

Photoinduced Volume Changes Associated with the Early Transformations of Bacteriorhodopsin: A Laser-Induced Optoacoustic Spectroscopy Study

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ABSTRACT Volume changes associated with the primary photochemistry of bacteriorhodopsin (BR) were measured by temperature-dependent laser-induced optoacoustic spectroscopy (LIOAS). Excitation was performed with 8-ns flashes establishing a photoequilibrium between the BR and the K states ($BR \rightleftharpoons {}^m K$). The concentration of K at the end of the laser pulse, which is an important parameter for the calculation of the volume change per molecule from the LIOAS data, was determined by flash photolysis with optical detection under the specific conditions (concentration, photon density) of the LIOAS experiment. Temperature-dependent measurements yielded a linear dependency of the ratio of the optoacoustic signals for BR and for a calorimetric reference ($CoCl_2$) with the cubic thermal expansion coefficient β of water. From the slope of this linear ratio a contraction of 11 cm^3/mol was determined.

INTRODUCTION

The retinal containing membrane protein bacteriorhodopsin (BR) of *Halobacterium halobium* transports protons across the cell membrane upon illumination (Stoeckenius et al., 1979). This conversion of light energy into electrochemical energy is based on the photochemistry of the retinal chromophore of BR. Essential for this biological function are interactions between the covalently bound chromophore, via a protonated Schiff base to lysine residue 216, and amino acid residues from the binding site which give rise to the strong red shift of the absorption band of BR ($\lambda_{max} = 568$ nm) (Nakanishi et al., 1980; Tavan et al., 1985; Mogi et al., 1988). The proton transport is accomplished during the photocycle of BR, which is initiated by a sub-ps all-*trans* $\rightarrow {}^h$ 13-*cis* photoisomerization of the retinal followed by a series of conformational changes of chromophore and protein and de- and reprotonations of the Schiff base and adjacent amino acids (Mathies et al., 1988; Mathies et al., 1991; Engelhard et al., 1985; Fodor et al., 1988). The high quantum yield of the primary photochemical and following thermal reactions and the rapid turnover of the photocycle, which is completed

within several ms, make BR a very efficient "light driven proton pump" (Oesterhelt and Krippahl, 1983; Lanyi, 1984).

Detailed information on the three-dimensional structure of BR and the interactions between protein and retinal chromophore was gained from a recent model derived from cryo-electron microscopy investigations (Henderson et al., 1990) and from mutagenesis experiments (Butt et al., 1989; Marinetti et al., 1989; Stern and Khorana, 1989) that allowed identification of amino acids participating in the proton pumping. The dynamic processes of chromophore and protein, which take place during the photocycle, and the proton transport have been characterized using several spectroscopic methods. (Mathies et al., 1991; Engelhard et al., 1985; Smith et al., 1985; Braiman et al., 1988; van den Berg et al., 1990; Rothschild 1988; Atkinson et al., 1989).

The investigation of the primary photoreaction, however, is complicated by the fact that the first photoproducts, J and K, can undergo photoreactions within the duration of the exciting flash inasmuch as both species exhibit absorption spectra that strongly overlap with that of ground state BR. This effect has invoked a broad and contradictory discussion on the quantum yield (Goldschmidt et al., 1977; Tokunaga et al., 1976; Grossjean and Tavan, 1988; Birge et al., 1989; Dioumaev et al., 1989; Schneider et al., 1989; Govindjee et al., 1990; Tittor and Oesterhelt, 1990; Xie, 1990; Rohr et al., 1992) and on the amount of energy that is stored in the first photocycle intermediates J and K (for discussion see Rohr et al., 1992). Also, the molecular processes that occur during the primary photoreaction are still a subject of debate (Fodor et al., 1988; van den Berg et al., 1990; Atkinson et al., 1989; Grossjean and Tavan, 1988; Sharkov et al., 1985; Polland et al., 1986; Brack and Atkinson, 1991; Schulten and Tavan, 1978).

The application of sub-ps laser pulses to prevent secondary photochemistry and the combination of such ultrashort flashes with acoustic detection of the heat released by the intermediates using laser-induced optoacoustic spectroscopy

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Abbreviations used in this paper: CHAPS, 3-[(Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate, c_p , heat capacity; n^s , number of Einstein absorbed; n_{BR} , n_K , number of mols BR at the end of the heat integration time; n_K , number of mols K after the laser pulse; α , fraction of "prompt heat"; β , cubic thermal expansion coefficient; ΔV_{th} , thermally induced volume change; ΔV_v , volume change originating from a molecular process other than heating; ΔV_R , volume change per mol; V_{BR} , V_K , V_L , molar volumes of BR, K and L; ρ , density; τ'_a , effective acoustic transit time.

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(LIOAS) was recently demonstrated to circumvent the above mentioned drawback and has allowed the calculation of the quantum yield of formation of K from BR ($\Phi_{\text{BR} \rightarrow \text{K}}$), independent of assumptions on the optical properties of K. Using ns pulses and a mathematical model, the value of $\Phi_{\text{K} \rightarrow \text{BR}}$ was also derived (Rohr et al., 1992).

Temperature-dependent optoacoustic measurements, based on the relation between the signal generation and the underlying thermodynamic parameters yield volume changes associated with the photochemical reaction of a particular system under study (Callis et al., 1972; Westrick et al. 1987). We here report on volume changes during the BR \rightarrow K photoreaction derived from time-resolved, temperature-dependent optoacoustic measurements using ns-flash excitation. The amount of K obtained after ns laser flash excitation was measured with optical detection.

MATERIALS AND METHODS

Sample preparation

BR containing membrane patches were isolated from *Halobacterium halobium* according to literature protocols (Oesterhelt and Stoeckenius, 1974) and were stored at -20°C in the dark until use. Samples were prepared from the stock solution by diluting aliquots with distilled water (pH 7.2). All BR samples were light adapted and were spectrally characterized before and after the measurements to ensure the intact state of the sample. Absorption spectra were measured with a Perkin-Elmer (Norwalk, CT) PE356 spectrophotometer. Samples of $A_{532} = 0.20$ ($A_{568} = 0.26$) were used, corresponding to approximately $4 \mu\text{M}$ ($\epsilon_{\text{max}}: 63000 \text{ M}^{-1}\text{cm}^{-1}$), allowing a simplified signal treatment (Patel and Tam, 1981).

Solubilization of the BR samples was performed by the addition of CHAPS to a final concentration of 15 mM. After shaking for 1 h the solution was centrifuged for 5 min at 15,000 g. The supernatant was characterized by the absorption spectrum before the LIOAS experiments. A small blue shift was observed in the absorption spectrum, in agreement with previous reports (Stark et al., 1984).

"9,12-phenylretinal"-containing BR that was used as an internal calorimetric reference was prepared according to the literature (Kölling et al., 1984).

Laser system

The laser system used was described in detail previously (Rohr et al., 1992) and was operating at 532 nm. A maximal pulse frequency of 5 Hz allowed completion of the BR photocycle before a new laser pulse arrived at the sample. The laser energy was continuously measured during the experiment with pyroelectric power meters (Laser Precision Corp. RJ7620 and RJP-735, Karlsruhe, Germany; Comlinear E103, Fort Collins, CO; and Tektronix 7912, Köln, Germany) before and after the sample to monitor possible changes in absorbance.

Optical detection

Using laser flashes of identical duration, wavelength, and photon density as those used in the LIOAS experiment, absorption flash photolysis measurements were carried out with a previously described detection system (Aramendía et al., 1987). The diameter of the excitation laser beam was magnified by a telescope to get a homogeneous photon density within an area of $5 \times 5 \text{ mm}$ on a fluorescence sample cuvette (pathlength = 5 mm). The detection wavelength was set to 628 nm to get a large difference in the absorption coefficients of BR and K. The analyzing light was filtered by an interference filter ($\lambda_{\text{max}} = 628 \text{ nm}$). The concentration of the sample was the same as in the LIOAS experiment.

LIOAS detection system and signal analysis

The LIOAS setup and piezoelectric resonant ceramic detector (PZT-type, Vernitron 4 mm \times 4 mm) were described previously (Rohr et al., 1992; Braslavsky and Heihoff, 1989). Taking into account the sound velocity in the medium the heat integration time (vide infra) was adjusted to 200 ns by means of a 0.3 mm pinhole.

The samples were thermostated between 6 and 27 (± 0.1) $^\circ\text{C}$ with a PT100 resistor placed directly into the sample cuvette. To improve the signal-to-noise ratio, all optoacoustic signals were averaged 200 times, amplified by a factor of 100 (Comlinear E103), and stored using a Tektronix 7912 transient recorder. Further signal treatment was performed by a RISC-workstation (DEC-station 5000/200) and a PC (IBM compatible 80386).

The analysis of the LIOAS signal was performed as described previously (Heihoff et al., 1987) with the exception that the difference between the first maximum and minimum was measured as the optoacoustic signal H to reduce the error for small signals at low temperatures. CoCl_2 solutions in water, matched in absorbance to those of BR were used as calorimetric reference (Braslavsky and Heihoff, 1989).

In LIOAS, the heat emitted by a sample after absorption of a laser pulse generates an optoacoustic signal that is based on the thermally induced volume change ΔV_{th} . This volume change gives rise to a pressure wave detectable with a piezoelectric transducer (Patel and Tam, 1981) and is proportional to the term $\beta/c_p\rho$ which describes the thermoelastic properties of the sample

$$\Delta V_{\text{th}} \propto \alpha \frac{\beta}{c_p\rho} E_{\text{exc}}(1 - 10^{-A}) = \alpha \frac{\beta}{c_p\rho} n^s E_\lambda \quad (1)$$

In Eq. 1 β is the cubic thermal expansion coefficient, c_p is the heat capacity at constant pressure, ρ is the density, and α is the fraction of absorbed energy $E_{\text{exc}}(1 - 10^{-A})$ dissipated into the medium as "prompt heat." E_{exc} is the energy of the laser pulse, A is the absorbance of the sample (Patel and Tam, 1981; Braslavsky and Heihoff, 1989), E_λ is the energy per einstein of the laser pulse, and consequently n^s is the number of einsteins absorbed. For short pulses, the prompt heat is integrated over a time that depends on the effective acoustic transit time ($\tau'_a = 2R/v_a$, with $2R$, beam diameter and v_a , velocity of sound in the solvent) of the sound wave traveling through the laser beam cross section. For aqueous systems, τ'_a is in the range of ns to μs , depending on R . The optoacoustic signal amplitude H is proportional to the heat dissipated to the solvent during τ'_a (Heihoff et al., 1987) through an instrumental constant k (Eq. 2).

$$H = k \alpha \frac{\beta}{c_p\rho} E_{\text{exc}}(1 - 10^{-A}) \quad (2)$$

In addition to ΔV_{th} , a volume change ΔV_r originating from a molecular process other than heating (i.e., conformational or solvation changes) may induce a pressure change and, consequently, an optoacoustic signal (Callis et al. 1972; Westrick et al., 1987; Ort and Parson, 1979; Strauss and Walder, 1988; Peters et al., 1991; Rudzki-Small et al., 1989; Braslavsky and Heibel, 1992). Thus, the equation describing the optoacoustic signal amplitude of a sample H^s has to be extended to account for ΔV_r ,

$$H^s = k[\Delta V_{\text{th}} + \Delta V_r] \quad (3)$$

Similar to α , describing the prompt heat in the thermal part of the signal, the quantum yield Φ of the induced process (amount of photoproduct) has to be taken into account for ΔV_r that renders Eq. 3 into Eq. 4 ($\Delta V_r = n^s \Phi \Delta V_R$, with ΔV_R as volume change per mol).

Thus

$$H^s = kn^s \left[\underbrace{(\beta/c_p\rho)\alpha E_\lambda}_{\text{temperature dependent}} + \underbrace{\Phi \Delta V_R}_{\text{temperature independent}} \right] \quad (4)$$

Because for a calorimetric reference the photon-to-heat conversion is unity, and in addition no photoinduced volume change is expected, ΔV_R in Eq. 4 drops to zero. Thus, the ratio of the energy-normalized signals for

sample, H_n^S , and reference, H_n^R , yields the following correlation

$$\frac{H_n^S}{H_n^R} = \alpha + \frac{\Phi \Delta V_R c_p \rho}{E_\lambda \beta} \quad (5)$$

The strong temperature dependence of the β -value between 4°C and room temperature (Weast, 1984) allows the evaluation of $(\Phi \Delta V_R / E_\lambda)$ of Eq. 5 in temperature-dependent LIOAS measurements.

This treatment implies that the species initially photoproducted by the laser pulse does not, in turn, absorb at the wavelength of the laser and that it decays in a time longer than the heat integration time (τ'_a), i.e., it is photo and thermally stable during the time window of the experiment. This is the case for the photoisomers of the carbocyanines DODCI and DOCl (Churio et al., 1994). In the case of BR, a photochromic equilibrium is established within the relatively long 8-ns pulse duration (Rohr et al., 1992). Consequently, the value of Φ in Eq. 5 describes a composite quantum yield that should take into account the forward and back photo-reactions. In addition, the relatively short lifetime of K in the case of BR requires the use of small values of τ'_a . In the following section a more general development is made.

Time-resolved measurements

In general, the measured volume change represents all changes taking place within the heat integration time, τ'_a , including the step $BR \rightarrow^{h\nu} K$ and in part the step $K \rightarrow^{h\nu} L$. Equation 6 describes the overall volume change during this time period.

$$\Delta V'_t = \int_0^{\tau'_a} \frac{dV}{dt} dt \quad (6)$$

Thus the measured value of $\Delta V'_t$ should reflect the difference between the final (according to the heat integration time τ'_a) and the initial volumes in the sample.

$$\Delta V'_t = V'_t - V'_0 = V_{BR} n_{BR}|_{\tau'_a} + V_K n_K|_{\tau'_a} + V_L n_L|_{\tau'_a} - V_{BR} n_{BR}|_{t=0} \quad (7)$$

V_{BR} , V_K , and V_L are the molar volumes of the intermediates BR, K, and L. The corresponding number of mols within the excited volume are given by

$$n_{BR}|_{\tau'_a} = n_{BR}|_l = n_{BR}|_0 - n_K|_l; \quad n_K|_{\tau'_a} = n_K|_l e^{-\tau'_a/\tau_K}; \quad (8)$$

$$n_L|_{\tau'_a} = n_K|_l (1 - e^{-\tau'_a/\tau_K})$$

The subscript τ'_a identifies the number of mols at the end of the heat integration time, and the subscript l describes the number of mols of BR and K at the end of the laser pulse. Therewith, the integrated volume change is given by Eq. 9, which now consists of a time-dependent and a time-independent term

$$\Delta V'_t = [(V_L - V_{BR}) + (V_K - V_L) e^{-\tau'_a/\tau_K}] n_K|_l \quad (9)$$

For $\tau'_a \ll \tau_K$

$$\Delta V'_t = (V_K - V_{BR}) n_K|_l \quad (10)$$

The photoequilibrium between BR and K requires that the value of $n_K|_l$ is measured optically, using identical photon density conditions as those in the LIOAS measurements. From the value of $n_K|_l$ obtained by flash photolysis with optical detection, it is possible to derive the value of $(V_K - V_{BR})$, i.e., the difference between the volumes of K and BR. For $\tau'_a = 200$ ns, less than 15% of K decayed to L within the heat integration time, and the above approximation is sufficient. Thus, Eq. 5 turns into Eq. 11 after replacement by Eq. 10

$$\frac{H_n^S}{H_n^R} = \alpha + (V_K - V_{BR}) n_K|_l \frac{1}{n^s E_\lambda} \frac{c_p \rho}{\beta} \quad (11)$$

RESULTS

The time-dependent absorbance of K was observed by flash photolysis with optical detection at 628 nm. The maximum amplitude of the absorbance change at the end of the laser pulse ΔA showed a linear relationship with the photon density up to 8 mJ cm⁻², corresponding to an excitation energy of about 5.7 μJ using a 0.3 mm pinhole in the LIOAS experiment (Fig. 1). Above this photon density saturation occurred. From the value of ΔA the number of mols K after ns excitation, $n_K|_l$, was calculated by Eq. 12

$$n_K|_l = \frac{\Delta A}{d(\epsilon_K - \epsilon_{BR})} V_{exc} \quad (12)$$

where $d = 5$ mm is the length of the sample cuvette and $V_{exc} = 0.25$ cm³ the excited volume. The difference of the absorption coefficients of BR and K at 628 nm is $(\epsilon_K - \epsilon_{BR}) \approx 23 \times 10^3$ M⁻¹cm⁻¹ (Gärtner, unpublished data). In the linear range, the ratio of $n_K|_l$ to the number of einsteins absorbed is therefore $n_K|_l/n^s = 0.09$.

The influence of the photoequilibrium $BR \rightleftharpoons K$ on the absorbance of the sample during the ns pulse and, therefore, on n^s was calculated using a model including the excitation of K and its photochemical back reaction to BR (Rohr et al., 1992). The absorbed energy, E_a , was calculated by Eq. 13,

$$E_a = E_{exc} \int_{pulse} (1 - 10^{-A(t)}) L(t) dt \quad (13)$$

where $A(t)$ is the total absorbance of the sample at time t and $L(t)$ is the temporal distribution of the laser beam with $\int L(t) dt = 1$. The quantum yield for the reaction $BR \rightarrow K$ was taken as 0.6 (Govindjee et al., 1990; Rohr et al., 1992). For the back reaction $K \rightarrow BR$, values between 0.6 (Rohr et al., 1992) and 1.0 (Govindjee et al., 1990; Xie, 1990; Bazhenov et al., 1992) were chosen. The lifetime of excited K was varied between 1 and 10 ps. The other rate constants were taken from the study by Rohr et al. (1992). Using these

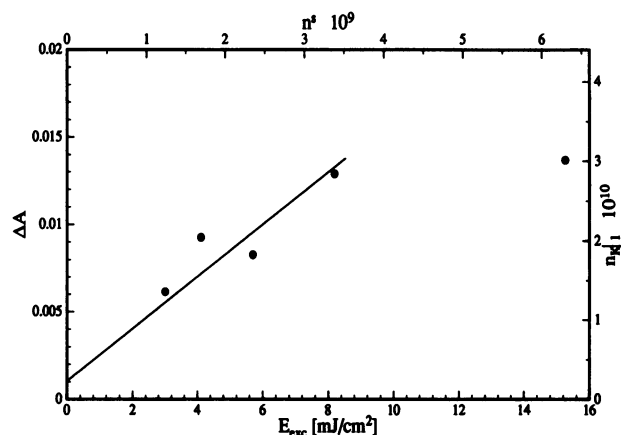


FIGURE 1 Amplitude of the absorbance change of BR at $\lambda_{obs} = 628$ nm and calculated concentration $n_K|_l$ of K after the laser pulse versus excitation energy density and number of einsteins absorbed, n^s ; $\lambda_{exc} = 532$ nm, $A_{532} = 0.20$.

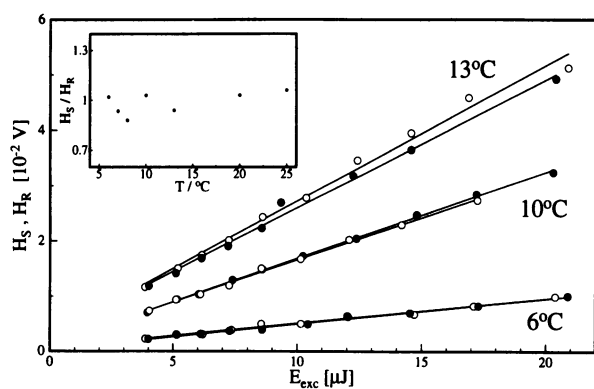


FIGURE 2 Amplitude of the LIOAS signals of 9,12-phenylretinal (*closed circles*) (see text), H_S , and CoCl_2 (*open circles*), H_R , of matched absorbance ($A_{532} = 0.15$) at the excitation wavelength (532 nm, beam diameter 1 mm) versus excitation energy. The inset shows the ratio of the signal amplitudes H_S/H_R at different temperatures.

parameters and the experimental conditions of the LIOAS experiment ($E_{\text{exc}} < 4 \mu\text{J}$, pinhole 0.3 mm, $\lambda_{\text{exc}} = 532 \text{ nm}$) the change in the absorbed energy E_a was less than 6% of the value of E_a calculated using the absorbance without excitation. This is within the error of the measurement. Changes in the absorbance because of the photoequilibrium can therefore be neglected under these conditions.

In the LIOAS experiment, CHAPS-solubilized BR solutions were used to produce monomeric BR (Stark et al., 1984; Casadio et al., 1980; Kropf, 1982) and, thus, to avoid interactions between patches.

The absorption coefficient of CoCl_2 is relatively low ($\epsilon_{\text{max}} \approx 3 \text{ M}^{-1}\text{cm}^{-1}$). The concentration needed to produce an absorbance of $A \approx 0.2$ may therefore change the value of β for pure water. However, at the wavelength used in this work ($\lambda = 532 \text{ nm}$), excitation of aqueous CoCl_2 solutions at temperatures between 6 and 27°C gave identical LIOAS signals as buffered (10mM TRIS buffer in H_2O) Evans blue and bromocresol purple solutions of matched absorbance. This indicates that the concentration of CoCl_2 is still low enough to preserve the thermoelastic properties of distilled water. However, at other wavelengths at which CoCl_2 absorbs less, this was not the case.

The possible influence of the protein surrounding the retinal chromophore on the value of β of water was examined. For this purpose, a retinal derivative (“9,12-phenylretinal”) was used as internal reference. This derivative reconstitutes a BR pigment but is sterically hindered in all-*trans* \rightarrow 13-*cis* isomerisation in the BR binding site (Kölling et al., 1984). In other words, no photoproduct is formed in the reconstituted BR. Because of the lack of photochemistry it acts as a calorimetric reference releasing all absorbed energy as prompt heat, whereas the chromophore environment is the same as for BR. Fig. 2 shows the linear energy dependence of the optoacoustic signals of this reference and CoCl_2 of matched absorbance at the excitation wavelength ($\lambda_{\text{exc}} = 532 \text{ nm}$). The inset contains the ratio of the signals at different temperatures. The LIOAS signal in water at temperatures

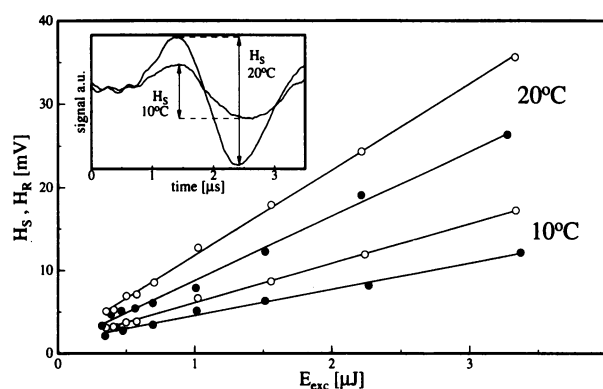


FIGURE 3 Amplitude of the LIOAS signals of BR (*closed circles*), H_S , and CoCl_2 (*open circles*), H_R , ($\lambda_{\text{exc}} = 532 \text{ nm}$, $A_{532} = 0.20$, beam diameter = 0.3 mm) versus excitation energy at 10 and 20°C. The inset shows the LIOAS signals of BR at 10 and 20°C; the arrows indicate the signal amplitude H_S .

between 6 and 25°C was the same for the BR-analogue as for CoCl_2 within the error of the measurement. Therefore, CoCl_2 is an appropriate reference for BR in water, and the thermoelastic parameters c_p , ρ , and β of distilled water can be used for both sample and reference. This result also confirms that the patches still present in this reference seem not to affect the thermoelastic parameters of the sample.

The LIOAS signal of BR, as well as the signal of CoCl_2 , were linear with excitation energy between 10 and 27°C (Fig. 3). The excitation energy was $E_{\text{exc}} < 4 \mu\text{J}$ corresponding to < 3 photons absorbed per molecule over the 8 ns duration of the pulse to guarantee for a proportionality $n_K |l| \propto E_{\text{exc}}$. At lower temperatures no reasonable signals could be detected because of the low signal-to-noise ratio at the low excitation energy used. At 4°C, no signal was detected because the volume change ΔV_r was too small because of the low total quantum yield $\Phi = (n_K |l|/n^s)$. The ratios of slopes for the various temperatures are plotted versus $c_p \rho / \beta$ (Fig. 4) according to Eq. 11. The slope of this resulting plot yields $\Delta V_r = 4.4 \times 10^{-6} \text{ cm}^3 \text{ J}^{-1}$, which, divided by the value of

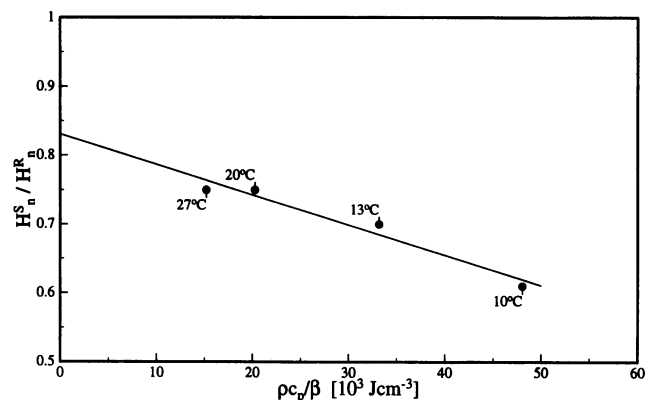


FIGURE 4 Ratio of energy-normalized LIOAS signals of BR and CoCl_2 , H_S^0/H_R^0 , versus $c_p \rho / \beta$, between 10 and 27°C, $\lambda_{\text{exc}} = 532 \text{ nm}$, $A_{532} = 0.20$, $\tau_\alpha = 200 \text{ ns}$.

$n_K |l/n^s = 0.09$ obtained from the flash photolysis experiment and E_λ , yields a value $\Delta V_R = V_K - V_{BR} = -11 \text{ cm}^3 \text{ mol}^{-1}$, i.e., a contraction.

DISCUSSION

The value of the molecular volume change, $\Delta V_R = -11 \text{ cm}^3 \text{ mol}^{-1}$, derived from the slope of Fig. 4, indicates that a contraction occurs during the photoreaction of BR. This result is of the same order of magnitude as that already reported as a contraction of $(7.8 \pm 3.2) \text{ cm}^3 \text{ mol}^{-1}$, derived from the pressure dependence of the all-*trans* \rightleftharpoons 13-*cis* equilibrium of dark-adapted BR (Tsuda and Ebrey, 1980).

Although at first sight the comparison between the two sets of data is tempting, we should note that several of the transients in the BR photocycle have a chromophore with a 13-*cis* conformation. However, the protein around the chromophore certainly adopts different conformations and suffers charge redistributions that may lead to volume changes in addition to the volume change induced by isomerization of the chromophore. Therefore, this comparison might not be of any significance.

Attempts to make the same type of temperature-dependent measurements using fs pulses to avoid the simultaneous excitation of BR and K have so far been impaired by technical difficulties. It has not yet been possible to use the shortest transit time, 200 ns, that would guarantee a small contribution from K decay to the heat signal. In particular, at lower temperatures (10 or 13°C) the signal-to-noise ratio after fs excitation was too low. Larger transit times cannot be used for these calculations, inasmuch as the energy difference between K and L is not known (Rohr et al., 1992). The value of $\alpha = 0.83 \pm 0.05$ extrapolated from Fig. 4 agrees with the value obtained for α under the condition of photoequilibrium determined for 532 nm in (Rohr et al., 1992).

One condition for the validation using Eq. 5 or Eq. 11 is that the decay $K \rightarrow L$ should not contribute to the heat emitted at any temperature. Using the value of $\tau'_\alpha = 200 \text{ ns}$ at 20°C, less than 15% K should decay within that time. This validates the use of Eqs. 5 and 11.

The value of ΔV_R should be attributed to a change in the protein arrangement around the chromophore concomitant with the photoinduced isomerization. Studies with model compounds have shown that relative large volume changes can be attributed to changes in the solvation sphere of photoisomers with different dipole moments (Churio et al., 1994). Nevertheless, changes in the protein conformation within such short periods also have been identified (Diller et al., 1991; Gat et al., 1992). Therefore, this contraction observed between BR and K in this paper need not necessarily be correlated only with the intrinsic changes in the chromophore but, rather, with the whole movement of the complex within 200 ns after excitation.

In conclusion, a contraction has been determined for the $BR \rightarrow K$ step in the BR photocycle by using laser-induced optoacoustics with ns excitation and a short heat integration time. The amount of K resulting at the end of the ns pulse

used and arising from the establishment of the $BR \rightleftharpoons K$ photoequilibrium within the pulse duration was determined by flash photolysis with optical detection.

A possible hypothesis for the found contraction is that it acts as a storage of energy that should be released at a later step of the BR photocycle, e.g., in a sum of expansions needed for the proton pumping. Measurements in longer time scales will help to support or disprove this hypothesis.

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