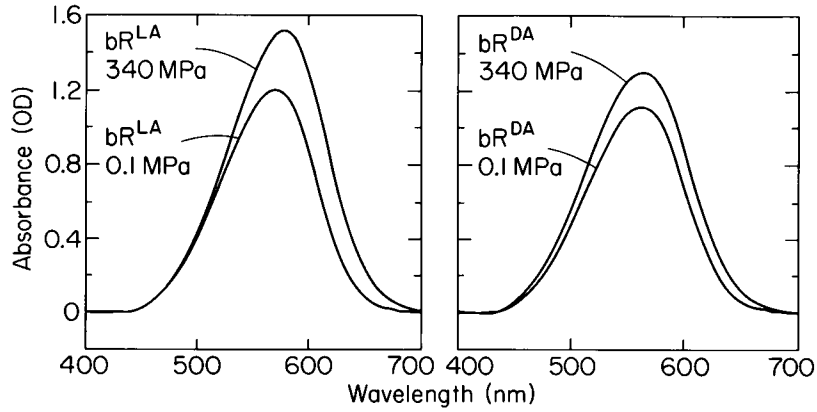

FIGURE 1 The effect of high pressure on the absorption band of light-adapted (bR^{LA}) and dark-adapted (bR^{DA}) bacteriorhodopsin at 290 K.

a transition between bR_T and bR_C. The simplest kinetic scheme for this process is

$$bR_T \underset{k_{CT}}{\overset{k_{TC}}{\rightleftharpoons}} bR_C. \qquad (2)$$

The two rate coefficients k_{TC} and k_{CT} generally depend on temperature, pressure, and viscosity. The kinetics of the transition are described by

$$f_T(t; P) = f_T(0; P) - \Delta f_T(P)(1 - e^{-kt}),$$
$$\text{with} \quad k = k_{TC} + k_{CT}. \qquad (3)$$

$f_T(t; P)$ denotes the mole fraction of bR molecules with all-trans retinal at time t and pressure P, and $\Delta f_T(P) = f_T(0; P) - f_T(\infty; P)$ is the total change in the mole fraction of bR_T during the relaxation.

The light-adapted form of bacteriorhodopsin bR^{LA} contains only bR_T. In the dark, it converts to the dark-adapted form bR^{DA} containing both bR_T and bR_C. The kinetics of this process can be modeled using Eq. 3 with $f_T(0; P) = 1$, and $\Delta f_T(P) = 1 - f_T^{DA}(P)$, where $f_T^{DA}(P)$ denotes the equilibrium mole fraction of bR_T in bR^{DA} at a particular pressure P. Pressure shifts the dark equilibrium towards bR_C. Immediately after a pressure increase that is fast compared to the inverse transition rates, the bR sample still contains the isomeric ratio that is characteristic for the low pressure. The subsequent approach towards the new equilibrium that is characteristic for the high pressure is also described by Eq. 3. For the pressure jump, $f_T(0; P) = f_T^{DA}(P_0)$, and $\Delta f_T(P) = f_T^{DA}(P_0) - f_T^{DA}(P)$. P_0 is the initial pressure prior to the pressure jump.

We now analyze the spectra with our kinetic model. The bR spectrum is a linear superposition of the bR_T and bR_C absorption spectra. Thus the band position $\bar{\lambda}$ can be

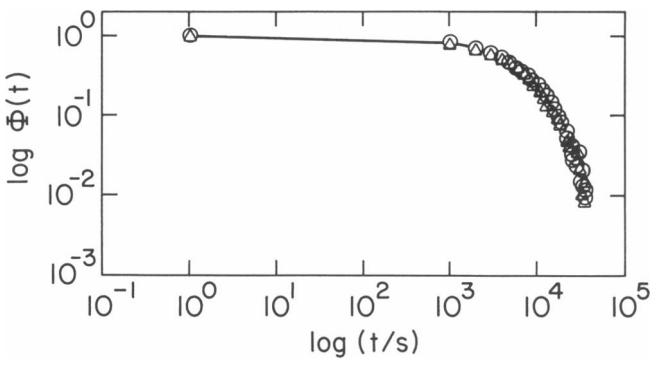

FIGURE 2 The relaxation function $\Phi(t)$ (normalized spectral shift) versus time t at 290 K. *Circles:* dark-adaptation at 340 MPa; *triangles:* pressure jump from 0.1 MPa to 340 MPa.

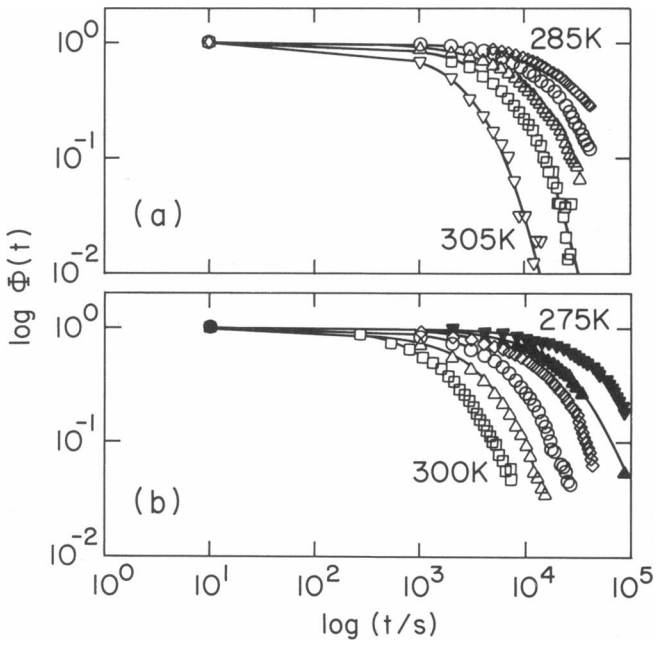

FIGURE 3 The relaxation function $\Phi(t)$ (normalized spectral shift) versus time t during the dark-adaptation at (*a*) 285, 290, 295, 300, and 305 K, at 0.1 MPa; (*b*) 275, 280, 285, 290, 295, and 300 K, at 340 MPa.

TABLE 1 **Rate coefficients for the dark-adaptation kinetics (Fig. 3)**

T	k	k_{CT}	k_{TC}	k	k_{CT}	k_{TC}
K	$\times 10^{-4}\ s^{-1}$, 0.1 *MPa*			$\times 10^{-4}\ s^{-1}$, 340 *MPa*		
275				0.20	0.04	0.16
280				0.39	0.09	0.30
285	0.31	0.16	0.16	0.63	0.14	0.49
290	0.50	0.25	0.25	1.30	0.29	1.01
295	0.84	0.41	0.41	2.60	0.57	2.03
300	1.53	0.77	0.77	5.58	1.22	4.35
305	3.63	1.82	1.82			

expressed as a linear combination of the band positions $\bar{\lambda}_T$ and $\bar{\lambda}_C$ for bR_T and bR_C, respectively:

$$\bar{\lambda} = \frac{f_T\bar{\lambda}_T + X(1-f_T)\bar{\lambda}_C}{f_T + X(1-f_T)}, \tag{4}$$

X is the ratio of the area of the bR_C absorption band to the area of the bR_T band. As the change of the area of the absorption spectrum is only ~8% at 0.1 MPa and 16% during dark-adaptation at 340 MPa, we fixed X to 1. With $X = 1$, Eq. 4 can be simplified to

$$\bar{\lambda} = f_T\bar{\lambda}_T + (1-f_T)\bar{\lambda}_C. \tag{5}$$

Evidently, the shift of the spectrum is proportional to the change in the mole fraction of the all-*trans* isomers, f_T. This linear relation justifies the use of the normalized relaxation function $\Phi(t)$ defined in Eq. 1 as a parameterization of the kinetics. In Fig. 3, the relaxation curves obtained from the fits of $\Phi(t) = \exp(-kt)$ are shown with the dark-adaptation data.

The kinetics of the dark-adaptation at 0.1 and 340 MPa, Fig. 3, indicate that high pressure increases the apparent rate coefficient, k, of this relaxation. The band positions of both light-adapted and dark-adapted forms, $\bar{\lambda}^{LA}$ and $\bar{\lambda}^{DA}$ are independent of temperature, as shown in Fig. 4. Therefore, f_T^{DA} does not change with temperature; the all-*trans* and 13-*cis* forms of bR have the same conformational enthalpies and volumes at high pressure. At 0.1 MPa, the same behavior has been observed for temperatures between 273 and 310 K (11). We use the results of Tsuda and Ebrey (10), f_T^{DA} (0.1 MPa) = 0.50, and f_T^{DA} (340 MPa) = 0.22, to separate the apparent rate coefficient k into the two rate coefficients k_{TC} and k_{CT} that govern the kinetics of the transition $bR_T \rightleftharpoons bR_C$. For our kinetic scheme, Eq. 2, the rate coefficients are given by

$$k_{CT} = k f_T^{DA}, \tag{6}$$

$$k_{TC} = k(1 - f_T^{DA}). \tag{7}$$

The rate coefficients are listed in Table 1 and plotted in Fig. 5 in an Arrhenius plot. The rate coefficient k_{TC} is significantly larger at 340 MPa than at atmospheric pressure.

DISCUSSION AND CONCLUSION

The parameters obtained from our kinetic experiments on the dark-adaptation explain the pressure response of the overall bR^{LA} and bR^{DA} bands. bR^{LA} contains only bR_T, and the band position of this conformation, $\bar{\lambda}_T$, shifts by ~7 nm to the red when raising the pressure to 340 MPa. bR^{DA} contains both bR_T and bR_C and shows a red-shift of 9 nm shortly after a pressure jump from 0.1 to 340 MPa. The subsequent blue-shift of the band position $\bar{\lambda}^{DA}$ from 566 to 559 nm indicates that the decrease of bR_T at 340 MPa compensates for the pressure-induced red-shift of the overall band position. It is also responsible for the fact that the maximum absorbance, λ_{max}, shifts to the blue.

By measuring the kinetics as a function of temperature and pressure we can study how these intensive thermodynamic variables affect the rate coefficients. Usually, the temperature and pressure dependencies are described using transition state theory (27), with

$$k = A \exp\left(-\frac{E^{\ddagger}}{RT}\right)\exp\left(-\frac{PV^{\ddagger}}{RT}\right). \tag{8}$$

Here, A is the preexponential, and $E^{\ddagger}$ is the activation energy. R denotes the universal gas constant, T the absolute temperature, P the pressure, and $V^{\ddagger}$ the activation volume of the reaction.

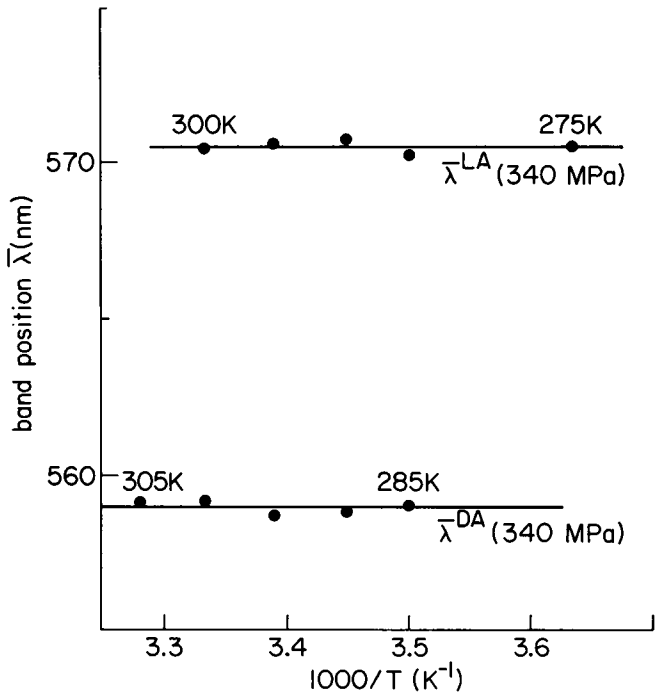

FIGURE 4 Temperature dependencies of the band positions, $\bar{\lambda}$, of bR^{LA} and bR^{DA} at 340 MPa.

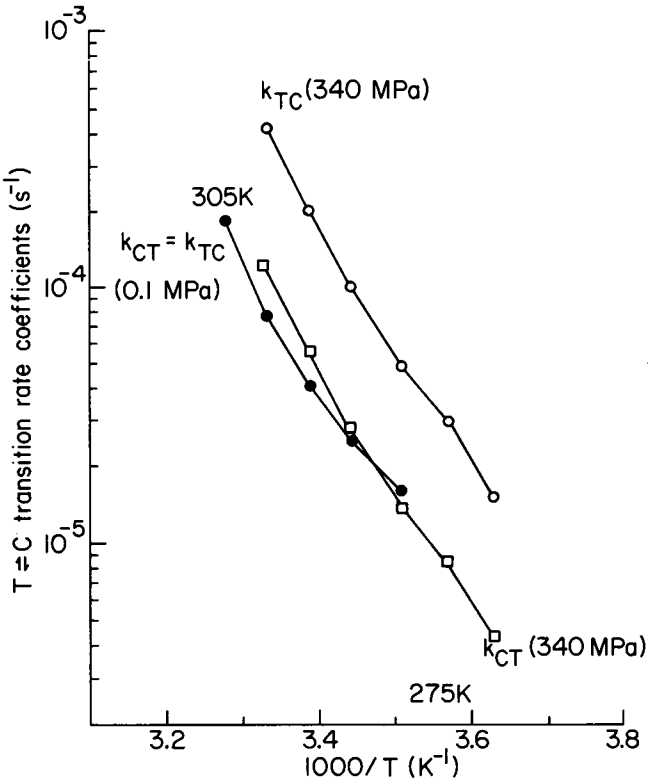

FIGURE 5 Arrhenius plots of the rate coefficients k_{CT} and k_{TC} of the $bR_T \rightleftharpoons bR_C$ transition at 0.1 and 340 MPa.

The application of transition state theory to protein reactions has been shown to be inappropriate (29, 30). The criticism focuses on the point that protein reactions depend on a large number of reaction coordinates, and not only on a few (or even a single one). The net effect of the many coordinates can be modeled as friction along the reaction coordinate. The first approach to introduce a frictional force into a rate theory is due to Kramers (28). In protein reactions, the friction is pressure- and temperature-dependent and related to both the intrinsic viscosity of the protein and the viscosity of the surrounding medium (membrane, solvent). Consequently, an Arrhenius plot will not give values for A and activation enthalpy $H^{\ddagger}$ that characterize solely the protein reaction. These coefficients will also include contributions from the viscosity and the dependence of the viscosity on temperature.

The dependence of the kinetics of the bR_T photocycle on the viscosity of the surrounding medium has been studied in detail (31). It was shown that the slow, later steps of the photocycle were slowed with increasing viscosity. A subsequent study of pressure effects on the kinetics of the photocycle showed a slowing with increasing pressure (23). It was argued that pressure effectively increases the viscosity of the membrane so as to slow down the photocycle kinetics.

The temperature and pressure dependencies of the rate coefficients of the dark-adaptation of bR are summarized in Fig. 5. The pressure dependence of the dark-adaptation is interesting: While the photocycle kinetics is slowed by increasing pressure, the rate coefficient k_{TC} of the isomerization reaction speeds up with pressure. We follow the argumentation by Marque and Eisenstein (23), that the viscosity of the protein environment (the membrane) increases with increasing pressure. Then we have to conclude that in the dark isomerization reaction, the viscosity effect is overcompensated due to a significantly larger preexponential A (see Table 2).

The conformational volumes of the bR_T and bR_C are identical since the isomeric ratio does not change with temperature at 340 MPa. The evaluation of activation volumes from the dark-adaptation kinetics at two different pressures is problematic since pressure not only shifts the $bR_T \rightleftharpoons bR_C$ equilibrium, but also the conformational substate distributions within either bR_T or bR_C to substates with smaller molar volumes. This statement is supported by the significant increase of the preexponential A at high pressures. Furthermore, the peak absorbance of bR^{DA} increases with time following the pressure jump. From the relaxation towards more bR_C isomer we would actually have expected a decrease of the peak absorbance. This effect was actually overcompensated by conformational relaxations within the two isomeric forms.

Finally, we want to point out some connections between the results presented here and our work on myoglobin (Mb). Experiments with myoglobin indicate that the conformational substates are arranged hierarchically in a number of tiers that are characterized by distinctly different free energy barriers between the substates (32). A large number of substates exists in the lower tiers of the hierarchy, whereas there are only a few "taxonomic substates" (32, 33) in the highest tier. In myoglobin, they are called the A substates, A_0, A_1, and A_3. Similarly, one may refer to bR_C and bR_T as two taxonomic substates of bacteriorhodopsin, and the pressure experiments also give evidence for substates in lower tiers. In Table 2, we compare the Arrhenius parameters of the interconversions between the bR_T and bR_C substates. The activation enthalpies $H^{\ddagger}$ are comparable in the two systems, but the preexponential factors A differ considerably. The large

TABLE 2 **Arrhenius parameters of the isomerization reaction in bacteriorhodopsin ($bR_C \rightleftharpoons bR_T$) and the A substate exchange in carbonmonoxy-myoglobin ($A_0 \rightleftharpoons A_1$)**

System	Reaction	log ($A_{\rightarrow}/s^{-1}$)	log ($A_{\leftarrow}/s^{-1}$)	$H^{\ddagger}$
bR (0.1 MPa)	$bR_C \rightleftharpoons bR_T$	10.9	10.9	86
bR (340 MPa)	$bR_C \rightleftharpoons bR_T$	14.5	15.0	106
MbCO	$A_0 \rightleftharpoons A_1$	18.5		74

For bR, we have fitted the temperature dependencies of k_{CT} and k_{TC} at 0.1 and 340 MPa (see Fig. 5) with the Arrhenius law. Data for MbCO (in 75% glycerol/25% buffer, by volume) have been obtained from flash photolysis experiments between 230 and 280 K (32, 34).

preexponential for Mb implies that the Arrhenius relation is inappropriate and that the Ferry or Vogel-Tammann-Fulcher relation should be used (21, 35). The nonlinearity of the Arrhenius plot and the dramatic change of the preexponential with pressure in bR also suggest that simple transition state theory is inappropriate. Although the kinetics of the $bR_C \rightleftharpoons bR_T$ is exponential, bR molecules fluctuate among many slightly different conformational substates at physiological temperatures, and the distribution of conformational substates responds sensitively to pressure and temperature. At cryogenic temperatures, conformational transitions are arrested, and the distribution is pressure- and temperature-independent. Experiments should be performed both below and above the glass transition temperature of the protein to examine the conformational volume effects and the reaction volume effects separately. This approach is discussed in detail in reference 21.

We will continue our kinetic investigations on these systems in order to understand the influence of external conditions, including temperature, pressure, pH, solvent chemistry, and solvent viscosity, on protein conformational transitions.

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