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Photoacoustic Calorimetric Study of the Conversion of Rhodopsin and Isorhodopsin to Lumirhodopsin†

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ABSTRACT: The enthalpy and volume changes for the conversion of rhodopsin and isorhodopsin to lumirhodopsin have been investigated by time-resolved photoacoustic calorimetry. The conversion of rhodopsin to lumirhodopsin is endothermic by 3.9 ± 5.9 kcal/mol and is accompanied by an increase in volume of 29.1 ± 0.8 mL/mol. The lumirhodopsins produced from rhodopsin and isorhodopsin are energetically equivalent.

The bleaching of the vertebrate photoreceptor rhodopsin initiates a series of enzymatic reactions leading to visual transduction (Fung et al., 1981). The first step in the enzymatic cascade is the association of a GTP-binding protein, transducin, with photoactivated rhodopsin (Baehr et al., 1982; Wessling-Resnick & Johnson, 1987). Rhodopsin is an integral membrane protein (39 kDa) located in disk membranes of the rod outer segment (ROS)[1] of the retina. This glycoprotein consists of an apoprotein, opsin, and a chromophore, 11-*cis*-retinal, attached via a protonated Schiff base. Following absorption of a photon, the chromophore undergoes an isomerization to the all-trans form, which causes the protein to thermally evolve through a series of conformational changes that are revealed as spectroscopically distinct metastable species (Birge, 1990). The dynamics of the decay of these metastable species have been well characterized at ambient temperatures (Applebury, 1984).

$$\underset{(498\ \text{nm})}{\text{rhodopsin}} + h\nu \xrightarrow{<6\ \text{ps}} \underset{(540\ \text{nm})}{\text{batho}} \xrightarrow{50\ \text{ns}} \underset{(497\ \text{nm})}{\text{lumi}} \xrightarrow{0.3\ \text{ms}} \underset{(478\ \text{nm})}{\text{metal}} \xrightarrow{10\ \text{ms}} \underset{(380\ \text{nm})}{\text{meta II}}$$

One of rhodopsin's intermediates, metarhodopsin II, is believed (Fung et al., 1981; Wessling-Resnick & Johnson, 1987) to be the active species that associates with transducin. The association of the two proteins effects an exchange of GTP for GDP on the α subunit of transducin. Subsequently, the α subunit of transducin binds to a phosphodiesterase, which leads to the hydrolysis of cGMP. The resulting decrease in cGMP concentration closes the Na^+ channels of ROS, causing a hyperpolarization of the plasma membrane (Fung et al., 1981).

The molecular basis by which photoactivated rhodopsin binds to transducin has not been formulated. Before a molecular model for the mechanism can be developed, the energetics of the reaction must be understood; thus a reaction energy profile for the bleaching of rhodopsin to form its various metastable species is required. In a very elegant series of photocalorimetry studies of the bleaching of rhodopsin, Cooper (1981) generated a reaction energy profile for the bleaching of rhodopsin and its evolution through the metarhodopsin II stage. The energetics of each intermediate were measured by trapping the metastable species at low temperatures where they were kinetically stable. For the first two intermediates, bathorhodopsin and lumirhodopsin, this entailed trapping in a low-temperature glycerol/water glass (Cooper, 1981; Schick et al., 1987).

In order for photolyzed rhodopsin to activate transducin, it is reasonable to speculate that the rhodopsin undergoes a series of structural changes within the portion of the protein that is exposed to the cytoplasm, which contains transducin (Kuhn et al., 1982). This may entail an increase or decrease in the volume of the protein. In addition, the change in conformation of the protein may lead to the exposure or the burying of charged groups. Thus, examining the energetics of rhodopsin's intermediates in a low-temperature glycerol/water glass may perturb the energetics of protein conformational changes as well as the energetics associated with the

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[1] Abbreviations: ROS, rod outer segment; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; CTAB, cetyltrimethylammonium bromide; BCP, bromocresol purple.

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change of charge at the protein surface. Ideally, a reaction energy profile for the bleaching of rhodopsin needs to be obtained under physiological conditions.

With the recent development of time-resolved photoacoustic calorimetry, the dynamics of enthalpy and volume changes associated with intermediates produced following photolysis of rhodopsin can be measured on the nanosecond–microsecond time scale at ambient temperatures (Peters & Snyder, 1988). The resolution of the present experiment is such that, for reactions which occur in less than 100 ns, the amplitudes of the enthalpy and volume changes are measured. For reactions occurring between 100 ns and 20 μs, the dynamics of the enthalpy and volume changes for the reaction can be obtained. Herein we report the enthalpy and volume changes associated with the formation of lumirhodopsin from both rhodopsin and isorhodopsin solubilized in the detergent dodecyl β-D-maltoside.

Experimental Procedures

Isolation of Rhodopsin. All manipulations were carried out at 4 °C and under dim red light unless otherwise stated. Bovine rod outer segments were prepared from frozen retinas (Hormel) as described (Smith et al., 1975). Rhodopsin was extracted into a 10 mM HEPES buffer, pH 7.0, containg 2% dodecyl β-D-maltoside detergent (CalBiochem) by stirring overnight at 4 °C followed by centrifugation (20000 rpm, 2 h, Sorvall SS-24 rotor).

Preparation of Isorhodopsin. Isolated ROS were suspended in HEPES buffer (10 mM, pH 7.0). A membrane pellet was obtained by centrifugation (15000 rpm, 20 min, Sorvall SS-34 rotor), resuspended (HEPES buffer containing 100 mM NH_2OH), and irradiated with orange light (>540 nm) at 25 °C until completely bleached. The membranes were collected as a pellet and washed ten times with HEPES buffer and two times with distilled water to remove the retinal oxime complex. The pellet was lyophilized to complete dryness, followed by five washes with petroleum ether to remove residual lipids and chromophore. The membranes were then solubilized into HEPES buffer containing 2% dodecyl β-D-maltoside detergent. A solution of 9-*cis*-retinal (Sigma) in ethanol was added in excess to solubilized opsin, and the resultant solution was incubated (20 h, 20 °C). The solution was applied to a concanavalin A–Sepharose 4B (Sigma) affinity column. Isorhodopsin was eluted with 0.5 M methyl α-D-mannoside. Ions, sugar, and the remaining free chromophore were removed by filtration (YM-30, Amicon).

Photoacoustic Calorimeter. The details of the photoacoustic calorimeter have previously been discussed (Rudzki et al., 1985). Briefly, a PRA nitrogen-pumped dye laser (LN1000/LN102) was used that produced a light pulse at 500 nm with a pulse width of 500 ps and energy of 15 μJ. The acoustic waves were detected by a 0.5-MHz lead zirconate–lead titanate piezoelectric transducer. The output was passed through a preamplifier (Panametric) and recorded by a Gould 4072 digital oscilloscope at 100 MHz. The data were stored in an IBM XT computer and analyzed on a VAX computer.

Sample Handling. The total sample volume in the photoacoustic cell was 2 mL. On each laser shot, the laser beam, with a beam diameter of 2 mm and an energy of 15 μJ, bleached approximately 7% of the sample in the cylinder defined by the laser beam. Between each shot the sample was thoroughly stirred. A total of 10 shots were utilized to obtain a photoacoustic spectrum at a given temperature. The total amount of the sample that was bleached with 10 laser shots was 1.3%. For each temperature a new sample was employed. The relationship between the laser intensity and the photoacoustic signal was examined over the energy range of 0–20 μJ and was found to be linear, which is characteristic of single photon chemistry.

Data Analysis

The photoacoustic signal, S, is produced by a change in volume, ΔV, of the irradiated sample due to an expansion or a contraction of the system (eq 1). The parameter K is a

$$S = K\Delta V \tag{1}$$

function of the instrument response. The expansion or contraction of the system can arise through two processes. The first process is an uptake or release of heat, Q, to the solvent by the reacting molecules. The consequent change in temperature induces a change in volume of the solvent through the relationship

$$\Delta V_{th} = (\beta/C_p\rho)Q \tag{2}$$

where β is the thermal expansion coefficient, C_p is the heat capacity, and ρ is the density of the system. A second process that can give rise to a volume change within the system is a change in the size of the molecular configuration, ΔV_{con}, such as an increase in the volume of a protein or the expansion/contraction of water about a charged group. Therefore

$$S = K(\Delta V_{th} + \Delta V_{con}) = K[(\beta/C_p\rho)Q + \Delta V_{con}] \tag{3}$$

In order to eliminate K, a calibration compound is used, which converts the energy of the photon, $E_{h\nu}$, into heat on a time scale faster than the response time of the instrument. This heat release has no volume component, ΔV_{con}. Thus

$$S_{cal} = K(\beta/C_p\rho)E_{h\nu} \tag{4}$$

The ratio of the amplitudes for the sample wave to the calibration wave, ϕ, is

$$\phi = S/S_{cal} = Q/E_{h\nu} + \Delta V/[(\beta/C_p\rho)E_{h\nu}] \tag{5}$$

Defining

$$F(T) = \beta/C_p\rho \tag{6}$$

leads to

$$E_{h\nu}\phi = Q + \Delta V_{con}/F(T) \tag{7}$$

For proteins in an aqueous environment the parameter $F(T)$ is highly temperature dependent whereas ΔV_{con} is expected to be independent of temperature over a small temperature range (Callis et al., 1972; Westrick et al., 1987). Thus, the correlation of $E_{h\nu}\phi$ with $1/F(T)$ yields both Q and ΔV_{con}. The overall enthalpy change for the reaction is then just

$$\Delta H = E_{h\nu} - Q \tag{8}$$

Results

For our initial photoacoustic calorimetry study of rhodopsin and isorhodopsin, we have chosen dodecyl β-D-maltoside as the detergent for protein solubilization. We initially examined rhodopsin solubilized in ammonyx LO, digitonin, cetyltrimethylammonium bromide (CTAB), and dodecyl β-D-maltoside. Dodecyl β-D-maltoside detergent was the optimal choice for the photoacoustic experiments mainly due to its high solubility at low temperatures. We found that both digitonin and CTAB precipitated from solution at temperature below 10 °C. With the possible exception of digitonin and CHAPS (Stark et al., 1984), the alkyl glucoside detergents have been shown to be better than other detergents in providing thermal stability to rhodopsin and in the efficiency of regeneration of rhodopsin from opsin and 11-*cis*-retinal (Stubbs et al., 1976).

In order to eliminate the instrument response parameter K (eq 1), the photoacoustic signal for the sample is normalized

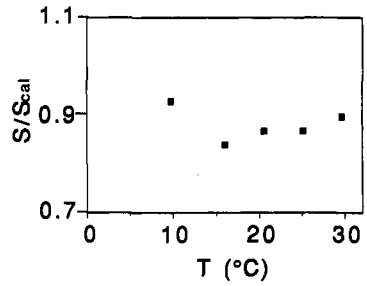

FIGURE 1: Temperature dependence of the ratio of the photoacoustic signal (S) from rhodopsin, OD_{266nm} = 0.20, in 0.01 M HEPES, pH 7.0, containing 2% dodecyl β-D-maltoside to the photoacoustic signal (S_{cal}) from bromocresol purple, OD_{266nm} = 0.20, in 0.1 M HEPES, pH 7.0, containing 2% dodecyl β-D-maltoside. Excitation wavelength λ_{exc} = 266 nm.

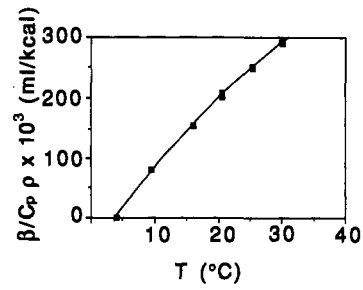

FIGURE 2: Temperature dependence of $\beta/C_p\rho$ for the solution 0.01 M HEPES, pH 7.0, containing 2% dodecyl β-D-maltoside.

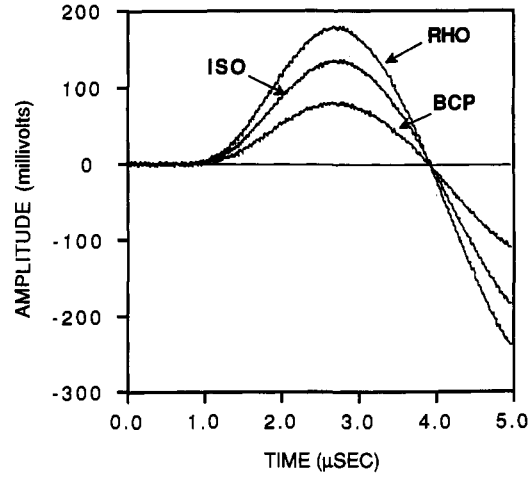

FIGURE 3: Photoacoustic spectra for rhodopsin (RHO), isorhodopsin (ISO), and bromocresol purple (BCP) in 0.1 M HEPES, pH 7.0, containing 2% dodecyl β-D-maltoside at 30 °C with an excitation wavelength λ_{exx} = 500 nm and OD_{500nm} = 0.20 for RHO, ISO, and BCP.

by the photoacoustic signal from a calibration compound (eq 5). This process of normalization assumes that the $F(T)$'s (eq 6) for the sample and the calibration compound are the same. In this study the sample wave results from the photolysis of either rhodopsin or isorhodopsin solubilized in HEPES buffer containing 2% dodecyl β-D-maltoside detergent. The calibration compound is bromocresol purple (Arata & Parson, 1981) dissolved in the same detergent buffer. The question which then arises is whether the $F(T)$ for rhodopsin dissolved in a detergent micelle is the same as the $F(T)$ for bromocresol purple dissolved in a 2% detergent solution. To address this concern, the photoacoustic signal from bromocresol purple in a 2% detergent solution, irradiated at 266 nm, is compared to the 266-nm photoacoustic signal from the protein opsin, prepared by bleaching rhodopsin, in a 2‰ detergent solution. Following irradiation, opsin will either convert the photon energy into heat or reemit the energy as fluorescence. The fraction of the 266-nm photon energy that is converted into heat for opsin will be less than 1.0 since tryptophan, tyrosine, and phenylalanine, which all absorb at 266 nm, will fluoresce. On the basis of the number of these amino acids in rhodopsin (Dratz & Hargrave, 1983; Applebury & Hargrave, 1986), their absorption extinction coefficients (Wetlaufer, 1962), and their fluorescence quantum yields (Cantor & Schimmel, 1980), the fraction of the photon energy that will be converted into heat is calculated to be approximately 0.86. Therefore, if detergent-solubilized opsin and bromocresol purple have the same F(T)'s, the ratio of photoacoustic signals should be approximately 0.86. The ratio of the photoacoustic signal for rhodopsin to bromocresol purple (S/S_{cal}) over the temperature range of 10–30 °C is shown in Figure 1. The average value over the entire temperature range is 0.90 ± 0.027, which is very close to the predicted value of 0.86. Therefore, we assume that the $F(T)$'s for the two solutions are the same so that bromocresol purple in detergent can be used as a calibration compound when rhodopsin is irradiated at 500 nm.

The value of $F(T)$ for bromocresol purple in a 2% detergent solution (BCP, 2%) is obtained from comparison of its acoustic wave amplitude to the acoustic wave amplitude for bromocresol purple in a lightly buffered (10 mM HEPES) solution (BCP). From eq 4, the ratio of the photoacoustic signals is

$$S_{BCP,2\%}/S_{BCP} = F(T)_{BCP,2\%}/F(T)_{BCP} \quad (9)$$

Within the error of our experiment it has been shown that $F(T)$'s for dilute buffers are the same as the F(T)'s for distilled water, whose absolute values are known (Westrick et al., 1990). Given $F(T)_{BCP}$ and the ratio of the acoustic wave amplitudes, the values for $F(T)_{BCP,2\%}$ can be calculated (Figure 2).

The photoacoustic spectra for rhodopsin and isorhodopsin are obtained over the temperature range of 29.7–4 °C. At 29.7 °C the amplitudes of the photoacoustic waves for both rhodopsin and isorhodopsin are greater than the calibration compound, bromocresol purple (Figure 3). Furthermore, the waves do not shift in time relative to the calibration compound as evidenced by the crossing of all three waves at 4 μs (Figure 3). This suggests that all the observed kinetic processes occur in less than 100 ns, the time resolution of the experiment. If kinetic processes were occurring between 100 ns and 20 μs, the sample acoustic waves would be shifted in time relative to the calibration wave, as observed in previous experiments (Westrick et al., 1987). When the temperature of the solution is reduced to 4 °C, the thermal expansion coefficient for the solution approaches zero. For a calibration compound that only converts the photon energy into heat and undergoes no changes in molecular configuration on the time scale of the experiment, the photoacoustic signal will go to zero. The acoustic wave for bromocresol purple disappears at 4 °C (Figure 4). However, at 4 °C both rhodopsin and isorhodopsin have significant positive acoustic wave amplitudes (Figure 4), which result from configurational changes within the protein.

The photoacoustic spectra for rhodopsin were examined as a function of temperature, ranging from 29.7 to 9.7 °C. A total of six separate experiments were carried out encompassing 25 data sets. The results of these experiments, ϕ, are shown in Figure 5, where the data are presented as a correlation of $E_{h\nu}\phi$ with $1/F(T)$ (eq 7). The data from 9.7 to 4 °C were not analyzed since the amplitude of the photoacoustic wave for the calibration compound is negligible through this temperature range. The energy released during the reaction, Q(RHO), is 54.4 ± 5.9 kcal/mol, and the accompanying

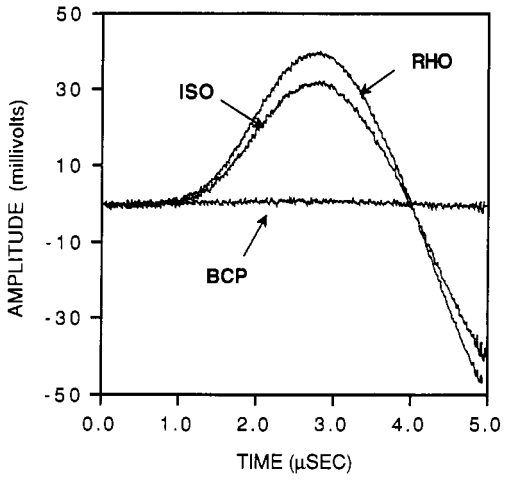

FIGURE 4: Photoacoustic spectra for rhodopsin (RHO), isorhodopsin (ISO), and bromocresol purple (BCP) in 0.1 M HEPES, pH 7.0, containing 2% dodecyl β-D-maltoside at 4 °C with an excitation wavelength λ_{exc} = 500 nm and OD_{500nm} = 0.20 for RHO, ISO, and BCP.

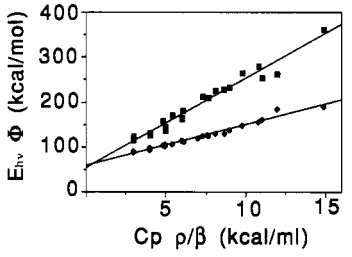

FIGURE 5: Plot of $\phi E_{h\nu}$ vs $C_p\rho/\beta$ for rhodopsin (squares) and isorhodopsin (diamonds) in 0.1 M HEPES, pH 7.0, containing 2% dodecyl β-D-maltoside with an excitation wavelength λ_{exc} = 500 nm over a temperature range of 29.7–9.7 °C.

volume change, ΔV_{con}(RHO), is 19.5 ± 0.8 mL/mol.

A similar series of experiments were carried out for isorhodopsin. The temperature was varied from 29.7 to 4 °C for four separate experiments with a total of 22 acoustic spectra. The data are shown in Figure 5. The energy change, Q(ISO), is 57.5 ± 2.1 kcal/mol, and the volume change, ΔV_{con}(ISO), is 8.5 ± 0.3 mL/mol.

Discussion

From the analysis of the photoacoustic data for rhodopsin, the species giving rise to the acoustic waves are formed in less than 100 ns; there are no observable enthalpy or volume changes within the 100-ns to 20-μs time scale. From laser flash photolysis data, upon absorption of a photon by rhodopsin, bathorhodopsin is formed in less than 6 ps, and in turn it decays in approximately 50 ns at room temperature to produce lumirhodopsin (Applebury, 1984; Birge, 1990). This latter species is stable on the 300-μs time scale at room temperature. Thus, the observed acoustic waves are attributed to the decay of the excited singlet state of rhodopsin to produce lumirhodopsin. The 100-ns time resolution of the photoacoustic calorimeter precludes the separation of the individual enthalpy and volume changes for the formation and decay of bathorhodopsin; only the overall enthalpy and volume changes for the formation of lumirhodopsin are revealed.

The photoacoustic experiment only measures the total enthalpy and volume changes for a photochemical reaction. If there is a partitioning into products along the reaction pathway, then the quantum yields for product formation must be factored into the enthalpy and volume change determinations. The quantum yield for the formation of lumirhodopsin from rhodopsin has been determined to be 0.67 (Birge, 1990); the remaining 0.33 represents the return of the excited-state rhodopsin back to ground-state rhodopsin. Similarly, at ambient temperatures, the quantum yield for the formation of lumirhodopsin from isorhodopsin is 0.22 ± 0.03 (Hurley et al., 1977).

The overall enthalpy and volume changes for the decay of the excited singlet state of rhodopsin to form lumirhodopsin are 54.4 ± 5.9 kcal/mol and 19.5 ± 0.8 mL/mol. With a quantum yield of 0.67 for lumirhodopsin formation and a photon enery of 57 kcal/mol, the overall enthalpy and volume changes for the conversion of ground-state rhodopsin to lumirhodopsin are 3.9 ± 5.9 kcal/mol and 29.1 ± 0.8 mL/mol, respectively. Similarly, the overall enthalpy and volume changes for the conversion of isorhodopsin to lumirhodopsin are −2.0 ± 2.1 kcal/mol and 38.6 ± 0.3 mL/mol, respectively.

To determine the enthalpy change for the formation of lumirhodopsin from rhodopsin and isorhodopsin, it is necessary to establish the enthalpy change for the conversion of rhodopsin to isorhodopsin. The relative energetics of rhodopsin and isorhodopsin have been measured by Cooper (1979) utilizing photocalorimetry. In rod outer segments the conversion of rhodopsin to isorhodopsin is an endothermic process by 5.2 ± 2.3 kcal/mol at 3 °C. If the same energetics occur for rhodopsin and isorhodopsin in 2‰ dodecyl β-D-maltoside, then the enthalpy change for the conversion of rhodopsin to lumirhodopsin, formed from isorhodopsin, is endothermic by 3.2 kcal/mol. This value is virtually identical with the value for rhodopsin/lumirhodopsin conversion, 3.9 kcal/mol, suggesting that the lumirhodopsin species formed from rhodopsin and isorhodopsin are the same.

The overall volume changes for the conversion of the excited singlet states of rhodopsin and isorhodopsin to lumirhodopsin are 19.5 ± 0.8 and 8.5 ± 0.3 mL/mol. Factoring in the appropriate quantum yields for lumirhodopsin formation, the volume changes for the rhodopsin–lumirhodopsin and isorhodopsin–lumirhodopsin conversions are 29.1 ± 0.8 and 38.6 ± 0.3 mL/mol. These volume changes may represent an increase in the molecular size of the protein upon formation of lumirhodopsin or may result from the removal of charged groups from the protein surface, which would have the effect of releasing bound water to the solvent, thereby causing an increase in volume for the system (Kauzmann, 1959). Assuming the two lumirhodopsins are the same molecular species, then isorhodopsin's volume is 9.5 mL/mol less than that of rhodopsin. With a molecular weight of 41 000 and a density of 1.2 g/mL, the overall volume of rhodopsin is estimated to be 34 000 mL/mol so that the difference of 9.5 mL/mol represents only a 0.03% change in volume of the protein.

The only other investigation of the energetic relationship between rhodopsin and lumirhodopsin has been the photocalorimetry study of Cooper (1981). When rod outer segments are suspended in a glycerol/pH 7.2 phosphate buffer (2:1 volume ratio) at −75 °C, irradiation of rhodopsin produces lumirhodopsin, which is kinetically stable. The enthalpy increase, under these conditions, is 26.3 ± 4.2 kcal/mol. This value is significantly greater than the 3.9 ± 5.9 kcal/mol determined by photoacoustic calorimetry. A possible rationale for this discrepancy is that the rhodopsins in the two experiments are in different environments. In the original photocalorimetry experiment rhodopsin was in its native lipid environment, while for the present experiment rhodopsin is in the detergent dodecyl β-D-maltoside. However, from thermal stability, regeneration, and circular dichroism studies, rhodopsin in dodecyl β-D-maltoside detergent appears not to be

significantly perturbed relative to rhodosin in the native membrane (Stubbs et al., 1976).

A more likely rationale for the difference in the two experiments is that the glycerol matrix perturbs the energetics of lumirhodopsin. When rhodopsin becomes photoactivated, it apparently undergoes a series of changes in the protein structure, as evidenced by changes in absorption spectra for each intermediate, so that it can activate the GTP-binding protein, transducin. The photoacoustic experiments reveal that the volume of the protein increases by 29.1 mL/mol upon lumirhodopsin formation. A frozen glass matrix may prevent the change in the molecular configuration within the protein that is responsible for this volume increase and may result in a perturbation of energetics. In addition, if these conformational changes result in a net increase or decrease of charge at the protein surface, then the frozen glass may not be able to reorient and stabilize the change in charge. Such an effect was found in our photoacoustic study of the photodissociation of carbon monoxide from carboxymyoglobin, where the dissociation process is accompanied by the breaking of a salt bridge that is apparently prevented from breaking in a low-temperature glass (Westrick et al., 1990).

In summary, photoacoustic calorimetry reveals that the formation of lumirhodopsin from rhodopsin, solubilized in 2% dodecyl β-D-maltoside, is accompanied by an increase in enthalpy of 3.9 ± 5.9 kcal/mol and an increase in volume of 29.1 ± 0.8 mL/mol at ambient temperatures. In addition, the lumirhodopsins produced from rhodopsin and isorhodopsin are energetically equivalent. Future studies will determine the effect that detergents and lipids have upon the energetics of lumirhodopsin formation.

Registry No. Dodecyl β-D-maltoside, 69227-93-6.

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