Decay of the tryptophan fluorescence anisotropy in bacteriorhodopsin and its modified forms

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ABSTRACT In this work we study the decay of the polarization of the Trp fluorescence in native bacteriorhodopsin (bR), deionized bR (dlbR), and the retinal-free form of bR, bacterioopsin (bO), using picosecond laser/streak camera system. Two types of depolarization processes are observed, one around 250 ps, which is temperature independent around room temperature, and the other in the 1–3-ns range, which is sensitive to temperature and certain bR modifications. This suggests the presence of at least two different environments for the eight Trp molecules in bR. Native bR and deionized bR gave the same depolarization decay times, suggesting that the removal of metal cations does not change the microenvironment of the emitting Trp molecules. The slow component is faster in bO than in bR, suggesting a change in the environment of the Trp molecules upon the removal of the retinal chromophore. All these results are discussed in terms of the different mechanisms of Trp fluorescence depolarization. A comparison between the depolarization decay in rhodopsin and bR is made.

INTRODUCTION

The mechanism and function of light-adapted bacteriorhodopsin (bR), the intrinsic membrane protein found in the cell wall of *Halobacterium halobium,* have been subject of a large number of studies during the last few years. It contains an all-*trans* retinal chromophore attached to Lys-216 via a protonated Schiff base linkage (PSB) (Stoeckenius and Bogolmoni, 1982). bR uses the light energy absorbed by its chromophore to pump protons from the inside to the outside of the cell. Upon absorption of a photon it undergoes a photochemical cycle of at least five intermediates on timescales varying from femto- to milliseconds (Lozier et al., 1975):

$$bR_{568} \rightarrow J_{625} \rightarrow K_{610} \rightarrow L_{550} \rightarrow M_{412} \rightarrow O_{640} \rightarrow bR_{568}.$$

The PSB is deprotonated after the $L_{550} \rightarrow M_{412}$ step, leading to the proton pumping process. The proton gradient directly drives some metabolic processes, such as ATP synthesis and other endergonic processes (Stoeckenius and Bogomolni, 1982, and references therein).

Bacteriorhodopsin contains eight tryptophan (Trp) and 11 tyrosine (Tyr) residues out of 248 amino acid residues in the polypeptide chain (Khorana et al., 1979; Ovchinnikov et al., 1979; Ovchinnikov, 1982). These Trp and Tyr residues absorb at a relatively long wavelength (~280 nm) compared with other amino acid residues. Therefore, they are very useful optical probes to study the protein structure and the interactions of the protein with the retinal and its photocycle intermediates. The fluorescence of bR and its retinal-free form bacterioopsin (bO) is characteristic of Trp only (Scherman, 1981, 1982).

Several studies have been performed on the Trp fluorescence of bR (Bogomolni et al., 1978; Kalisky et al., 1981; Fukumoto et al., 1981; Sherman, 1981, 1982; Acuna et al., 1984; Palmer and Sherman, 1985; Polland et al., 1986; Jang et al., 1988). Energy transfer from Trp to retinal causes quenching of the fluorescence (Sherman, 1982; Palmer and Sherman, 1985) and leads to a photocycle identical with that excited at 568 nm, the visible absorption band of the retinal (Kalisky et al., 1981). The quenching of Trp in bR was further studied by Jang et al. (1988) under different perturbations, e.g., removal of the retinal and metal cations, M_{412} formation, etc.

The dynamic properties of proteins are of interest for studying the physical properties of these macromolecules. Again, Trp residues can function as optical probes. Their rotational freedom can be detected through a study of the time-resolved and lifetime-resolved anisotropies. Some proteins that have been studied in this way are azurin, nuclease B and human serum albumin (Munro et al. 1979), alcohol dehydrogenase and adrenocorticotropin (Alexander Ross et al., 1981*a* and *b*), *N*-acetyl-L-tryptophanamide (Nordlund and Podolski, 1983), and myoglobin (Hochstrasser and Negus, 1984). For an extensive review see Beecham and Brand (1985). Fluorescence anisotropy decay studies can be used to reveal both motions within proteins and motion of the protein as a

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whole. Wheras the former usually occur on a subnanosecond timescale, the latter generally take several nanoseconds or even microseconds and longer, when the protein is embedded in a membrane. All studies mentioned above supported the concept of very fast dynamic motions in proteins on a picosecond–nanosecond timescale.

A very interesting study related to the work presented here was that of rhodopsin and its intermediates (Lubert et al., 1978). Rhodopsin is a visual pigment closely related to bR. They both have the same retinal chromophore, although rhodopsin contains only five Trp residues, compared with eight in bR. For almost all intermediates of rhodopsin a depolarization of the Trp fluorescence was observed on the nanosecond timescale (ranging from 1.3 to 2.3 ns). It is interesting to note that only for metarhodopsin I a much longer rotational correlation time of 10.5 ns was found. Because the most significant rearrangement in the internal structure of the rhodopsin occurs in the step from lumirhodopsin to metarhodopsin I (Erhardt et al., 1966), Lubert et al. (1978) concluded that a conformational change induced during the formation of metarhodopsin I apparently gives rise to a much more stable conformation. The observation of very small values of the initial anisotropy implied the presence of very fast internal motions on a picosecond or even faster timescale.

In this work we present a study of the decay of the anisotropy of the Trp fluorescence in native bR, deionized bR (dIbR), and bO with a picosecond laser/streak-camera system. The change in microenvironment of some of the different fluorescent Trp molecules that occurs after bR perturbation can best be examined by this sensitive technique.

MATERIALS AND METHODS

Materials

Bacteriorhodopsin (bR) was extracted from the ET1-001 strain of *Halobacterium halobium*, which was generously provided to us by Professor W. Stoeckenius and Professor R. Bogomolni. The purple membrane was grown and purified according to a combination of the methods outlined by Oesterhelt and Stoeckenius (1974) and Becher and Cassim (1975). Typical bR concentrations used were ~80 μM. The deionized sample was prepared by flowing bR through a cation exchange column and washing several times with doubly deionized water. The bleached membrane, bacterioopsin (bO), was prepared according to the method of Crouch et al. (1986). The retinal oxime was extracted with hexane after lyophilizing. The absorption at 362 nm (retinal oxime) was monitored to assure full removal of the retinal. The dried sample was subsequently resuspended in doubly deionized water. We checked that addition of free retinal completely regenerated bR in its native form, i.e., the sample of bO studied in this work did not contain any denatured protein. All data reported here were measured in unbuffered suspensions in water (pH ~5–6).

Emission decay kinetics measurements

The samples were excited by the fourth harmonic (266 nm) of a passively/actively mode-locked model 471 Nd/YAG laser (Quantel International, Santa Clara, CA), which produces a ~35-ps, ~1-mJ, 1,064-nm fundamental pulse. The excitation pulses at a typical energy of ~70 μJ were focused to a ~2-mm spotsize. The fluorescence wavelength was selected using UV transmitting filters while blocking the 266-nm excitation pulses effectively with a $CuSO_4$ solution filter.

The detector consisted of a model C979 streak camera (Hamamastu Corp., Middlesex, NJ) with 10-ps time resolution, coupled to an intensified 1420 reticon with a 1218 multichannel controller (Princeton Applied Research, Princeton, NJ). This was interfaced to a model LSI 11/23 computer (Digital Equipment Corp., Maynard, MA). Nonlinear responses of the streak camera/reticon system in time and intensity were calibrated. The temporal instrument response function with the employed 50-μm slit has been determined as 40 ps fwhm on the fastest streak rate. A 532-nm prepulse was used as a time marker and as an alignment for the signal averaging procedure. All the emission collecting lenses were quartz. The UV collecting lens assembly of the streak camera was homemade. The exposure of the samples to the UV excitation was minimized (<1,000 shots) to prevent photobleaching. A half-wave plate (coated for 266 nm) and a clean-up polarizer were used just in front of the sample to provide vertically polarized light.

The temperature-dependent measurements were performed in a thermostated cuvette (Beckman Instruments Inc., Fullerton, CA) between 3 and 39°C. At low temperatures a nitrogen flow prevented condensation on the cuvette. Temperature determination was accurate to within 0.5°C.

Analysis of the fluorescence and anisotropy decays

The experimental anisotropy, $r(t)$, was defined as

$$r(t) = [I_{\parallel}(t) \cdot G - I_{\perp}(t)]/[I_{\parallel}(t) \cdot G + 2 \cdot I_{\perp}(t)], \quad (1)$$

in which $I_{\parallel}(t)$ and $I_{\perp}(t)$ are the components of the fluorescence parallel and perpendicular, respectively, to the vertically polarized excitation light. G is a correction factor for the efficiency of the detector towards vertically and horizontally polarized light. The G-value is the ratio of the vertical to horizontal emission, when the excitation is horizontally polarized. The two components $I_{\parallel}(t)$ and $I_{\perp}(t)$ were recorded independently. They were scaled by using the 532-nm prepulse as an "intensity marker." Several scans were taken to check the reproducibility of the intensities and decays. Data analysis was performed using a nonlinear least-squres computer program (Bevington, 1967).

Because $[I_{\parallel}(t) \cdot G + 2 \cdot I_{\perp}(t)]$ represents the total fluorescence intensity, we checked whether the decays obtained by us agreed with those found by Jang et al. (1988). In all cases a satisfactory agreement was found, in that all previously determined components in the emission decay could be resolved.

RESULTS AND DISCUSSION

Emission characteristics of Trp in bR under different perturbations

It has been shown that both metal cations and retinal cause quenching of the Trp fluorescence in bR (Jang et

al., 1988). Addition of retinal to bO even reduces the emission intensity by a factor of ~3. Whereas four components of the Trp emission were found in native and deionized bR (with values of 100, 200, 800, and 2,200 ps for native bR, and 100, 200, 1,000, and 2,200 ps for deionized bR), only two are present in bO. The fluorescence decay curves that were obtained for bO are presented as an example in Fig. 1. They represent the parallel and perpendicular decay of bO. As was mentioned above for bR, in accordance with the earlier work of Jang et al. (1988) for bO two different decay components are present in the total fluorescence, one of 200 ps and a longer one of 2,200 ps.

The fluorescence anisotropy decay of Trp in bR under different perturbations

In Fig. 2 *a* a plot of the anisotropy, $r(t)$, of bO at 19°C is given in two different dynamic windows of the streak camera. Clearly two different decay components can be distinguished, one ~265 ps and one ~1,200 ps, with respective preexponential factors of 0.17 and 0.14. Note that the decays are exponential in time over five orders of magnitude. Two other samples were studied, native bR and deionized bR. Similar plots as those of Fig. 2 *a* are shown in Fig. 2, *b* and *c*, for native bR at 25°C and deionized bR at 19°C, respectively. All results have been summarized in Table 1. The uncertainties given are standard deviations based on four to eight experiments. As can be seen from this table, in all samples and at all temperatures studied two decay components could be distinguished. The fast one is for all samples around 260 ps and is independent of temperature between 3 and 39°C. The slower one, which varies with temperature between ~800 and ~3,000 ps, is the same for native and deionized bR, but different for bO.

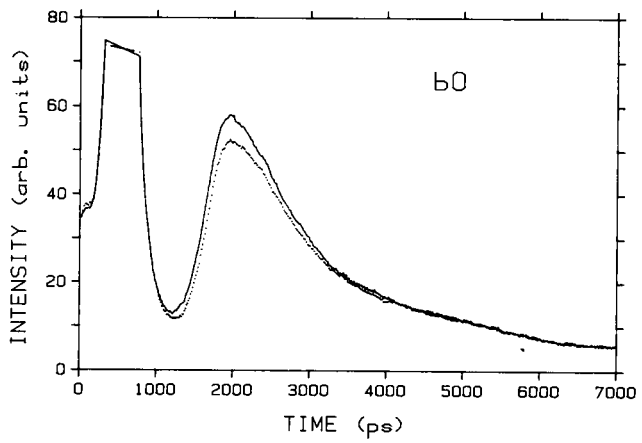


FIGURE 1 Trp emission kinetics of bO suspensions in water. Sample excitation was made at 266 nm, and the emission was collected from 290 to 300 nm. The temporal resolution for this time window is ~400 ps. The two curves represent the parallel (*upper*) and perpendicular (*lower*) decay of bO with respect to the vertically polarized excitation light.

Although it was shown before that the Trp emission decay in bR can be resolved into four components, corresponding to Trp-residues in four different environments (Jang et al., 1988), this does not necessarily mean that there should be also four components in the anisotropy decay. It has been shown in several studies that Trp residues can be virtually immobilized in their protein environment, e.g., the Trp in nuclease B and human serum albumin (Munro et al., 1979), and the two Trps in horse liver alcohol dehydrogenase (Alexander Ross et al., 1981*a* and *b*). The presence of two anisotropy decay components could be explained either by two different types of environments for the emitting Trp molecules or by the presence of two different emitting states in the same environment. The fact that the two components have very different uncorrelated temperature dependencies strongly argues for the former. The difference in environment could suggest a difference in viscosity. In this case the anisotropy decay is a result of rotational depolarization. Depending on the activation energy to rotation, the anisotropy decay could have different temperature dependencies in the two different environments. Of course the difference in environment could also reflect differences in average distances between the different Trp molecules. In this case the decay of the anisotropy reflects the competition between Trp–Trp intermolecular energy transfer and fluorescence. This mechanism should have a small, if any, temperature dependence. It is possible that the two observed mechanisms for the decay of the anisotropy are either due to rotational depolarization or else that one of them (the 260-ps component) results from energy transfer while the nanosecond component results from rotational depolarization.

A study of the single Trp in holazurin, which is buried deep within the protein, clearly showed the rotational depolarization mechanism (Munro et al., 1979). The anisotropy decay showed a very fast component of 0.51 ns, which was interpreted in terms of a free rotation of the Trp molecule, and a slow decay of 11.8 ns, which corresponds to a rotation of the protein as a whole. The fast decay indicated that the protein has a fluidlike interior.

For membranes and micelles the concept of microviscosity η has been introduced, the average viscosity which opposes rotation of probe molecules embedded in them. It has been shown to decrease with temperature in an Arrhenius-type fashion:

$$\eta = \eta_0 \cdot \exp(E_a/kT). \quad (2)$$

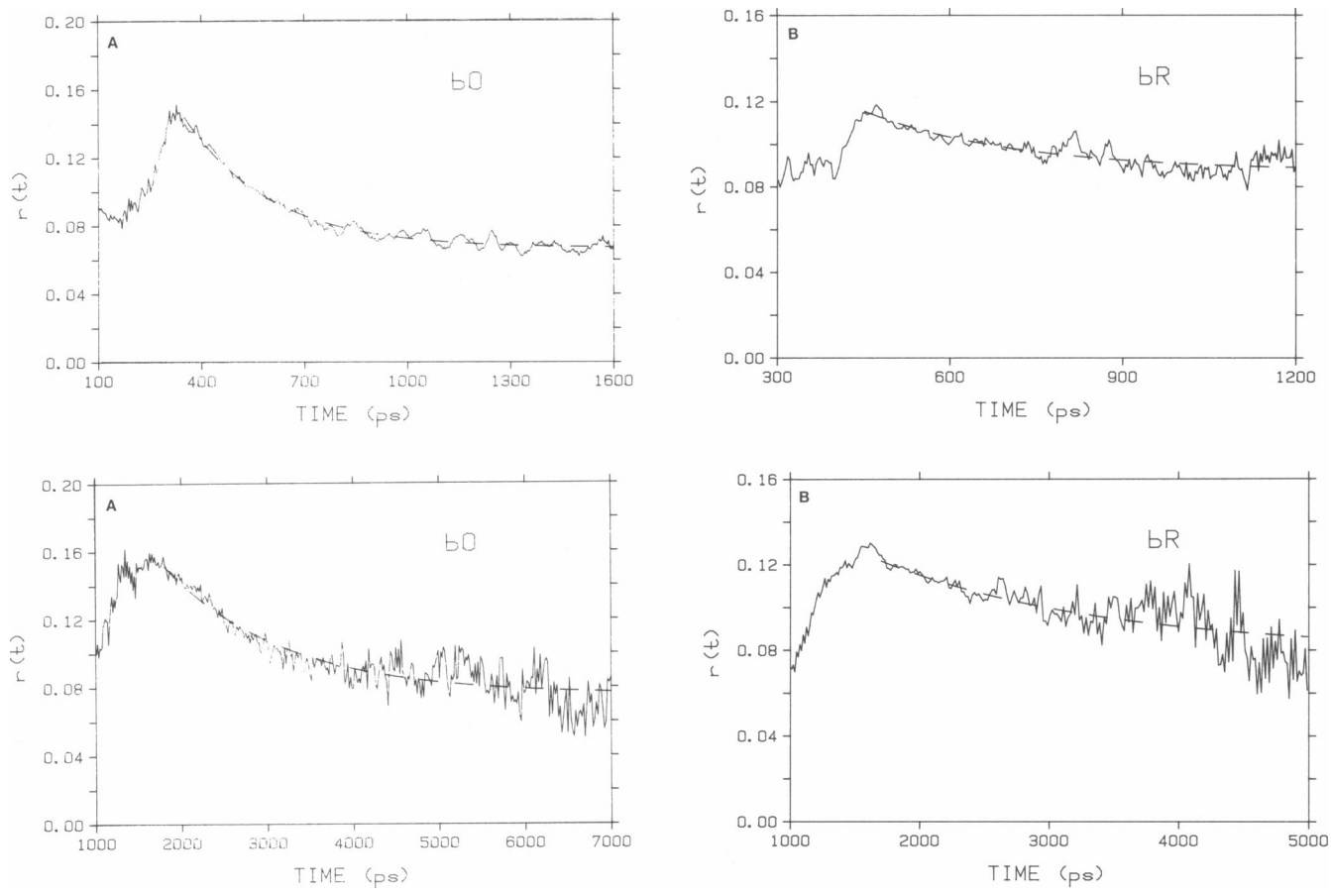
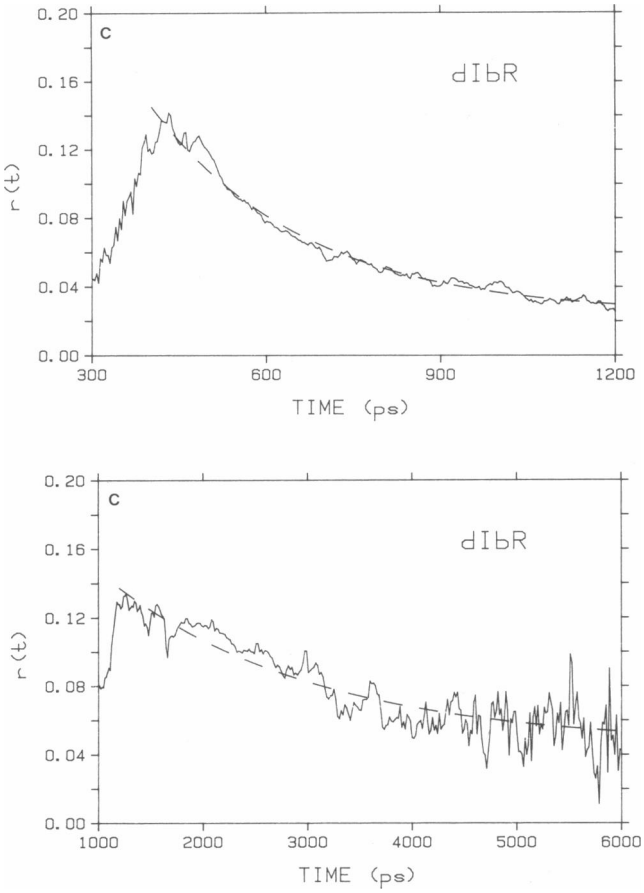


FIGURE 2 Anisotropy decay of suspensions of bO (*A*), bR (*B*), and dIbR (*C*) in water at temperatures of 19, 25, and 18°C, respectively, measured in two different dynamic windows of the streak camera to show that there are two different decay components. Dashed lines represent the fits with the parameters found in Table 1. Sample excitation was made at 266 nm, and the emission was collected from 290 to 300 nm. The temporal resolutions of the windows *A* and *B* are 90 and 400 ps, respectively. The deconvoluted rotational relaxation times are 265 ps and 1.2 ns for bO, 239 ps and 1.7 ns for bR, and 265 ps and 2.0 ns for dIbR.

Because it is well known that ϕ is proportional to the microviscosity according to the Perrin equation (Yguerabide et al., 1970; Lakowicz et al., 1983), we have directly plotted log ϕ as a function of 1/T in Fig. 3. Both for native and dIbR and bO straight lines were obtained with activation energies of 4.5 and 3.4 kcal/mol, respectively. These activation energies are somewhat lower than literature values obtained for the rotation of fluorophores in membranes and micelles, which in general fall in the range of 6–10 kcal/mol (Shinitzky et al., 1971; Shinitzky and Barenholz, 1978). This means that there exists a considerable freedom for at least part of the protein. The fact that bO shows an even smaller activation energy can be understood in terms of an opening of the protein structure when retinal is removed. It was found already that the wavelength of Trp emission maximum shifts from 323 nm in native bR to 335 nm in bO (Jang et al., 1988). This indicates that upon removal of the retinal from the hydrophobic portion of the protein (Seiff et al., 1985), the Trp environments become more hydrophilic, suggesting an opening of the protein structure.

The zero-time value of the fluorescence anisotropy

The anisotropy $r(t)$ is related to the orientational correlation time of the transition dipoles in the ensemble. When the absorption and emission dipoles of the excited molecule are parallel, a zero-time value of 0.4 is expected for $r(t)$. When on the other hand they are perpendicular, $r(0)$ should be -0.2. Although for Trp the former applies, the value of 0.4 is not reached because Trp has two absorbing and emitting states in the UV region, ^1L_a and ^1L_b, and $r(0)$ will depend on the relative fraction of each excited (Valeur and Weber, 1977). Furthermore, $r(0)$ will



decrease because of the emission from states which relax on a timescale too fast to be resolved.

In all samples and at all temperatures studied the $r(0)$ values varied between 0.10 and 0.16. These values are in accordance with those found for the emission of myoglobin (Hochstrasser and Negus, 1984) and *N*-acetyl-L-tryptophanamide (Nordlund and Podolski, 1983), and are also close to those reported for frozen solutions of indole and tryptophan (Valeur and Weber, 1977). For bO the $r(0)$ values are 20–30% larger, probably reflecting the

TABLE 1 Rotational relaxation times, ϕ, and zero-time anisotropies, $r(0)$, of the samples studied

Sample	t	ϕ_1	$r_1(0)$	ϕ_2	$r_2(0)$
	°C	*ns*		*ps*	
bR/dIbR	3	3.0 ± 0.6	0.11 ± 0.02	249 ± 45	0.12 ± 0.02
	18	2.1 ± 0.3	0.10 ± 0.02	240 ± 36	0.12 ± 0.01
	25	1.7 ± 0.3	0.11 ± 0.01	270 ± 40	0.11 ± 0.02
	35	1.3 ± 0.3	0.11 ± 0.02	230 ± 25	0.12 ± 0.02
bO	11	1.3 ± 0.1	0.10 ± 0.02	240 ± 60	0.15 ± 0.05
	19	1.1 ± 0.2	0.14 ± 0.02	265 ± 72	0.17 ± 0.06
	30	0.90 ± 0.06	0.13 ± 0.02	274 ± 23	0.14 ± 0.02
	39	0.79 ± 0.12	0.12 ± 0.02	271 ± 33	0.14 ± 0.03

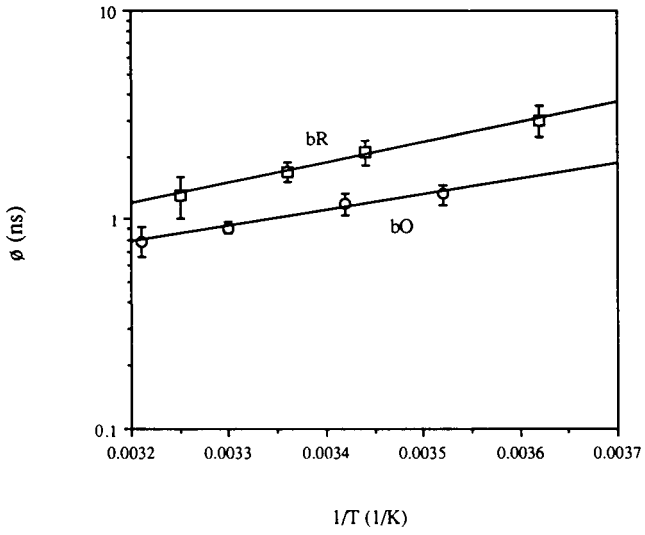


FIGURE 3 Semilogarithmic plot of the rotational relaxation time ϕ vs. $1/T$ for native/deionized bR (*upper line*) and bO (*lower line*). Straight lines are obtained, indicating an exponentially activated behavior of ϕ on temperature. The respective activation energies are 4.5 and 3.4 kcal/mol.

fact that in native and deionized bR the anisotropy is decreased because of energy transfer to the retinal chromophore, which has been shown to be an energy sink for the Trp emission in bR (Palmer and Sherman, 1985; Jang et al., 1988). A slight rotation of a Trp residue might bring it in a more favorable position for energy transfer to occur to the retinal.

It is interesting to note that the $r(0)$ values observed for bR are a factor of 3 larger than those found for rhodopsin (Lubert et al., 1978) when excited at a similar wavelength. In the latter, very fast internal motions or rapid intermolecular energy transfer processes on a femtosecond–picosecond timescale apparently cause a decrease of $r(0)$. This would imply that some Trp residues in rhodopsin have either a considerably greater freedom, or a shorter average Trp–Trp distance. A comparison of the fluorescence anisotropy of the retinal chromophore in rhodopsin (Cone, 1972) and bR (Heyn et al., 1977) on a much longer timescale one sees a similar behavior. Whereas the rotational relaxation time of bR in a membrane is ~500 μs, that of rhodopsin is ~20 μs.

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