J. Braz. Chem. Soc., Vol. 6, No. 2, 167-171, 1995.
Printed in Brazil.

0103 - 5053 $5.00 + 0.00

Photothermal Studies with Biological Photoreceptors. The Bacteriorhodopsin Photocycle

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Received: October 21, 1994; November 14, 1994

A determinação da variação fotoiniciada de volumes moleculares em sistemas biológicos complexos usando técnicas fototérmicas é ilustrada por estudos optoacústicos e de deflecção fototérmica de feixe induzidos por laser, em bacteriorhodopsin (BR) em membranas púrpura. É detectada uma pequena contração na janela temporal de 200 ns depois do pulso do laser (correspondendo ao processo BR $\rightarrow$ K), enquanto são observadas duas expansões consecutivas maiores (correspondendo às etapas K $\rightarrow$ L, 1.3 μs e L $\rightarrow$ M, 90 μs, respectivamente), seguidas por uma contração final (correspondendo à reformação da BR). As variações de volume são interpretadas com base em uma reorganização por etapas do meio ao redor do cromóforo isomerizado, com distribuição de carga diferente da BR.

The determination of photoinitiated molecular volume changes in complex biological systems with photothermal techniques is illustrated by the use of laser-induced optoacoustic and photothermal beam deflection studies with bacteriorhodopsin (BR) in purple membrane. A small contraction is detected with a time window of 200 ns after the laser pulse (corresponding to the process BR $\rightarrow$ K), while two consecutive larger expansions (corresponding to the steps K $\rightarrow$ L, 1.3 μs and L $\rightarrow$ M, 90 μs, respectively) followed by a final contraction (corresponding to the reformation of BR) are observed. The volume changes are interpreted on the basis of stepwise medium reorganization around the isomerized chromophore with a charge distribution different from that of BR.

Keywords: *optoacoustics, photoacoustics, phototermal beam deflection, laser-induced photoreceptors, photocycle, volume changes, bacteriorhodopsin*

Introduction

The nature of the chromophore-protein interactions in biological photoreceptors is of basic importance since it is intimately related to the specific function of each of the photoreceptors. In all photoreceptors the photoinduced reaction of the chromophore starts a chain of reactions implying in many cases (*e.g.*, in rhodopsin, bacteriorhodopsin, and phytochrome) a series of conformational movements in the apoprotein, eventually leading to the signal transduction. Time-resolved photothermal methods offer an alternative to the commonly used optical methods in order to study the time-resolved enthalpy changes and protein movements triggered by the photoinduced reactions, in addition to the kinetics. Callis *et al.*[1] have applied pulsed excitation with acoustic detection for the first time for the determination of the volume changes in *Chromatium* chromatophores. Various biological photoreceptors were subsequently studied using this method by Parson's research group[2,3]. In more recent times, the availability of lasers and more advanced electronics has given a new impulse to the use of photothermal methods.

With most biological photoreceptors (*e.g.*, bacteriorhodopsin and phytochrome), primary quantum yields are difficult to determine by optical methods, due to the spectral overlap between the parent molecule and the first intermediate(s). In these cases, photothermal methods can be applied with great advantage.

Essentially, the photothermal effect is based on the fact that, after pulse excitation, a volume change takes place in the medium. The volume change may be detected by a rapid pressure transducer and in this case we call the method laser-induced optoacoustic spectroscopy (LIOAS)[4]. This method is applicable to events in the range of few ns to *ca.* 10 μs. The photoinduced volume change may be composed of two terms: (i) the change (in most solvents an expansion)

Invited lecture presented at the 4th Latin-American Photochemical Meeting, April 1994, Valparaiso, Chile.

due to the release of heat by radiationless processes from the excited molecules and (ii) the possible volume change due to photoinduced movements concomitant with the photoreaction. While the first contribution is temperature dependent, since it depends on the ratio of thermal expansion coefficient and specific heat of the medium (β/c_p), the second generally is not, specially in the relatively small temperature range analysed. Thus, both contributions can be separated by temperature dependent measurements[1,5,6]. This fact is of special interest in aqueous solutions, since the value of that ratio, and particularly of β, in these solutions strongly depends on temperature.

As an alternative to pressure detection, the volume change may be detected by the refractive index (n) changes induced in the medium. These changes are monitored with a continuous wave (CW) beam parallel to the excitation beam and of a wavelength far apart from the absorption of reactants, intermediates and products. This method has been called photothermal beam deflection (PBD)[7] and is related to thermal lensing. PBD could be also called thermal prism, since the CW beam is deflected as a result of the photoinduced changes in n. With PBD, the time range available for the studies is longer than with LIOAS, since thermal diffusion is slow and the changes in n induced in the solution remain for relatively long times. Thus, using LIOAS and PBD, the time range from few ns to several ms can be studied. The ms time range is particularly interesting for biological photoreceptors, since within these times large protein movements related to their physiological function are expected.

When monitoring time-resolved changes in n, in addition to the two contributions to the volume changes described above, a third contribution (iii) should be considered, determined by absorbance changes taking place at wavelengths far apart from that of the monitoring beam. This is due to the fact that the refractive index spectrum is much broader than the absorbance spectrum of a solution. Both quantities (absorbance and n) are related through the Kramers Kronig relationship[8]. This contribution to the changes in n has been sometimes called "population lens"[9].

We have performed temperature-dependent LIOAS studies with several photoreceptors, in order to time-resolve the enthalpic and molecular movements triggered by light and taking place in few microseconds[10-12].

A prerequisite for the application of PBD to photoreceptors in the longer time ranges, is the development of a procedure which leads to the separation of contribution (iii) (*vide supra*) to the photoinduced time-resolved refractive index changes. Schulenberg has now carried out the necessary analysis of the PBD signal and applied it to BR in aqueous suspensions of membrane patches[13]. As far as we know, this is the first application of PBD to a biological photoreceptor.

The results of the LIOAS and PBD measurements with BR are summarized in the next section. The application of both methods has enabled us to determine accurately the primary quantum yield for the transformation of BR to the first intermediate, K, the energy of this intermediate, to evaluate the quantum yield of the photoinduced back reversion of that intermediate, and to time-resolve the molecular volume changes in the complete photocycle of BR.

The bacteriorhodopsin photocycle

The intrinsic membrane protein bacteriorhodopsin (BR) of *Halobacterium halobium* is a photoreceptor which converts the energy of absorbed light into electrochemical energy, which is stored in form of a proton gradient across the cell membrane[14]. Essential for this process is the retinal chromophore of BR which is covalently bound to the apoprotein via a protonated Schiff base formed with the lysine residue 216. Light absorbed by the retinal molecule initiates a reaction cycle which starts with a subpicosecond all-*trans* $\rightarrow$ 13-*cis* photoisomerization of the retinal molecule and is completed by the re-formation of BR after several milliseconds. During the photocycle several spectrally discernible intermediates (named K, L, M, N, and O) are formed. The Schiff base is deprotonated during the L $\rightarrow$ M transition and reprotonated during the M $\rightarrow$ N transition. The knowledge of the time course of the conformational changes of the protein during the photocycle is of great interest.

Due to the spectral overlap between BR and K, and taking into account the fact that K is produced within few ps after excitation[15], a photochromic equilibrium between BR and K is built within the duration of pulses longer than few picoseconds. Using pulses of 585 nm and < 500 fs duration, in order to avoid the build-up of this photochromic equilibrium, the extent of K formation after the pulse was obtained by LIOAS from the heat stored after excitation[16].

On the basis of the LIOAS measurements with BR using 8 ns pulses of variable fluence and wavelength, a mathematical description of the system BR $\leftrightarrow$ K $\rightarrow$ L was developed, which yielded an energy content of 160 kJ/mol for K, *i.e.*, 30 kJ/mol lower than the 0-0 level of BR. With this value, combined with the fs LIOAS data, it was possible to calculate the quantum yield for the BR $\rightarrow$ K photoinduced process: $\Phi_{BR \rightarrow K} = 0.6$. Values for the quantum yield of the back photoconversion, K $\rightarrow$ BR, $\Phi_{K \rightarrow BR} = 0.6$ and for the lifetime of excited K, τ_{K^*} = 4-10 ps, also resulted from the calculations[16].

The volume change associated with the BR $\rightarrow$ K phototransformation was determined using temperature-dependent LIOAS measurements. As outlined in a previous paragraph, the amplitude of the photoinduced acoustic signal produced within the heat integration time (*i.e.*, the

transit time of the acoustic wave front through the laser beam diameter, in our case 200 ns) from a sample (S) undergoing photochemistry, H^S, results from a volume change composed of two terms, a thermal one arising from energy relaxation, ΔV_{th}, and a change arising from molecular processes other than heating, $\Delta V_r = n^S \Phi V_R$, with n^S the number of einsteins absorbed, Φ the quantum yield of the reaction, and ΔV_R the volume change per mol. Thus,

$$H^S = k\ (\Delta V_{th} + \Delta V_r) \quad (1)$$

The first term on the right side of Eq. 1 is directly proportional to the absorbed energy, $E_a = n^S E_\lambda$ (E_λ is the energy of each einstein of wavelength λ), to the fraction of absorbed energy promptly (within the heat integration time) dissipated as heat, α, and to the ratio of thermoelastic parameters of the medium, β/c_p (β, volume expansion coefficient; c_p, heat capacity; ρ, mass density). Thus, Eq. 2 results:

$$H^S = k\ n^S [(\beta/c_p \rho)\ \alpha E_\lambda + \Phi \Delta V_R] \quad (2)$$

In order to calibrate the system and eliminate k from Eq. 2, a calorimetric reference[4] is used for which $\alpha = 1$ and $\Delta V_R = 0$. Thus, for the reference, Eq. 3 is obtained

$$H^R = k\ n^R [(\beta/c_p \rho)\ E_\lambda] \quad (3)$$

Calorimetric references for the LIOAS work with BR were aqueous $CoCl_2$ solutions between 6 and 27 °C, which gave identical results as buffered evans blue and bromocresol purple. Measurements with "9,12-phenyl-retinal-BR", which carried a retinal derivative with an impeded all-*trans* → 13-*cis* isomerization[17] as calorimetric reference, yielded identical results as those with the other references. Thus, the thermoelastic parameters of the protein solution should be the same as those for water[18].

The ratio of the energy-normalized (care was taken to work always in the linear energy range) signals, S, for sample and reference (Eqs. 2 and 3) in the same solvent and measured under identical conditions is therefore expressed as in Eq. 4[5,6].

$$S = \alpha + (\Phi \Delta V_R / E_\lambda)\ (c_p \rho / \beta) \quad (4)$$

The strong dependence of the β value between 4 °C and room temperature in water and aqueous solutions, allows the evaluation of the term $(\Phi \Delta V_R / E_\lambda)$ in Eq. 4. This treatment of the signals implies that the intermediate species produced by the laser pulse do not, in turn, absorb the laser light within the pulse duration and that they do not decay within the heat integration time of the experiment. This is the case for the photoisomers of the carbocyanines DODCI and DOCI, which we used as models for isomerizable polyenes[19]. Since in the case of BR, the photoproduced species, K, absorbs also the laser radiation within the duration of the 8 ns laser pulse, the value of Φ describes a composite quantum yield. The actual number of molecules converted to K at the end of the 8 ns pulse was thus measured using flash photolysis with optical detection[18]. The heat integration time of the LIOAS experiment was kept very short (200 ns) in order to keep negligible the heat delivered by the decay of K.

Under the above conditions, using Eq. 4 and measuring in the temperature range 4 - 27 °C, a molecular volume contraction of $\Delta V_R = -11$ cm^3/mol was determined for the BR → K process in detergent-treated BR (the BR membrane patches were dissolved in the detergent CHAPS)[18]. Based on our studies with the isomerizable model compounds DODCI and DOCI[19], we attribute the contraction observed for the process BR → K (equivalent to 18 Å^3 molec^{-1}) to a change in protein arrangement around the chromophore, concomitant with the photoinduced isomerization.

Laser-induced PBD with 8 ns pulse excitation was used to time-resolve *n* changes taking place during the BR photocycle in aqueous suspensions of purple membrane. Monitoring was by a non-absorbed CW probe beam (λ = 825 nm). Calibration was performed using bromocresol purple. Also in the longer time ranges studied by PBD, measurements with suspensions of the "9,12-phenyl-retinal-BR" yielded the same results as bromocresol purple solutions, indicating that the thermoelastic parameters for the protein suspension can be taken as those of water, for every time range[13].

For the two time ranges analysed (1 - 200 µs and 200 µs to 10 ms) the PBD signal was well fitted to a sum of exponential decays, *i.e.*, $S(t) = a_0 + \Sigma a_i [1 - \exp(-k_i t)]$. For each decay the preexponential factor a_i was taken as a sum of the three contributions to the refractive index change, *i.e.*, (i) the heat release due to relaxation, (ii) molecular volume changes concomitant with the formation of the i intermediate, and (iii) absorbance changes. Contribution (i) is isotropic and (ii) can be taken as such, while contribution (iii) was studied using linear dichroic measurements, *i.e.*, exciting the sample with a polarized laser pulse and monitoring the transient absorbance with light polarized parallel ($\Delta A_\parallel$) or perpendicular ($\Delta A_\perp$) to the polarization of the exciting beam[13]. The development of each preexponential factor in the three additive terms (i), (ii), and (iii) mentioned above, was made under the assumption that for the multiexponential decay, for the time constants it holds that $\tau_i << \tau_{i+1}$, and that enthalpy, volume, and absorbance changes are of comparable size for all steps.

The value of $(\Delta A_\parallel / \Delta A_\perp)$ for BR in aqueous suspensions of purple membrane remained unchanged up to 200 µs and changed < 10% up to 10 ms, indicating that the membrane particles rotate very slowly. This lack of time dependence in the anisotropy of the membrane patches with BR was a

key factor which allowed the separation of contribution (iii) to the refractive index change. On the contrary, when the patches were destroyed by the addition of detergent CHAPS, the anisotropy rapidly decayed[13].

Under the educated assumption that the ratio of polarized absorbance changes is identical to the ratio of the anisotropic part of polarized PBD measurements, contribution (iii) was evaluated from PBD measurements with polarized excitation and detection and subtracted from the experimental a_i values. Since for all time ranges the a_i factors were constant with temperature, for the range 10-30 °C, within the experimental error, term (i) was always much smaller than terms (ii) plus (iii)[13]. Thus, the volume change accompanying each step was the remaining part of the signal. Subsequent to the contraction of 11 cm^3/mol for the process BR → K, an expansion of 60 (K → L) followed by another expansion of 145 cm^3/mol (L → M) were observed. A contraction of 185 cm^3/mol was determined for the 6 ms M →→ BR decay[13].

Within the experimental error, the balance of volume changes is (10 ± 75) cm^3/mol, although the contraction determined by LIOAS was for detergent-treated BR. In any case, the contribution of the BR → K contraction is relatively small compared to the subsequent expansions and recontraction.

Since the isomerization of the retinal chromophore determines strong changes in the electrostatics and structure of the protein crevice, we attribute at least part of the overall volume changes (which obviously imply a density change) to changes in the tertiary structure of the protein, concomitant with the electrostatic changes determined by the chromophore isomerization.

Our present data are compatible with recent electron diffraction[20] data on structural differences between BR and the M state, in which the protein opens to the cytoplasmatic side. These data indicate that 9 water molecules are displaced between these two states[21]. Since our results show a density change, rather than just a structural change between the various states, they indicate a netto density change equivalent to *ca.* 10-11 water molecules.

The structural volume changes we observe correlate linearly with the absorption maxima of the transients which, in turn, correlate with the C=C stretching as determined from time-resolved resonance Raman studies[22]. A contraction is determined on going from blue to red absorption maximum (higher to lower stretching frequencies), while an expansion results when going in the opposite direction. Albeit with only four points, this indicates that all volume changes, up to the ms times, correlate with changes influencing also the chromophore spectrum. A larger delocalization (decreased bond order) in the chromophore correlates with a contraction, while decreased conjugation (increased bond order) correlates with volume expansions.

The 20 μs intermediate detected by PBD, in addition to the 1.3 and 90 μs observed by time-resolved absorption[23], arose only from contribution (iii) being thus only determined by absorbance changes integrated over the whole spectrum and more sensitively detected by PBD. The large contributions of the volume changes to the total a_i values impaired the evaluation of the relative energy levels of the various intermediates. The energy differences between the various intermediates after K result to be very small.

For the photoreceptor phytochrome in buffer (a dimer with a MW of 248 kDa), the anisotropy was lost in the first μs. This has hindered, so far, PBD measurements with this photoreceptor in soluble form.

Acknowledgments

The work on BR is the result of a fruitful team work with Dr. W. Gärtner, P.J. Schulenberg, and Dr. M. Rohr. I thank Professor A. Holzwarth and his group for the collaboration in the area of fs excitation, P. Schmidt for his contribution to the polarized light measurements, and J. Straßburger for the preparation of the BR samples. Discussions with the co-authors (coworkers and colleagues) of our papers in the photothermal area led to many of the conclusions exposed. I am deeply indebted to G. Koç-Weier, D. Lenk, S. Pörting, W. Schlamann, and G. Wojciechowski for able technical assistance. My thanks also go to Professor Kurt Schaffner for his constant support.

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