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Energy Storage in the Primary Photochemical Events of Rhodopsin and Isorhodopsin

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ABSTRACT: The energetics associated with the photoequilibrium

$$\text{rhodospin (R)} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{bathorhodopsin (B)} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{isorhodopsin (I)}$$

are measured at 77 K by using pulsed-laser photocalorimetry and a range of excitation wavelengths and relative starting concentrations. Enthalpies for the photochemical transformations R $\xrightarrow{h\nu}$ B and I $\xrightarrow{h\nu}$ B are measured to be $\Delta H_{RB} = 32.2 \pm 0.9$ kcal mol^{-1} and $\Delta H_{IB} = 27.1 \pm 3.2$ kcal mol^{-1}, respectively. Although the value of ΔH_{RB} is slightly lower than that reported previously by Cooper of 34.7 ± 2.2 kcal mol^{-1} [Cooper, A. (1979) *Nature (London) 282*, 531–533], the two values are in agreement within experimental error. The energy difference $\Delta H_{RB} - \Delta H_{IB} = 5.1 \pm 3.3$ kcal mol^{-1} is identical within experimental error with the difference in enthalpies of isorhodopsin and rhodopsin [5.2 ± 2.3; Cooper, A. (1979) *FEBS Lett. 100*, 382–384]. We suggest that this result is consistent with the theory that bathorhodopsin is a single, common photochemical intermediate connecting rhodopsin and isorhodopsin.

The primary event in the vertebrate vision process involves absorption of light by the membrane glycoprotein rhodopsin (R) and its conversion to a metastable intermediate, bathorhodopsin (B). The chromophore of R is the protonated Schiff base of 11-*cis*-retinal, which isomerizes to the all-trans form during the primary event (Honig, 1978; Ottolenghi, 1980; Birge, 1981). The photoreaction is endothermic, and the energy stored by B is used to promote subsequent thermal reactions in the protein bleaching sequence and in the subsequent transducin cycle. At physiological temperatures, the protein decays from B through several intermediates and eventually ($\sim 10^3$ s later) dissociates into *all-trans*-retinal and opsin. Complex dark reactions within the transducin cycle then serve to regenerate rhodopsin, and the cycle continues toward the eventual hydrolysis of cyclic GMP to 5′-GMP. This hydrolysis results in a constriction of the flow of Na^+ ions through the rod outer segment, thus hyperpolarizing the retinal rod cell. Vision is produced when this electrical signal is communicated to the other end of the retinal rod cell and to the other cells of the retina (Stryer, 1986).

Bathorhodopsin is stable at 77 K and forms the central species of a three-component photoequilibrium (Yoshizawa & Wald, 1963):

$$\text{R} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{B} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{I} \qquad \text{(1a)}$$

where isorhodopsin (I) is the label given to the 9-*cis*-retinyl isomer of the protein. The energetics of this photoequilibrium are very important in understanding light transduction in the visual process. The origin of the energy storage remains a subject of active study. Various mechanisms have been proposed whereby energy is stored in the form of either charge separation (Rosenfeld et al., 1977; Honig et al., 1979) or a mixture of charge separation and protein strain (Birge & Hubbard, 1980; Warshel & Barboy, 1982) as a result of the 11-cis to 11-trans photoisomerization of the retinyl chromophore. The energetics of the 11-cis (R) → 11-trans (B) photoconversion have been measured (Cooper, 1979a; Boucher & Leblanc, 1985), but these values are more than double the comparable energy storage associated with the primary event

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in bacteriorhodopsin (Birge & Cooper, 1983). Since different experimental methods were used, it is important to determine whether this difference is real or is due to a difference in experimental method. Furthermore, no energy storage measurements of the 9-cis (I) → 9-trans (B) photoconversion have been reported. This measurement should provide new insights into the nature of bathorhodopsin (see below) and the mechanism of energy storage. This study was prompted by the above considerations.

There is continued discussion regarding whether B (bathorhodopsin) is a singular molecular species forming a common intermediate between R and I. It has been suggested (Waddell et al., 1984; Spalink et al., 1983) that there are, in fact, two forms of bathorhodopsin, leading to a photoequilibrium of the form

$$\mathrm{R} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \mathrm{B}' \underset{(\Delta)}{\overset{(\Delta)}{\rightleftharpoons}} \mathrm{B}'' \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \mathrm{I} \qquad (1b)$$

where B′ and B″ are labels given to the batho products of R and I, respectively. One approach to resolving this controversy is to measure independent values of the ground-state energy differences between R and I and their respective bathoproducts. If B′ and B″ are indeed different species, their ground-state energies would be presumed to be different.

We report here enthalpy differences between the R, B, and I forms of bovine rhodopsin as measured by pulsed-laser photocalorimetry. Previous studies have determined that the enthalpy differences between R and B, ΔH_{RB}, is ~35 kcal mol^{-1} (Cooper, 1979a) and between R and I, ΔH_{RI}, is ~5 kcal mol^{-1} (Cooper, 1979b). We extended this work by measuring reaction enthalpies for a range of wavelengths and for a range of relative starting concentrations of R, B, and I. The data are used to determine values of ΔH_{RB} and ΔH_{IB} with well-defined standard deviations.

Materials and Methods

Preparation of Rhodopsin. Purified rhodopsin rod outer segments (ROS) were prepared by the step-wise sucrose gradient method described by Hong et al. (1982), which is a modification of the earlier procedures described by Hong et al. (1975) and Ebrey (1971). The dark-adapted bovine retinas were purchased from George-Hormel Co. (Austin, MN) and stored at dry ice temperatures. The entire preparation was done in a dark room under dim red lamps (Kodak filter no. 1A). All components that came in contact with the ROS membranes [pH 6.6 0.1 M potassium phosphate buffer, solutions of 1.02 M and 1.38 M sucrose (Schwarz/Mann) in phosphate, Teflon–glass homogenizer, centrifuge tubes, mortar and pestle, Finn pipets, rotor, etc.] were kept on ice or refrigerated prior to use in order to keep the ROS temperature below 4 °C at all times. Centrifugation was carried out at 2 °C on a Beckman ultracentrifuge equipped with a TI-30 fixed-angle rotor. Decantation steps were done very carfully in order to not disturb the pellet. Prior to homogenization the pellet was resuspended in small amounts of 1.02 M sucrose/0.1 M buffer with slight stirring of the pellet within the tube. Each of the centrifuge tubes was "rinsed" to collect all of the pellet. The suspended pellets were diluted to 40 mL with 1.02 M sucrose/0.1 M buffer solution prior to homogenization with a glass–Teflon homogenizer. The homogenizing was done manually and slowly (10 cycles) to minimize shearing strains on the protein (DeGrip et al., 1980). The 1.02 M sucrose flotation, homogenization, and pelleting cycle was repeated for a total of three cycles. All steps were monitored on a Cary 17D UV–visible spectrophotometer by taking absorption spectra of aliquots of the supernatant, the suspended floating material, and the pellet for each centrifugation step. The final ROS pellets were washed (suspended and centrifuged at 43000*g* for 15 min) with water and dilute buffer (15 mM) and then lyophilized. We will refer to the ROS preparation as "rhodopsin" or "R" in the remainder of the paper.

Prior to photocalorimetry the rhodopsin was solubilized in 67% glycerol/2% digitonin/10 mM potassium phosphate buffer at pH 7.0 to give an optical density of ~8.0 OD in a 1-cm path-length cell (~1×10^{-4} M). The sample was introduced into the stainless steel photocalorimeter cell (3-mL capacity) and cooled slowly following the procedures of Birge and Cooper (1983).

Preparation of Isorhodopsin. Isorhodopsin (I) was prepared at 77K in situ by irradiation of rhodopsin (R) (or a mixture of R, B, and I) at 580 nm with the apparatus shown in Figure 1. This apparatus, which is also used to collect the photocalorimetry data, provides for preirradiation of the sample by a 100-W xenon lamp (Z, Figure 1) with wavelength selection accomplished by a chemical filter (3 g of $K_2Cr_2O_7$ in 100 mL of H_2O, 10-cm path length) followed by an interference filter (580 nm, 4-nm FWHM band-pass) (see Figure 1). The photochemical processes associated with this procedure are described below. This technique has been used by Yoshizawa and Wald (1963) and by Mao et al. (1980) to generate isorhodopsin identical spectroscopically with direct incorporation of 9-*cis*-retinal into opsin.

The absorption spectra of R, B, and I at 77 K are shown in Figure 2a, and the edge behavior is shown with a log ϵ vs. λ axes in Figure 2b. One notes a small wavelength window from 577 to 585 nm where I has virtually no absorptivity ($\epsilon < 1$) and R and B both have absorptivities of $\epsilon > 100$. The absorptivities at 580 nm are shown with a dashed line in Figure 2b and yield $\epsilon_R^{580} = 490$, $\epsilon_B^{580} = 35\,200$, and $\epsilon_I^{580} = 2.2 \times 10^{-3}$ L M^{-1} cm^{-1}. The relative rate of photoconversion for a constant flux of monochromatic radiation is given by the product of the quantum yield and the molar absorptivity, measured at the wavelength of irradiation. The quantum yields for the various photoconversions at 580-nm excitation and 77 K as reported by Birge and Callender (1987) are

$$\mathrm{R} \underset{0.49}{\overset{0.67}{\rightleftharpoons}} \mathrm{B} \underset{0.089}{\overset{0.076}{\rightleftharpoons}} \mathrm{I}$$

which yield relative rates of

$$\mathrm{R} \underset{1}{\overset{2 \times 10^{-2}}{\rightleftharpoons}} \mathrm{B} \underset{1 \times 10^{-8}}{\overset{0.16}{\rightleftharpoons}} \mathrm{I}$$

While it is clear that isorhodopsin is a "kinetic sink", the preparation of I with 580-nm irradiation is not a "rapid" process. The approximate formation kinetics of I on the basis of the initial conditions of pure R and the apparatus shown in Figure 1 are graphed in Figure 2c. A photoequilibrium containing 16 parts B to 980 parts R is established rapidly (~1 s), but the small fractional amount of B prevents rapid formation of I despite a favorable B → I rate. After ~300 s, >98% I is formed, and this concentration is indistinguishable from pure I for the present experimental study. All experiments reporting initial starting concentrations of pure isorhodopsin were carried out following in situ 580-nm preirradiation times greater than 5 min.

Photocalorimetry. The pulsed dye laser photocalorimeter is schematically shown in Figure 1 and has been described in detail elsewhere (Cooper et al., 1984). The use of this apparatus to measure ΔH(bR → K) for the primary step in the bacteriorhodopsin photocycle has been reported (Birge & Cooper, 1983). Briefly, a pulsed, tunable dye laser (<1 mJ, 0.4-μs pulse width) sends a single "packet" of monochromatic

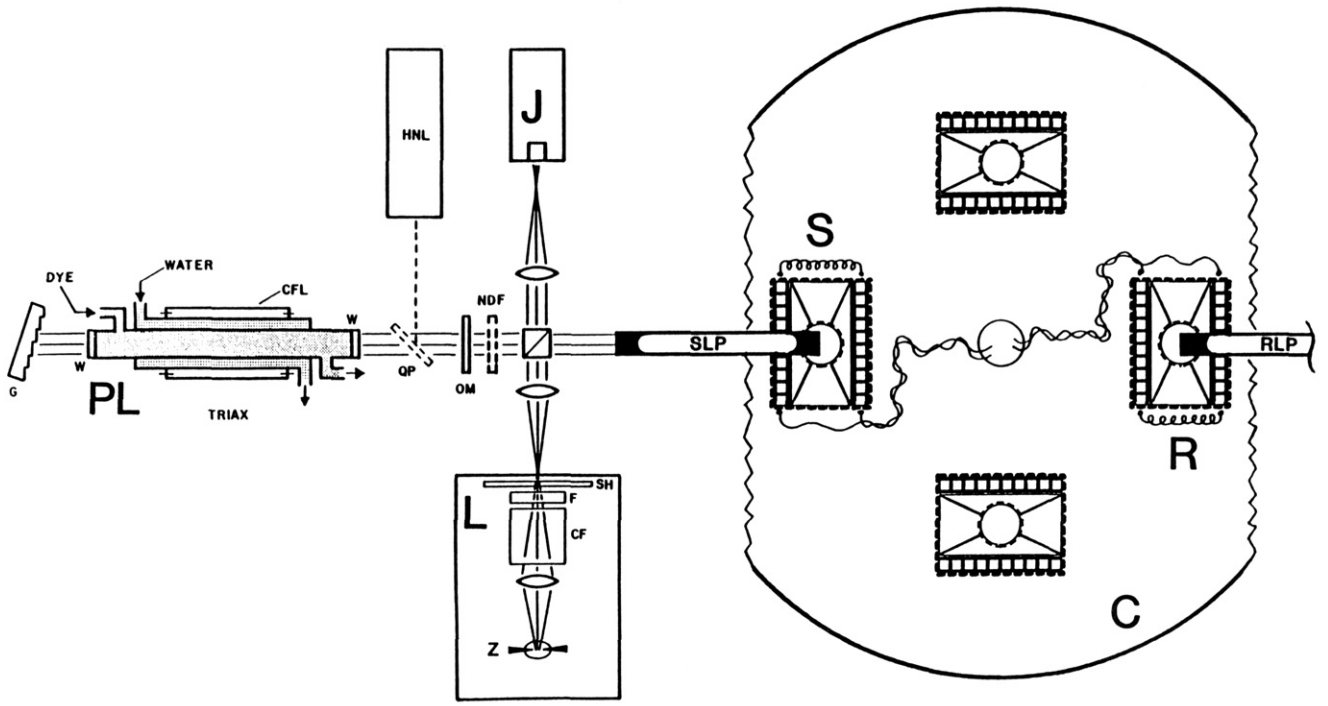

FIGURE 1: Overall schematic of pulsed tunable dye laser low-temperature photocalorimeter. The pulsed laser (PL) is a Phase-R Corp. DL 2100C triaxial (TRIAX) flashlamp-pumped tunable dye laser. The pulse energy is monitored with a Molectron Model J3 pyroelectric joulemeter (J). The desired photostationary state mixture is obtained with the xenon lamp and filter combination (L) (Table II) or with the laser (Table III). Light is directed into the sample cell (S) of the calorimeter (C) by a fiber optics light guide (SLP). An identical light guide (RLP) is inserted into the reference cell (R) but is capped at the external input to prevent light from entering. The calorimeter is placed into a 10-L Dewar (not shown), and the Dewar is filled with liquid nitrogen. A copper Faraday cage surrounds the entire Dewar, and the light guides and the shielded cables from the sample and reference cells exit out of the top of the Dewar assembly through a large brass heat sink, which is also immersed in liquid nitrogen. The remaining symbols are used to represent the following components: G = sine bar diffraction grafting, W = antireflection coated windows, CFL = coaxial flash lamp, QP = quartz plate, HNL = helium–neon alignment laser, OM = 50% reflecting output mirror, NDF = neutral density filter(s), SH = shutter (closed prior to firing the laser), F = band-pass or interference filter, CF = broad-band chemical filter to remove light with wavelengths less than 400 nm or greater than 800 nm, and Z = 100-W xenon lamp.

radiation via a fiber optic waveguide to a rhodopsin solution held at 77 K. The concentration of the solution (1×10^{-4} M total R + B + I) is such that >95% of the excitation light is adsorbed by the reaction mixture. The heat produced by the decay of the excited states back to their ground states is determined by measuring the heat flow across peltier receivers following the excitation pulse. The heat flow is measured as an output voltage and, after amplification, is integrated to give the total heat produced:

$$I_v = \int_{t_1}^{t_1+3\tau} V(t)\, dt \tag{2}$$

Here $V(t)$ is the observed voltage response of the peltier receivers and the integration is performed from the time the laser is fired (t_1) to the end of heat flow [$t_1 + 3\tau$, where τ (=25 s) is the photocalorimeter time constant].

The energetics of the reaction are related to the calorimeter signal by (Birge & Cooper, 1983; Cooper et al., 1984)

$$I_v = kNE_{ex}^{\lambda}\Gamma^{\lambda} \tag{3}$$

where k is the efficiency of the calorimeter, N is the number of moles of photons absorbed, E_{ex}^{λ} is the energy per mole of photons for wavelength λ, and Γ^{λ} is the dimensionless "molecular response function" (Birge & Cooper, 1983). This latter parameter is interpreted as the photochemical light-to-heat conversion efficiency of the molecular system ($\Gamma^{\lambda} < 1$ for an endothermic reaction; $\Gamma^{\lambda} > 1$ for an exothermic reaction). The relationship in eq 3 is applicable only to systems that decay via radiationless processes (all decay channels produce heat). For reaction mixtures in which the exciting radiation initiates more than one photochemical reaction, Γ^{λ} will contain contributions from each of the individual reaction paths. In this experiment we are monitoring the energetics for the equilibrium

$$R \underset{\phi_2}{\overset{\phi_1}{\rightleftharpoons}} B \underset{\phi_4}{\overset{\phi_3}{\rightleftharpoons}} I \tag{4}$$

where ϕ_1 through ϕ_4 are the quantum yields for the individual photoconversions. Therefore, absorption of light by the system will, in general, initiate the four photoreactions $R \xrightarrow{h\nu} B$, $I \xrightarrow{h\nu} B$, $B \xrightarrow{h\nu} R$, and $B \xrightarrow{h\nu} I$. These processes are depicted schematically in Figure 3.

The functional form of Γ^{λ} for the photoequilibrium shown in eq 4 may be derived by considering individually the contributions from each of the processes depicted in Figure 3. If the reaction mixture is initially 100% R in concentration, then Figure 3a alone represents the photoinduced reaction. By assuming that the initial concentration condition is not altered appreciably during the event, the heat produced by the system as a result of the absorption of 1 mol of photons is given by

$$\Delta H_{\text{reaction}}\ ([R] = 100\%) = E_{ex}^{\lambda}\Gamma_R^{\lambda} = E_{ex}^{\lambda} - \phi_1 \Delta H_{RB}$$

where ΔH_{RB} is the enthalpy difference $H_B - H_R$. The molecular response function for this system is, therefore

$$\Gamma_R^{\lambda} = 1 - \phi_1 \frac{\Delta H_{RB}}{E_{ex}^{\lambda}} \tag{5}$$

where the subscript on Γ_R^{λ} is used to emphasize that this molecular response function is applicable only when the initial concentration is 100% R. By use of analogous reasoning for an initial concentration of pure isorhodopsin, [I] = 100%

$$\Gamma_I^{\lambda} = 1 - \phi_4 \frac{\Delta H_{IB}}{E_{ex}^{\lambda}} \tag{6}$$

where ΔH_{IB} is defined as $H_B - H_I$. For a system with the initial condition [B] = 100%, the heat produced by absorption of light would be

$$\Delta H_{reaction}\ ([B] = 100\%) = E_{ex}^{\lambda}\Gamma_B^{\lambda} = E_{ex}^{\lambda} + \phi_2\Delta H_{RB} + \phi_3\Delta H_{IB}$$

so that

$$\Gamma_B^{\lambda} = 1 + \phi_2\frac{\Delta H_{RB}}{E_{ex}^{\lambda}} + \phi_3\frac{\Delta H_{IB}}{E_{ex}^{\lambda}} \quad (7)$$

The general form of Γ^{λ} in eq 3 is then obtained as the sum of the contributions Γ_R^{λ}, Γ_I^{λ}, and Γ_B^{λ} (eq 5–7), each weighted by partitioning the absorption of radiation by the mixture

$$\Gamma^{\lambda} = 1 + F_{RB}\frac{\Delta H_{RB}}{E_{ex}^{\lambda}} + F_{IB}\frac{\Delta H_{IB}}{E_{ex}^{\lambda}} \quad (8)$$

where

$$F_{RB} = \alpha_B^{\lambda}\phi_2 - \alpha_R^{\lambda}\phi_1 \quad (9a)$$

$$F_{IB} = \alpha_B^{\lambda}\phi_3 - \alpha_I^{\lambda}\phi_4 \quad (9b)$$

and the α_i^{λ} are the partition functions for excitation:

$$\alpha_i^{\lambda} = \frac{\epsilon_i(\lambda)[i]}{\epsilon_R(\lambda)[R] + \epsilon_B(\lambda)[B] + \epsilon_I(\lambda)[I]} \quad (10)$$

where $\epsilon_i(\lambda)$ is the extinction coefficient for component i at wavelength λ. In eq 8, ΔH_{RB} and ΔH_{IB} will both be positive for the case where $E_B^{\circ} > E_I^{\circ} > E_R^{\circ}$ (E_i° is the ground-state energy for component i).

When the mixture reaches a photostationary equilibrium, the molecular response function, Γ^{λ}, equals unity ($F_{RB}\Delta H_{RB} = -F_{IB}\Delta H_{IB}$). This observation results in a convenient means by which the photocalorimeter may be calibrated. When $\Gamma^{\lambda} = 1$, eq 3 becomes

$$I_v = kNE_{ex}^{\lambda}$$

An experimental value for Γ^{λ} may therefore be determined by

$$\Gamma_{obsd}^{\lambda} = \frac{(I_v/N)_{obsd}}{(I_v/N)_{ctrl}} \quad (11)$$

where $(I_v/N)_{ctrl}$ is the laser-intensity-normalized calorimeter signal obtained for a photostationary mixture. Between five and ten independent measurements of Γ_{obsd}^{λ} are collected in order to determine a statistically relevant average and standard deviation.

Inherent in eq 11 is the assumption that k, the photocalorimeter efficiency factor (see eq 3), is invariant to the formation of the photostationary state. This is to say that the difference in the optical density (OD) of the solution (at the wavelength of excitation) between the initial and stationary-state concentrations does not affect the measurement efficiency. There are possible scenarios for this difference to become appreciable, but all may be avoided experimentally. First, light entering the sample cell from the waveguide will be reflected by the meniscus and will escape through the waveguide without being detected. This is a general problem associated with cryogenic photocalorimetry. Our method of calibration removes this problem completely. Since the solution is frozen, the surface of the meniscus will not change optically, and the change in OD of the solution will not affect the reflectivity at the air/frozen solution interface (Harrick, 1967). Therefore, the meniscus reflectivities associated with the observed and control samples will be identical. Another problem arises if there is a large change in the transmission characteristics of the solution. Light propagating through the sample can be absorbed by the 95%-reflecting walls of the calorimeter or can escape, after reflection, through the waveguide. Both of these possibilities will produce systematic errors if the light is transmitted by different amounts through the initial and stationary-state mixtures. In our experiments, we avoid these problems by chosing appropriate excitation wavelengths such that less than 5% of the exciting radiation will be transmitted to the calorimeter walls on a single pass by either the initial or photostationary solutions.

Suzuki and Callender (1981) have reported values for the quantum yields ϕ_1, ϕ_2, ϕ_3, and ϕ_4 as 0.67, 0.5, 0.054, and 0.1, respectively. Recently, Birge and Callender (1987) have reported a wavelength dependence for ϕ_4 in agreement with a previous study by Hurley et al. (1977). Values of ϕ_4^{λ} range from ~0.09 at 560-nm excitation to ~0.17 at 440 nm. This information was used by Birge and Callender to reoptimize the values of ϕ_2 and ϕ_3 to yield respective values of 0.486 and 0.073. For analysis of the photocalorimetry data reported here, the values of ϕ_1, ϕ_2, ϕ_3, and ϕ_4 are taken from Birge and Callender (1987).

Initial concentrations of R, B, and I were generated as follows. For experiments in which [R] = 1×10^{-4} M (α_R^{λ} = 1.0), native rhodopsin from the procedure described above was used. For the remaining experiments, the initial concentrations were generated in situ by irradiating with pulsed-laser excitation (mixtures of R, B, and I) or the xenon lamp and filter combination (>98% I). The individual concentrations [R], [B], and [I] were then determined according to the procedures described in Birge and Callender (1987) with the restriction that [R] + [B] + [I] = 1×10^{-4}. Extinction coefficients were taken from the spectra shown in Figure 2.

Results

The experiments conducted to determine the enthalpy differences between R, B, and I are classified according to initial concentrations. Since the interconversion R $\rightleftarrows$ I is not a direct photoevent, experiments with $\alpha_R = 1.0$ or $\alpha_I = 1.0$ (initial concentrations of totally R or totally I) can be used to determine directly the enthalpies for R $\xrightarrow{h\nu}$ B or I $\xrightarrow{h\nu}$ B, respectively (see eq 8). Several different combinations of initial concentrations and excitation wavelengths were used to determine experimental values of ΔH_{RB} and ΔH_{IB}. Experiments with initial concentrations involving mixtures of R, B, and I were also carried out to check the consistency of the ΔH_{RB} and ΔH_{IB} values as measured by the first two types of experiments.

The results for the $\alpha_R^{\lambda} = 1.0$ experiments are given in Table I. These data can be used to determine directly ΔH_{RB} for the photoisomerization R $\xrightarrow{h\nu}$ B. In this case, eq 8 reduces to

$$\Delta H_{RB} = (1 - \Gamma_{obsd}^{\lambda})(E_{ex}^{\lambda}/\phi_1) \quad (12)$$

Values for ΔH_{RB} for the individual experiments are given in the table. The average value is calculated to be $\Delta H_{RB}^{av} = 32.2 \pm 0.9$ kcal mol^{-1}. This value is in agreement statistically with the previously reported value of 34.7 ± 2.2 kcal mol^{-1} obtained by Cooper (1979a). More recently, a similar value, 34.7 ± 1.5 kcal mol^{-1}, has been inferred from photoacoustic studies (Boucher & Leblanc, 1985). Despite the remarkable agreement between the earlier two studies, we are confident of the statistical validity of our slightly lower value.

The results for the $\alpha_I^{\lambda} = 1.0$ experiments are given in Table II. From these data, the ΔH_{IB} values shown in the table are computed by using an equation analogous to eq 12:

$$\Delta H_{IB} = (1 - \Gamma_{obsd}^{\lambda})(E_{ex}^{\lambda}/\phi_4) \quad (13)$$

where ϕ_4 is the wavelength-dependent quantum yield for the

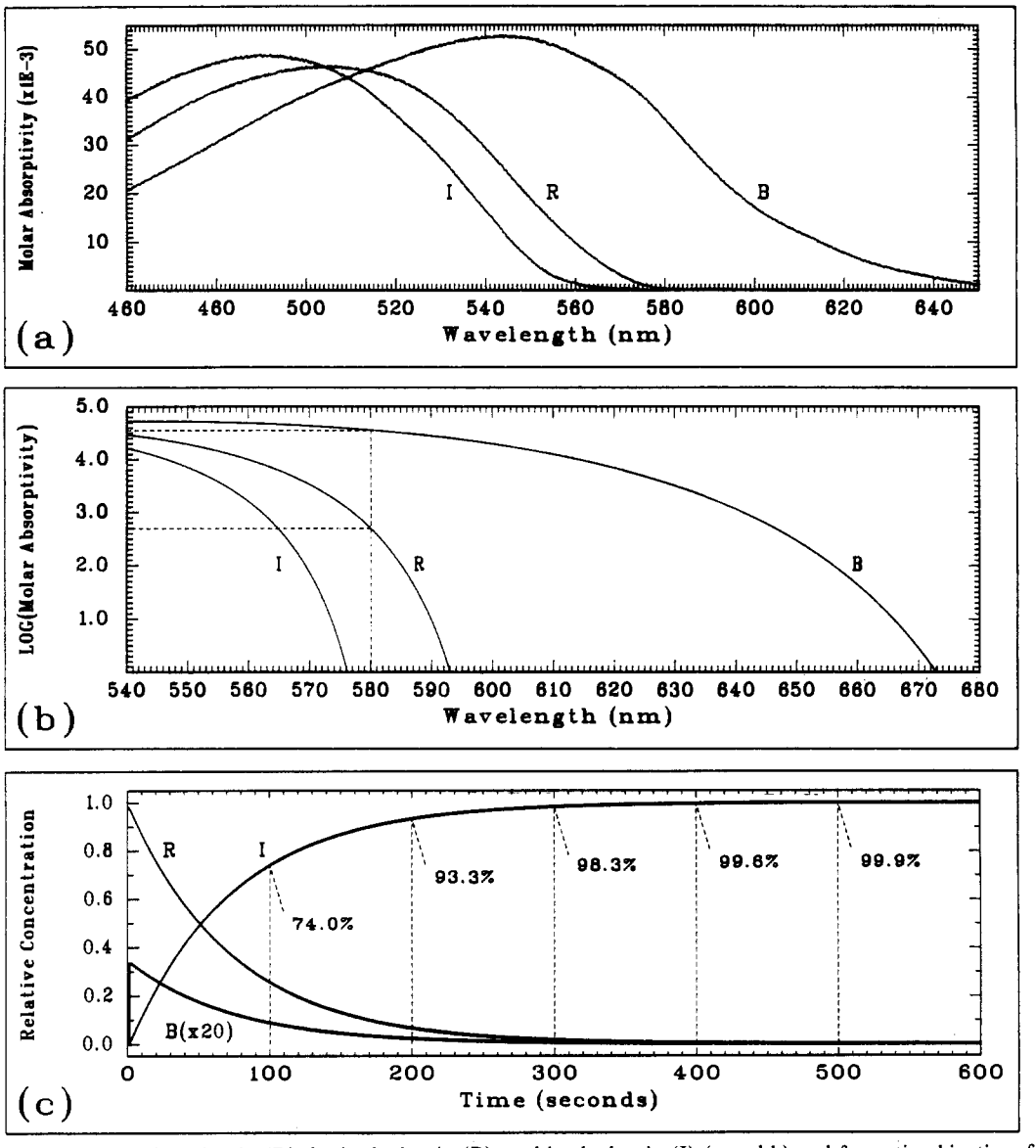

FIGURE 2: Absorption spectra of rhodopsin (R), bathorhodopsin (B), and isorhodopsin (I) (a and b) and formation kinetics of isorhodopsin (c) at 77 K (see text). Note that the time dependence of [I] presented in (c) assumes pure rhodopsin at time = 0 and irradiation from the xenon lamp and filter combination shown in Figure 1. The bathorhodopsin concentration, [B], is multiplied by 20.

Table I: Measurement of the Enthalpy of Reaction for Photochemical Conversion of Rhodopsin to Bathorhodopsin at 77 K

	experiment					
	1	2	3	4	5	6
$[R]^{a,b}$	1.000	1.000	1.000	1.000	1.000	1.000
$[B]^{b}$	0.000	0.000	0.000	0.000	0.000	0.000
$[I]^{b}$	0.000	0.000	0.000	0.000	0.000	0.000
λ_{ex} (nm)^{c}	440	461	479	520	541	565
$\epsilon_R(\lambda_{ex})^{d}$	23.9	31.6	40.9	43.7	28.2	6.1
$\epsilon_B(\lambda_{ex})^{d}$	12.2	21.0	30.0	47.8	52.4	46.4
$\epsilon_I(\lambda_{ex})^{d}$	36.2	39.7	46.9	36.2	15.3	0.6
$\alpha_R^{\lambda e}$	1.000	1.000	1.000	1.000	1.000	1.000
OD^{f}	4.01	5.32	6.87	7.34	4.75	1.02
$F_{RB}{}^{g}$	−0.670	−0.670	−0.670	−0.670	−0.670	−0.670
$F_{IB}{}^{g}$	0.000	0.000	0.000	0.000	0.000	0.000
$\Gamma_{obsd}^{\lambda}{}^{h}$	0.662 ± 0.027	0.657 ± 0.021	0.632 ± 0.025	0.614 ± 0.022	0.574 ± 0.036	0.613 ± 0.064
ΔH_{RB} (kcal/mol)^{i}	32.7 ± 2.8	31.8 ± 1.9	32.8 ± 2.2	31.7 ± 1.8	33.6 ± 2.8	29.2 ± 4.8
ΔH_{RB}^{av} (kcal/mol)				32.2 ± 0.9		

[a] Initial concentrations were generated by isolation and purification procedures (see text). [b] Initial concentrations of rhodopsin ([R]), bathorhodopsin ([B]), and isorhodopsin ([I]) (units of 10^{-4} M). [c] Excitation wavelength used for photocalorimetry measurement. [d] Extinction coefficients for λ_{ex} (units of 10^3 M^{-1} cm^{-1}). [e] Absorption partition function computed with eq 10. [f] Optical density for reaction mixture assuming 1-cm path length. [g] Terms involving $\alpha_i(\lambda_{ex})$ and ϕ_i in eq 8. The values for ϕ_1, ϕ_2, ϕ_3, and ϕ_4^{λ} are as reported by Birge and Callender (1987). [h] Mean and standard deviation based on eq 11 and five to ten measurements. [i] Computed with eq 12.

I $\xrightarrow{h\nu}$ B photoisomerization (Birge & Callender, 1987). Weighted averaging of the individual ΔH_{IB} values gives ΔH_{IB}^{av} = 27.1 ± 3.2 kcal mol^{-1}. The difference between ΔH_{IB}^{av} and ΔH_{RB}^{av} (5.1 ± 3.3 kcal mol^{-1}) is in excellent agreement with

Table II: Measurement of the Enthalpy of Reaction for Photochemical Conversion of Isorhodopsin to Bathorhodopsin at 77 K

	experiment			
	1	2	3	4
λ_{prep} (nm)[a]	580	580	580	580
[R][b]	0.000	0.000	0.000	0.000
[B][b]	0.000	0.000	0.000	0.000
[I][b]	1.000	1.000	1.000	1.000
λ_{ex} (nm)[c]	440	479	498	541
$\epsilon_R(\lambda_{ex})$[d]	23.9	40.9	45.9	28.2
$\epsilon_B(\lambda_{ex})$[d]	12.2	30.0	39.6	52.4
$\epsilon_I(\lambda_{ex})$[d]	36.2	46.9	47.9	15.3
α_R^{λ}[e]	0.000	0.000	0.000	0.000
α_B^{λ}[e]	0.000	0.000	0.000	0.000
α_I^{λ}[e]	1.000	1.000	1.000	1.000
OD[f]	6.08	7.88	8.05	2.57
F_{RB}[g]	0.000	0.000	0.000	0.000
F_{IB}[g]	−0.168	−0.165	−0.163	−0.108
Γ_{obsd}^{λ}[h]	0.928 ± 0.015	0.921 ± 0.015	0.931 ± 0.018	0.945 ± 0.026
ΔH_{IB} (kcal/mol)[i]	27.8 ± 5.8	28.6 ± 5.4	24.3 ± 6.3	27 ± 13
ΔH_{IB}^{av} (kcal/mol)			27.1 ± 3.2	

[a] Wavelength used to generate isorhodopsin (see text). [b] Initial concentrations of rhodopsin ([R]), bathorhodopsin ([B]), and isorhodopsin ([I]) (units of 10^{-4} M). [c] Excitation wavelength used for photocalorimetry measurement. [d] Extinction coefficients for λ_{ex} (units of 10^3 M^{-1} cm^{-1}). [e] Absorption partition function computed with eq 10. [f] Optical density for reaction mixture assuming 1-cm path length. [g] Terms involving $\alpha_i(\lambda_{ex})$ and ϕ_i in eq 8. The values for ϕ_1, ϕ_2, ϕ_3, and ϕ_4^{λ} are as reported by Birge and Callender (1987): ϕ_4(440 nm) = 0.168; ϕ_4(479 nm) = 0.165; ϕ_4(498 nm) = 0.163; ϕ_4(541 nm) = 0.108. [h] Mean and standard deviation based on eq 11 and five to ten measurements. [i] Computed with eq 13.

Table III: Measurement of the Molecular Response Functions Associated with the Mixed Photoreactions Rhodopsin $\overset{h\nu}{\rightleftarrows}$ Bathorhodopsin $\overset{h\nu}{\rightleftarrows}$ Isorhodopsin

	experiment			
	1	2	3	4
λ_{prep} (nm)[a]	461	565	565	565
[R][b]	0.281	0.082	0.082	0.082
[B][b]	0.583	0.013	0.013	0.013
[I][b]	0.136	0.905	0.905	0.905
λ_{ex} (nm)[c]	565	440	461	479
$\epsilon_R(\lambda_{ex})$[d]	6.1	23.9	31.6	40.9
$\epsilon_B(\lambda_{ex})$[d]	46.4	12.2	21.0	30.0
$\epsilon_I(\lambda_{ex})$[d]	0.6	36.2	39.7	46.9
α_R^{λ}[e]	0.059	0.056	0.067	0.073
α_B^{λ}[e]	0.938	0.005	0.007	0.009
α_I^{λ}[e]	0.003	0.939	0.926	0.919
OD[f]	4.836	5.855	6.521	7.755
F_{RB}[g]	0.416	−0.035	−0.041	−0.044
F_{IB}[g]	0.068	−0.157	−0.145	−0.151
Γ_{obsd}^{λ}[h]	1.293 ± 0.010	0.917 ± 0.029	0.920 ± 0.018	0.916 ± 0.013
Γ_{calcd}^{λ} ($\Gamma_{calcd}^{\lambda} - \Gamma_{obsd}^{\lambda}$)[i]	1.301 (0.008)	0.917 (0.000)	0.915 (−0.005)	0.908 (−0.008)

[a] Wavelength for which photostationary state was produced to effect initial concentrations. [b] Initial concentrations of rhodopsin ([R]), bathorhodopsin ([B]), and isorhodopsin ([I]) (units of 10^{-4} M). [c] Excitation wavelength used for calorimetry measurement. [d] Extinction coefficients for λ_{ex} (units of 10^3 M^{-1} cm^{-1}). [e] Absorption partition function computed with eq 10. [f] Optical density at λ_{ex} for reaction mixture assuming 1-cm path length. [g] Terms involving $\alpha_i(\lambda_{ex})$ and ϕ_i in eq 8. The values for ϕ_1, ϕ_2, ϕ_3, and ϕ_4^{λ} are as reported by Birge and Callender (1987): ϕ_4(440 nm) = 0.168; ϕ_4(461 nm) = 0.157; ϕ_4(479 nm) = 0.165; ϕ_4(565 nm) = 0.089. [h] Mean and standrad deviation based on eq 11 and five to ten measurements. [i] Calculated with eq 8 with values for ΔH_{RB}^{av} and ΔH_{IB}^{av} from Tables I and II.

the previously reported value of 5.2 ± 2.3 kcal mol^{-1} obtained by Cooper (1979b). Since Cooper measured the enthalpy difference between R and I, the similarity of these two values indicates that the batho products of R and I have the same ground-state energy. This is consistent with B being a single, common photoproduct of both R and I.

The data presented in Table III are complicated by the necessity to consider both ΔH_{RB} and ΔH_{IB} in the interpretation. However, in light of the indication that B is a common product connecting R and I, these data can be used to support further this hypothesis. If B is a common intermediate, then the values of ΔH_{RB} and ΔH_{IB} determined in Tables I and II can be used in eq 8 to reproduce the values of Γ_{obsd}^{λ} appearing in Table III. As shown in the latter table, values of Γ_{calcd}^{λ} match Γ_{obsd}^{λ} to within the statistical uncertainties.

Discussion

The preceding analysis indicates that ~32 kcal mol^{-1} of energy is stored during the primary event R $\overset{h\nu}{\rightarrow}$ B in the (bovine) rhodopsin photobleaching process. This value is in agreement, to within experimental uncertainty, with the value ~35 kcal mol^{-1} reported by Cooper (1979a) and later by Boucher and Leblanc (1985). The value reported here has been determined by using a range of excitation wavelengths and has a statistical uncertainty (±0.9 kcal mol^{-1}) lower than those of the previous studies (±2.2 kcal mol^{-1} by Cooper; ±1.5 kcal mol^{-1} by Boucher and Leblanc). Although all three values overlap after consideration of reported error, our results suggest that the true value of ΔH_{RB} is slightly lower than that reported previously. A weighted average of the current and literature values gives ΔH_{RB} = 33.1 ± 0.7 kcal mol^{-1}.

The value reported here for ΔH_{IB} is 27.1 ± 3.2 kcal mol^{-1}. The statistical uncertainty is relatively large due to the low quantum yield for the I $\overset{h\nu}{\rightarrow}$ B conversion. This low quantum yield results in values of Γ_{obsd}^{λ} near 1.0 (the value for a photostationary mixture), so small fluctuations in the mea-

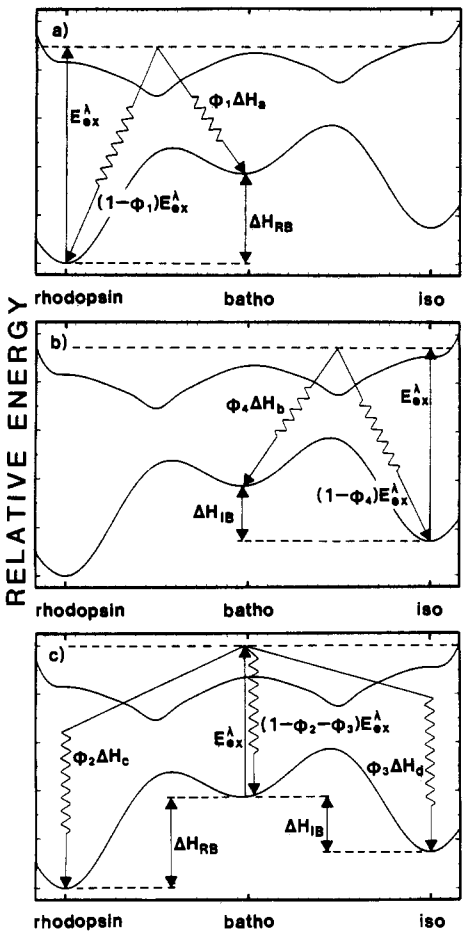

FIGURE 3: Schematic diagram of the photoreactions initiated by excitation of (a) rhodopsin, (b) isorhodopsin, and (c) bathorhodopsin at 77 K. The "reaction coordinate" is an arbitrary label and corresponds to the 11-cis → all-trans isomerization of the retinal chromophore for rhodopsin → bathorhodopsin and to 9-cis → all-trans isomerization for isorhodopsin → bathorhodopsin. The energy surfaces are qualitative and are displayed on a relative energy scale. The energy of excitation is labeled as E^{λ}_{ex}, and each decay channel is labeled by the energy it releases during the process. The ϕ_i values are quantum yields for the photoisomerizations as defined in eq 4. Other symbols are as follows: $\Delta H_a = E^{\lambda}_{ex} - \Delta H_{RB}$; $\Delta H_b = E^{\lambda}_{ex} - \Delta H_{IB}$; $\Delta H_c = E^{\lambda}_{ex} + \Delta H_{RB}$; $\Delta H_d = E^{\lambda}_{ex} + \Delta H_{RB}$.

surement of Γ^{λ}_{obsd} propagate into large uncertainties in ΔH_{IB}. The difference between this value and that for ΔH_{RB} is ΔH_{RI} = 5.1 ± 3.3 kcal mol^{-1}. The value of ΔH_{RI} is identical within experimental error with that reported by Cooper (1979b), 5.2 ± 2.3 kcal mol^{-1}. The comparison between ΔH_{RI} (Cooper, 1979b) and $\Delta H_{RB} - \Delta H_{IB}$ reported here supports further the theory that bathorhodopsin is a common intermediate between rhodopsin and isorhodopsin (Yoshizawa & Wald, 1963; Rosenfeld et al., 1977; Hurley et al., 1977). Mao et al. (1980) and Kliger et al. (1984) have reported that the absorption spectra of B′ and B″ (the photoproducts of R and I, respectively) are identical. They concluded that B′ and B″ are the same species, on the basis of the assumption that identical absorption spectra reflect identical species. The present photocalorimetry experiments indicate that if B′ and B″ exist as separate entities, they are energetically equivalent. Since the work of Mao et al. (1980) and Kliger et al. (1984) demonstrate that the "Franck–Condon surfaces" of the "two species" are identical, we conclude that bathorhodopsin formed from rhodopsin is identical with that formed from isorhodopsin. Hence, eq 1a, rather than eq 1b, represents the photochemical pathway coupling rhodopsin and isorhodopsin.

Registry No. Retinal, 116-31-4.

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