Direct Observation of the Thermal Equilibria among Lumirhodopsin, Metarhodopsin I, and Metarhodopsin II in Chicken Rhodopsin†

Hiroo Imai, Taku Mizukami, Yasushi Imamoto,‡ and Yoshinori Shichida*

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606-01, Japan

Received July 26, 1994; Revised Manuscript Received September 13, 1994⊗

ABSTRACT: Using low-temperature time-resolved spectroscopy, we have directly observed thermal back reaction of metarhodopsin I (meta I) to lumirhodopsin (lumi) and that of metarhodopsin II (meta II) to meta I in chicken rhodopsin to demonstrate the presence of thermal equilibria among lumi, meta I, and meta II. The back reaction from meta I to lumi was observed when the rhodopsin sample irradiated at −35 °C was warmed to −20 °C, while that from meta II to meta I was observed when the sample irradiated at −10 °C was cooled to −20 °C. Thermodynamic parameters of lumi, meta I, and meta II were calculated from the equilibrium constants estimated by analyzing the spectra of the equilibrium states at temperatures ranging from −30 to −10 °C. The results showed that meta I has an enthalpy and an entropy considerably smaller than those of lumi and meta II, while the difference in thermodynamic parameters between lumi and meta II is not so large. These results suggest that meta I is a crucial stage of conversion of the light energy captured by the chromophore into restricted conformations of the chromophore and/or protein, from which a large conformational change of the protein starts to form meta II.

Rhodopsin, a visual pigment present in retinal rod cells, contains 11-*cis*-retinal chromophore bound via a protonated Schiff base linkage to Lys-296 of the apoprotein opsin, whose secondary structure is predicted to contain seven transmembrane α-helices (Schertler et al., 1993). Light initiates a cis−trans isomerization of the chromophore to form the primary intermediate, photorhodopsin (Shichida et al., 1984). Subsequent thermal reactions result in the formation of several intermediates, each of which has a specific absorption spectrum, and finally lead to the formation of an enzymatically active intermediate, metarhodopsin II (meta II)[1] (Matthews et al., 1963). The meta II intermediate then activates the retinal G-protein, transducin, which triggers the enzymatic cascade system in rod cells (Fung et al., 1981; Bennet et al., 1982; Fukada & Yoshizawa, 1981). Since the light energy stored in a highly twisted chromophore in photorhodopsin (Mizukami et al., 1993) induces the changes in conformation of the protein moiety, it is important to investigate how the stored energy in the chromophore transfers to the protein moiety.

Accumulated evidence has now suggested that the highly twisted all-trans chromophore in photorhodopsin converts to a relaxed form through a series of changes in the chromophore−opsin interaction near the Schiff base, β-ionone ring, and 9-methyl group of the chromophore (Shichida et al., 1987, 1991; Albeck et al., 1989; Ganter et al., 1989; Okada et al., 1991). Then the Schiff base of the chromophore is deprotonated during the meta I to meta II transition (Matthews et al., 1963). According to calorimetric (Cooper & Converse, 1976) and spectroscopic (Matthews et al., 1963; Parkes & Liebman, 1984) studies, the enthalpy and entropy of meta II are much larger than those of meta I, suggesting that the reaction from meta I to meta II proceeds with large conformational changes in the protein moiety. Therefore, the light energy stored in the chromophore changes the conformation of the protein moiety during the meta I to meta II transition. Thus, it is of interest to investigate whether or not such a conformational change is unique for the conversion process from meta I to meta II.

This question might be answered by measurement of the entropy change during the lumi to meta I transition. Almost all of the experimental results except one (Applebury et al., 1984) suggested that the transition from lumi to meta I is unidirectional and that there is no equilibrium state between lumi and meta I. This has hampered determination of the entropy change by spectroscopic measurements, by which the entropy change during the meta I to meta II transition was estimated (Matthews et al., 1963). However, our recent work on chicken rhodopsin by means of low-temperature time-resolved spectroscopy (Imai et al., 1992) indicated that meta II was produced from a mixture of lumi and meta I even at −10 °C. This temperature is considerably higher than the transition temperature (−40 °C) for lumi to meta I transition reported by conventional low-temperature spectroscopy (Hubbard et al., 1959; Yoshizawa & Wald, 1963), suggesting that meta I forms an equilibrium state with lumi before converting to meta II. Therefore, we have tried to obtain direct evidence for the presence of an equilibrium state between lumi and meta I by measurement of the thermal back reaction from meta I to lumi. We have also tried to observe a thermal back reaction from meta II to meta I to confirm the generally accepted idea that meta I forms a

† This work was supported in part by Grants-in-Aid for Scientific Research and JSPS fellows from the Japanese Ministry of Education, Culture and Science and by the Ciba-Geigy Foundation (Japan) for the Promotion of Science.

* Author to whom correspondence should be addressed.

‡ Present address: Department of Earth and Space Science, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1994.

[1] Abbreviations: lumi, lumirhodopsin; meta, metarhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; KIU, kallikrein inhibitor units; PC, L-α-phosphatidylcholine from fresh egg yolk; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/94/0433-14351$04.50/0

thermal equilibrium state with meta II (Matthews et al., 1963; Parkes & Liebman, 1984; Straume et al., 1990). Current findings clearly show that the back reaction of meta I to lumi was observed when the irradiated chicken rhodopsin sample was warmed, while that of meta II to meta I was observed when it was cooled. From these results, we were able to estimate simultaneously the entropy and enthalpy changes during the lumi to meta I and meta I to meta II transitions, suggesting the unique properties of meta I.

MATERIALS AND METHODS

Sample Preparation. Chicken rhodopsin was extracted from chicken retinas with CHAPS as a detergent and purified by the method previously reported (Okano et al., 1989). Briefly, visual pigments in photoreceptor outer segments isolated from about 2000 chicken retinas were extracted with buffer A [50 mM HEPES, 140 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 4 μg/mL leupeptin, 50 KIU/mL aprotinin, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ (pH 6.6)] supplemented with 0.75% CHAPS and 1 mg/mL PC. After the CHAPS and PC concentrations of the extract were lowered to 0.6% and 0.8 mg/mL, respectively, by adding 1/5 vol of buffer A, it was applied to a ConA-Sepharose (Pharmacia) column (16 × 300 mm) that had been equilibrated with buffer A supplemented with 0.6% CHAPS and 0.8 mg/mL PC (buffer B). After elution of iodopsin, chicken blue, and chicken violet from the column with buffer B supplemented with 1.5 mM methyl α-mannoside, a mixture of rhodopsin and chicken green was eluted with buffer C (buffer B with a NaCl concentration of 10 mM) supplemented with 200 mM methyl α-mannoside. Glycerol was added to make a final concentration of 15.8% (v/v). The mixture of rhodopsin and chicken green was then loaded on a DEAE-Sepharose CL-6B (Pharmacia) column (16 × 300 mm) that had been equilibrated with buffer D (buffer C further supplemented with 15.8% (v/v) glycerol). Rhodopsin bound to the column was eluted with buffer B supplemented with 15.8% (v/v) glycerol. For low-temperature absorption spectroscopy, glycerol was added to make a final concentration of 56% (v/v) for the experiments carried out above −40 °C and 72% (v/v) for those below −40 °C to avoid freezing the sample.

Spectroscopy. Low-temperature absorption spectroscopy is a powerful technique used to identify the intermediates present in the photobleaching processes of visual pigments (Yoshizawa & Wald, 1963; Yoshizawa & Shichida, 1982). However, previous experiments did not pay much attention to measuring the precise kinetics of intermediates at constant temperature, because experimental limitations hampered their measurement. We have improved the technique to measure the thermal behavior of intermediates at constant temperature. We call this method low-temperature time-resolved spectroscopy. In this experiment, a spectrophotometer (Shimadzu MPS−2000) interfaced to a personal computer (PC-9801RA, NEC) was used for the measurements of absorption spectra of the samples. An optical cryostat (CF1204, Oxford) with a thermocontroller (ITC-4, Oxford) was used to keep the temperature of the sample within 0.1 °C. The rhodopsin sample was put into an optical cell (10 mm path length) in the cryostat. Irradiation of the sample was carried out by a 1-kW tungsten−halogen lamp (Rikagaku-seiki) with a glass cutoff filter (VR59 or VY52, Toshiba). Thermal reactions of intermediates are monitored by recording absorption spectra at intervals of 2−30 min until the reactions were saturated, and the data were analyzed by computer (PC9801RA, NEC).

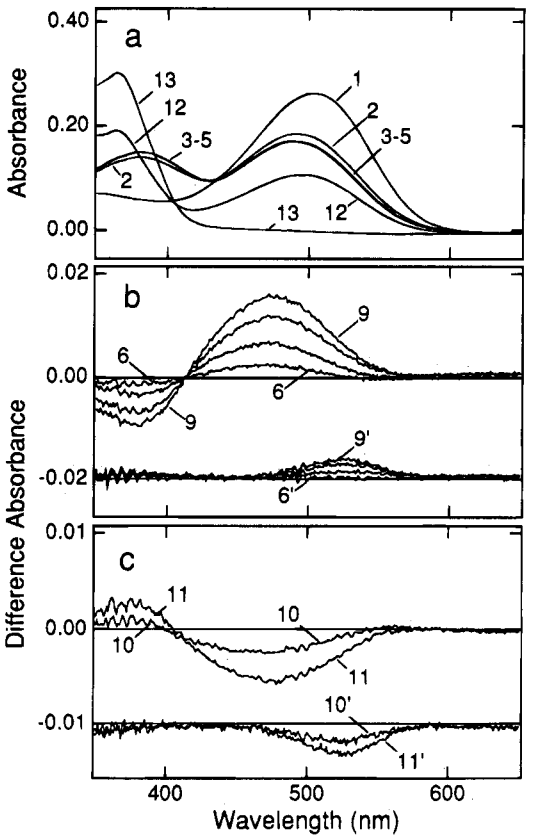

FIGURE 1: Temperature dependence of the equilibrium state between meta I and meta II. (a) All of the spectra shown in this panel were recorded at −10 °C. A rhodopsin−56% glycerol mixture (curve 1) was irradiated with a >520 nm light for 30 s (curve 2) at −10 °C and incubated for a total of 2.5, 5, and 10 min (curves 3−5). After the sample was cooled to −20 °C and rewarmed to −10 °C (the spectral measurements are shown in b and c), it was warmed at 20 °C, followed by the addition of 1/50 vol of 1 M hydroxylamine solution in buffer B supplemented with 56% (v/v) glycerol (curve 12). The sample was then irradiated with >500 nm light for 20 min at 0 °C (curve 13). (b) Difference spectra between the spectra before and after cooling the irradiated (upper panel) and unirradiated (lower panel) rhodopsin samples at −10 to −20 °C. The times after cooling are 5, 10, 15, and 20 min (curves 6−9 and 6′−9′ in the upper and lower panels, respectively). (c) Difference spectra between the spectra before and after rewarming the irradiated (upper panel) and unirradiated (lower panel) rhodopsin samples at −10 °C. The times after rewarming are 5 and 10 min (curves 10 and 11 and 10′ and 11′ in the upper and lower panels, respectively).

RESULTS

Direct Observation of the Thermal Back Reaction of meta II to meta I. To confirm the presence of a thermal equilibrium state between meta I and meta II, we tried to observe a thermal back reaction of meta II to meta I by cooling the irradiated chicken rhodopsin sample at −10 to −20 °C (Figure 1).

When chicken rhodopsin (curve 1 in Figure 1) was irradiated with >520 nm light for 30 s at −10 °C and incubated at this temperature for 15 min, a mixture of meta I and meta II was formed (curve 5 in Figure 1). The temperature of the sample was then lowered to −20 °C, and the changes in the spectra were recorded (upper panel in

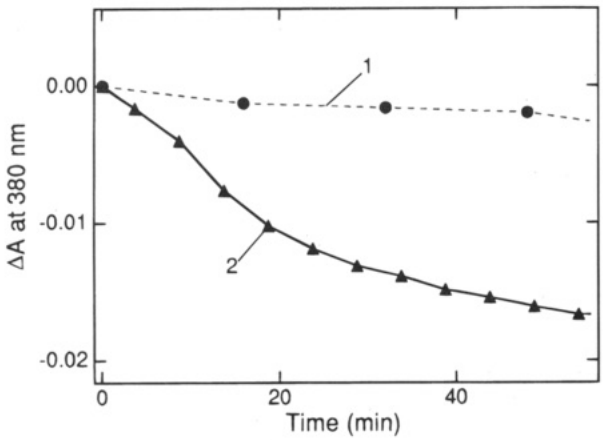

FIGURE 2: Course of the conversion from meta II to meta III at −20 °C. A rhodopsin−56% glycerol mixture was irradiated with >570 nm light for 30 s at −20 °C and incubated at this temperature. Changes in absorbance at 380 nm due to the decay of meta II to meta III are plotted against the incubation times (curve 1, ●). In comparison, the changes in absorbance at 380 nm due to the conversion from meta II to meta I observed in Figure 1b are shown as curve 2 (▲).

Figure 1b). Since the temperature of the sample reached −20 °C within 10 min under our experimental conditions, the spectra recorded at an early stage of cooling could represent the sample at temperatures intermediate between −10 and −20 °C. However, it is clear that lowering the temperature resulted in an increase in absorbance at about 480 nm and a decrease in absorbance at about 380 nm, suggesting that some fraction of meta II formed at −10 °C converts to meta I when the temperature of the sample is lowered. When the sample was rewarmed to −10 °C, a reverse spectral change was observed (upper panel in Figure 1c).

It should be noted that the spectral changes observed by cooling the sample from −10 to −20 °C (upper panel in Figure 1b) were somewhat different from those observed by warming the sample from −20 to −10 °C (upper panel in Figure 1c). This may originate from temperature-dependent changes in absorption spectra of meta I and meta II, as well as rhodopsin in the sample. The lower panels of Figure 1 (b and c) show the temperature dependence of absorption spectrum of rhodopsin, indicating that lowering the temperature increased the absorbance at wavelengths longer than the maximum, but the increase was not as large. Although we were not able to estimate the temperature dependence of the absorption spectra of intermediates, it is reasonable to speculate that they are similar to that of rhodopsin. Therefore, the difference in spectral shape observed between warming and cooling experiments is likely due to the temperature dependence of the absorption spectra of intermediates and rhodopsin.

Since it is well established that meta II converts to the following intermediate, meta III, one might speculate that the results shown in the upper panel of Figure 1b could originate from the conversion process of meta II to meta III. However, the time course of conversion from meta II to meta III monitored at −20 °C (curve 1 in Figure 2) was significantly slower than that of the formation of meta I by cooling (curve 2 in Figure 2). Therefore, we concluded that the thermal back reaction from meta II to meta I was really observed under our experimental conditions.

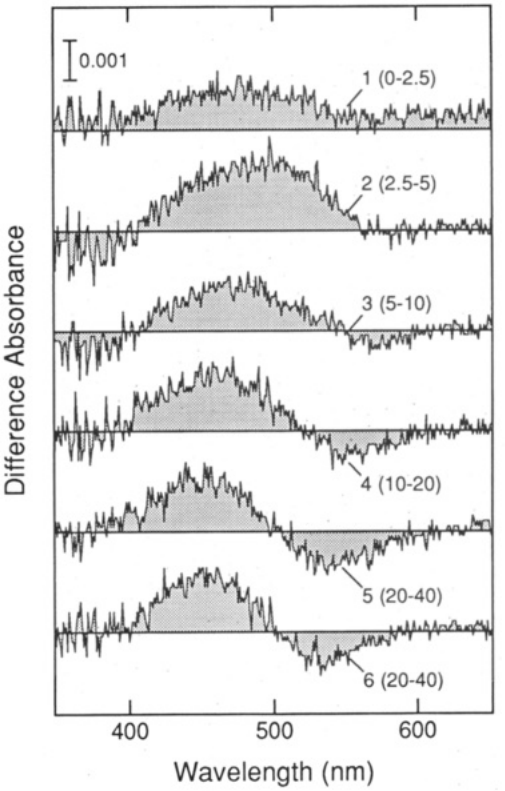

FIGURE 3: Changes in the spectrum of the mixture of lumi, meta I, and meta II from cooling. A mixture of lumi, meta I, and meta II was produced by irradiation of a rhodopsin−56% glycerol mixture with >520 nm light for 30 s at −20 °C, followed by incubation for 80 min. The spectra then were recorded 2.5, 5, 10, 20, 40, and 80 min after cooling to −30 °C. In the figure, the difference spectra between two successive spectra are shwon (curves 1−6). The values in parentheses are the times (min) of incubation after cooling.

Direct Observation of the Thermal Back Reaction of meta I to lumi. Next we lowered the temperature of the irradiated rhodopsin sample at −20 to −30 °C to observe a thermal back reaction of meta I to lumi. The experimental procedures are the same as those for Figure 1, except for the temperatures.

Figure 3 shows the spectral changes observed when a mixture of lumi, meta I, and meta II, produced by the irradiation of rhodopsin at −20 °C, was cooled to −30 °C. The spectral changes could be separated into three stages: At the first stage, an increase in absorbance at about 490 nm was observed (curve 1), which may be due to the temperature shift in the absorption spectrum of the mixture containing mainly meta I. Second, an increase in absorbance at about 470 nm and a decrease in absorbance at about 380 nm were observed (curves 2 and 3), indicating that meta II thermally reverted to meta I. Third and importantly, the subsequent increase in absorbance at about 470 nm with a decrease in absorbance at about 560 nm were observed (curves 4−6). Since the third spectral changes could be explained only by the conversion process of lumi to meta I, we concluded that some fraction of lumi contained in the mixture thermally converted to meta I when the temperature of the sample was lowered. The reverse reactions were also observed when the sample was rewarmed to −20 °C (data not shown).

We further investigated the reversal reaction from meta I to lumi (Figure 4). First, a rhodopsin sample was cooled to

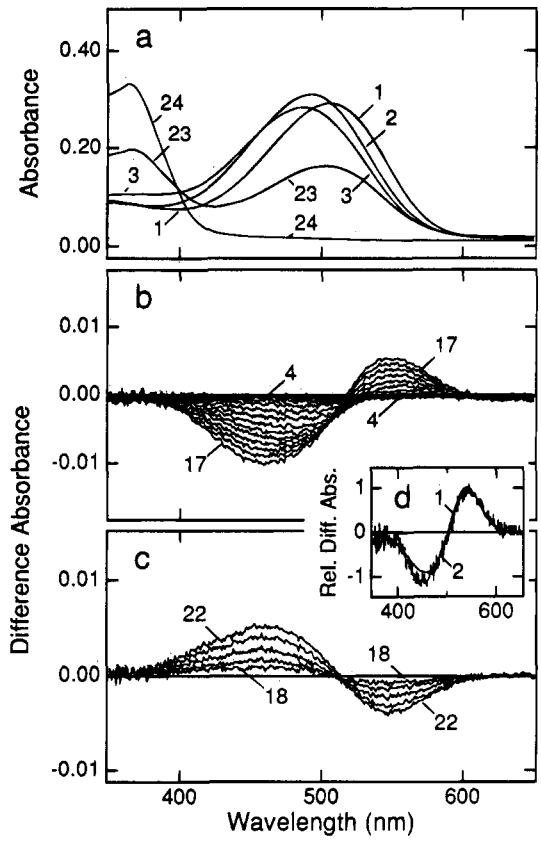

FIGURE 4: Thermal back reaction from meta I to lumi. (a) All of the spectra shown in the panel were recorded at −35 °C. A rhodopsin−56% glycerol mixture (curve 1) was irradiated with >570 nm light for 30 s (curve 2) at −35 °C and incubated for 34 h (curve 3). After the sample was warmed to −20 °C and recooled to −35 °C (the spectral measurements are shown in b and c), it was warmed at 20 °C, followed by the addition of 1/50 vol of 1 M hydroxylamine solution in buffer B supplemented with 56% (v/v) glycerol (curve 23). The sample was then irradiated with >500 nm light for 20 min at 0 °C (curve 24). (b) Difference spectra between the spectra before and after warming the irradiated rhodopsin samples at −35 to −20 °C. The times after warming are 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28 min (curves 4−17). (c) Difference spectra between the spectra before and after rewarming the irradiated rhodopsin samples to −20 °C. The times after rewarming are 2, 4, 6, 8, and 10 min (curves 18−22). (d) Curve 1 (relatively noisy line) is the difference spectrum between curves 17 and 13 in b. Curve 2 (relatively smooth line) is the difference spectrum between those of lumi and meta I obtained at −50 °C. These spectra are normalized at 570 nm.

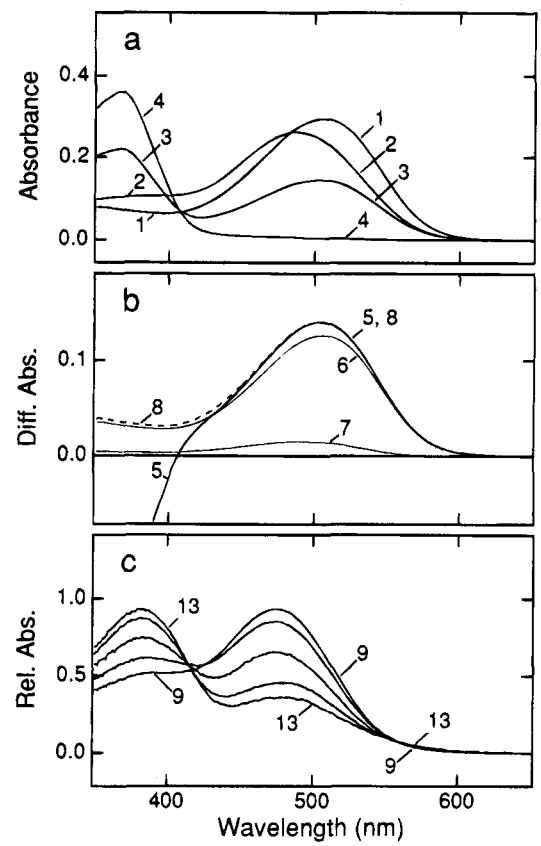

FIGURE 5: Estimation of the percentages of residual rhodopsin and isorhodopsin in the rhodopsin sample irradiated at −30 °C. (a) All spectra were recorded at −30 °C. A rhodopsin−56% glycerol mixture (curve 1) was irradiated with >570 nm light for 30 s at −30 °C, followed by incubation for 17 h (curve 2). After the sample was warmed to 20 °C, 1/50 vol of 1 M hydroxylamine solution in buffer B supplemented with 56% (v/v) glycerol was added, and the sample was incubated for 2 h (curve 3). The sample was then irradiated with >500 nm light for 20 min at 0 °C to bleach the residual rhodopsin and isorhodopsin in the sample (curve 4). (b) The difference spectrum (curve 5) calculated from curves 3 and 4 in a was simulated with the spectra of rhodopsin (curve 6) and isorhodopsin (curve 7). The resulting spectrum is shown as a broken line (curve 8). (c) Spectra of the equilibrium states at −30, −25, −20, −15, and −10 °C (curves 9−13, respectively). The calculation procedures are described in the text.

−35 °C and irradiated with >570 nm light for 30 s (curve 2 in Figure 4a), followed by incubation at this temperature for 34 h until a small amount of meta II was formed (curve 3 in Figure 4a). Then the sample was warmed to −20 °C and spectral changes were recorded (Figure 4b). The results clearly showed that after a decrease in absorbance at about 500 nm due to the temperature shift of the absorption spectrum of the mixture, meta I thermally reverted to lumi. The difference spectrum calculated from the later stage of the conversion (curve 1 in Figure 4d) was identical in spectral shape with the lumi/meta I difference spectrum (curve 2 in Figure 4d), which was calculated from the experiments at −50 °C at which only the conversion from lumi to meta I was observed (data not shown). When the sample was cooled again to −35 °C, the opposite reaction from lumi to meta I was observed (Figure 4c). Therefore, it is clear that meta I forms a thermal equilibrium state with lumi and that lowering the temperature shifts the equilibrium toward meta I.

Estimations of the Relative Thermodynamic Parameters of lumi, meta I, and meta II. We confirmed that the three intermediates, lumi, meta I, and meta II, form thermal equilibrium states at temperatures ranging from −30 to −10 °C. Now we have estimated the contents of these intermediates in the equilibrium states to calculate the thermodynamic parameters of these intermediates. Since the observed spectrum at each temperature is a composite of the spectra of the equilibrium state, residual rhodopsin, and isorhodopsin, we first estimated the percentages of rhodopsin and isorhodopsin present in the sample to calculate the absorption spectrum of the equilibrium mixture at each temperature. Next, to calculate the percentage of each intermediate present in the equilibrium state, we estimated the absorption spectra of intermediates.

Estimation of the Amounts of Rhodopsin and Isorhodopsin Present in the Sample. Figure 5a shows typical experiments to estimate the amounts of residual rhodopsin and isorhodop-

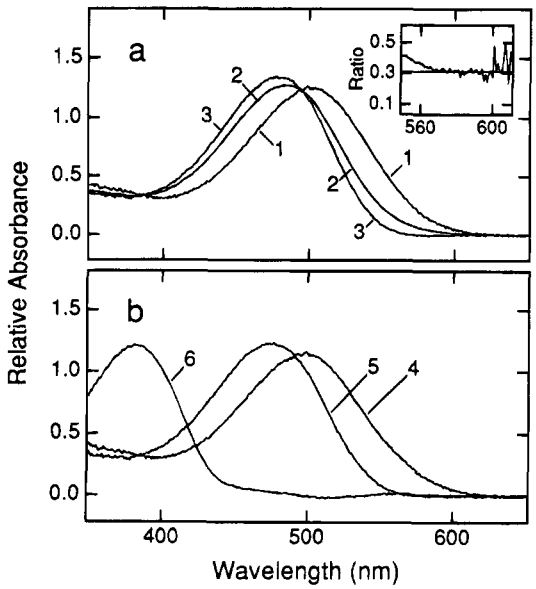

FIGURE 6: Estimation of the absorption spectrum of meta I and −80 °C and the spectra of lumi, meta I, and meta II at −15 °C. (a) Curve 1 is the absorption spectrum of lumi at −80 °C, which was estimated by conventional low-temperature spectroscopy. Curve 2 is the spectrum of the mixture of lumi and meta I. Curve 3 is the estimated absorption spectrum of meta I at −80 °C. Details of the calculations are described in the text. Inset: Ratio of the absorbance of the spectrum of the mixture to that of lumi over the wavelength region 540−630 nm. (b) Absorption spectra of lumi (curve 4), meta I (curve 5), and meta II (curve 6) at −15 °C.

sin present in the sample at −30 °C. The experimental procedures are described in the legend of Figure 5a. The difference spectrum (curve 5 in Figure 5b) between the mixture of residual rhodopsin and isorhodopsin and their bleaching products at −30 °C was calculated by subtracting curve 4 from curve 3 in Figure 5a. It was then simulated with the spectra of rhodopsin (curve 6 in Figure 5b) and isorhodopsin (curve 7 in Figure 5b) at −30 °C. The spectrum of isorhodopsin at −30 °C was calculated by the procedure described previously (Yoshizawa & Shichida, 1982), and the simulation was performed at wavelengths longer than 500 nm. The simulated curve (curve 8 in Figure 5b) was the composite of the spectra of 90% rhodopsin and 10% isorhodopsin. Thus, the spectrum of the equilibrium mixture (curve 9 in Figure 5c) was calculated by subtracting curve 8 from the observed spectrum (curve 2 in Figure 5a). The spectra of the equilibrium mixture at −25, −20, −15, or −10 °C were also calculated by a similar procedure using the experimental data obtained at each temperature. Resulting spectra are shown in Figure 5c, in which the magnitude of each spectrum is normalized to represent the same amount of rhodopsin (absorbance at λ_{max} = 1.0 at 0 °C) photoconverted.

Estimation of the Absorption Spectra of lumi and meta I. It is well known that bovine lumi is stable at −80 °C (Yoshizawa & Wald, 1963). Our experiments confirmed that chicken lumi is also stable at −80 °C, because the spectrum of the mixture composed of lumi, rhodopsin, and isorhodopsin, which was produced by irradiation of chicken rhodopsin at −80 °C, barely changed during incubation at this temperature for more than 30 h (data not shown). Therefore, the absorption spectrum of lumi at −80 °C (curve 1 in Figure 6a) was calculated by a similar method described in the previous section using the spectrum of the mixture at −80 °C. On other hand, it was difficult to calculate the spectra of meta I and meta II, because the irradiation of rhodopsin at any temperature ranging from −50 to −10 °C produced a mixture of intermediates. However, a mixture of only lumi and meta I was obtained by the irradiation of rhodopsin below −50 °C, because an increase in absorbance at 380 nm due to the formation of meta II was not observed even after incubation of the irradiated samples for 30 h at these temperatures (data not shown). Therefore, we were able to calculate the spectrum of meta I using the following procedures.

To obtain an absorption spectrum of a mixture containing only lumi and meta I, in addition to residual rhodopsin and isorhodopsin, we irradiated a rhodopsin sample with >570 nm light for 30 s at −60 °C and then incubated it at −50 °C for 30 h. The absorption spectrum of this mixture was recorded at −80 °C, and a calculation similar to that described earlier was performed to obtain the spectrum of a mixture of lumi and meta I (curve 2 in Figure 6a). Since meta I has an absorption maximum at shorter wavelength than lumi, the spectrum of the mixture at longer wavelengths should originate from only the lumi spectrum. Next we calculated the ratio of the absorbance of the spectrum of a mixture to that of lumi at −80 °C (inset of Figure 6a). From this panel, the ratios are constant in the wavelength region above 580 nm, while at shorter wavelengths they increased. These results indicated that the spectrum of the mixture at wavelengths longer than 580 nm originated from only the lumi spectrum, and the percentage of lumi contained in the mixture was estimated from the ratio. Since the ratio in the constant region was 0.31, the percentage of lumi was 31% of total intermediates present in the mixture. Thus, the absorption spectrum of meta I was calculated by subtracting the spectrum of 31% lumi from that of the mixture (curve 3 in Figure 6a).

To estimate the absorption spectra of lumi and meta I at each temperature ranging from −30 to −10 °C, we assumed that the temperature effects on the absorption spectra of lumi and meta I are similar to that of rhodopsin. First, the difference spectrum due to the temperature effect on the spectrum of rhodopsin was calculated by subtracting the spectrum recorded at each temperature from that at −80 °C; afterward these spectra were plotted against wavenumber scales, and they were translocated along the wavenumber axis to compensate for the difference in wavenumber between the maxima of rhodopsin and lumi or meta I at −80 °C. Finally, the translocated spectrum was added to the spectrum of lumi and meta I at −80 °C to produce the spectrum at each temperature.

Estimation of the Relative Amounts of Intermediates in the Equilibrium Mixture and Thermodynamic Parameters of Intermediates. Since it is reasonable to assume that meta II has no absorbance in the wavelength region longer than 480 nm, we can estimate the amounts of lumi and meta I present in the equilibrium mixture by simulating the longer wavelength region of the spectrum of the equilibrium mixture with the spectra of lumi and meta I. The amount of meta II was estimated by subtracting the amounts of lumi and meta I from the total amount of intermediates. The resulting values are listed in Table 1. These simulations also produced the absorption spectra of meta II at the respective temperatures. In Figure 6b, the absorption spectrum of meta II at −15 °C

Table 1: Contents of lumi, meta I, and meta II Intermediates in the Equilibrium Mixtures Produced at Various Temperatures[a]

temperature (°C)	lumi (%)	meta I (%)	meta II (%)
−30	11.0	62.1	26.9
−25	12.1	54.7	33.2
−20	12.9	39.2	47.9
−15	15.1	23.2	61.7
−10	16.0	14.6	69.4

[a] Standard deviations are 2.1%, 5.8%, and 5.9% for lumi, meta I, and meta II, respectively, estimated from six independent experiments.

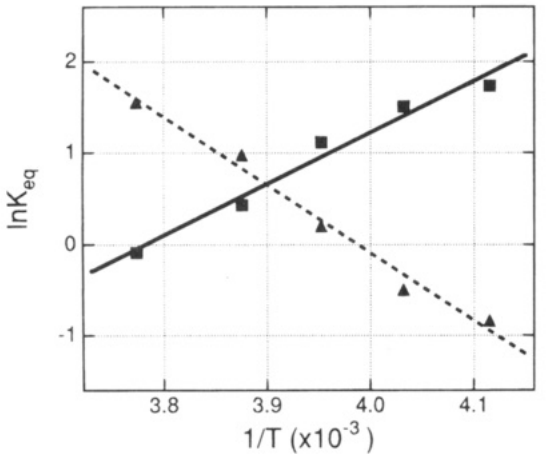

FIGURE 7: Temperature dependence of the equilibrium constants. Equilibrium constants between lumi and meta I (■) and between meta I and meta II (▲) were plotted against the inverse of the temperatures at which they were estimated. The solid and broken lines are the fitting lines calculated in a least-squares manner.

estimated from the simulations is shown together with those of lumi and meta I.

Using the estimated values listed in Table 1, equilibrium constants between the two intermediates, respectively, were calculated and plotted against the inverse of the temperature ($1/T$) (Figure 7). Thermodynamic parameters were then calculated from the following equations:

$$\Delta H = -R\ \mathrm{d}(\ln K)/\mathrm{d}(1/T)$$

$$\Delta G = -RT \ln K$$

$$\Delta S = (-\Delta G + \Delta H)/T$$

where ΔH, ΔG, ΔS, and K are the difference enthalpy, the difference free energy, the difference entropy, and the equilibrium constant, respectively, and R and T are the gas constant and temperature, respectively. In Table 2, calculated values are listed together with those previously reported (Matthews et al., 1963; Cooper, 1981).

DISCUSSION

In the present study, we have newly demonstrated the presence of a thermal equilibrium between lumi and meta I and calculated, for the first time, the ΔS value between them. In addition to the confirmation of the thermal equilibrium between meta I and meta II, the thermodynamic parameters other than the ΔS value between lumi and meta I were also calculated. They are qualitatively in good agreement with those reported previously (Matthews et al., 1963; Cooper, 1981), and the small differences might be due to the different species of rhodopsin (chicken and bovine) or to the different environments of the rhodopsins, such as detergent, glycerol

Table 2: Thermodynamic Parameters of lumi, meta I, and meta II Intermediates

	lumi → meta I		meta I → meta II	
	ΔH (kcal/mol)	ΔS (cal/mol/deg)	ΔH (kcal/mol)	ΔS (cal/mol/deg)
chicken Rh[a]	−11 (1.1)	−42 (3.0)	+15 (1.1)	+59 (3.9)
bovine Rh[b]			+13	+93
bovine Rh[c]	−9.5		+10	

[a] Values in parentheses are estimated standard deviations. Rh: rhodopsin. [b] Matthews et al., 1963. [c] Cooper, 1981.

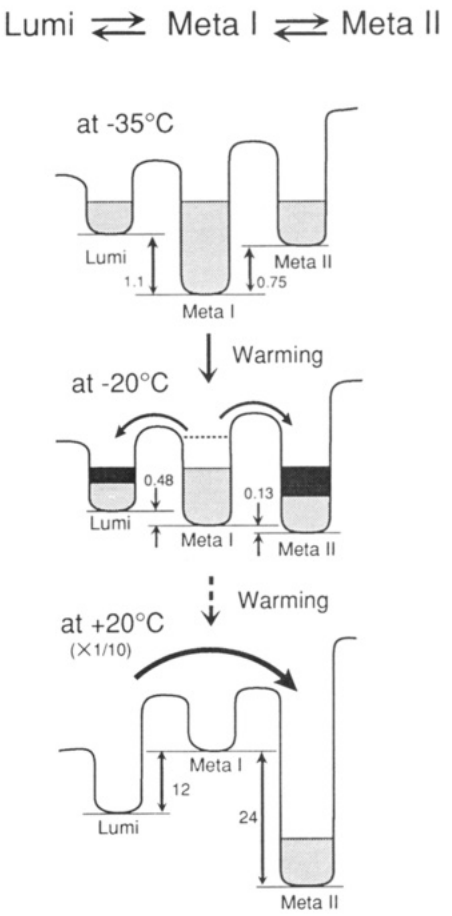

FIGURE 8: Schematic drawing of the temperature-dependent ΔG values between lumi and meta I and between meta I and meta II. ΔG values were estimated from the thermodynamic properties shown in Table 2.

concentration, and pH. Since the meta II formed under our experimental conditions can bind to bovine transducin and enhance the GDP−GTP exchange reaction (Okada et al., 1994; Tachibanaki et al., unpublished results), it is evident that our results give important information about the physiological properties of these intermediates. It is of interest to discuss what kinds of information could be derived from the thermodynamic parameters of these intermediates.

Thermal Behavior of Intermediates. Since meta I has an entropy considerably smaller than those of lumi and meta II (Table 2), the free energy level of meta I relative to those of lumi and meta II is largely temperature dependent. From the results, the ΔG value between lumi and meta I and that between meta I and meta II become small upon warming from −35 to −20 °C (Figure 8). These facts clearly explain why the thermal back reaction of meta I to lumi was observed when the equilibrium mixture was warmed, while that of

meta II to meta I was observed when it was cooled. Extrapolation of the free energy levels to higher temperature further suggests the apparent difference in the bleaching process of rhodopsin between low and room temperatures. Since meta I has the highest free energy level at 20 °C (Figure 8), it is barely accumulated at this temperature. Our preliminary calculations using the estimated equilibrium constants and the apparent rate constants obtained by laser photolysis ($\lambda_1 = 200\ \mu$s and $\lambda_2 = 12$ ms at 20 °C; Okada et al., unpublished observation) indicate that the thermal back reaction from meta I to lumi at 20 °C is about 1 order of magnitude faster than the forward reactions from lumi to meta I and from meta I to meta II, while the latter two reactions have similar rate constants. Thus, the formation of meta I would be barely detected at 20 °C, which is consistent with the experimental data obtained by laser photolysis (Thorgeirsson et al., 1992, 1993).

It should be noted that our experimental results are in contrast to those previously reported (Applebury, 1984): the former shows more production of lumi at higher temperature, while the latter shows more production of meta I.

Molecular Events during the Processes from lumi to meta II. From the thermodynamic parameters shown in Table 2, it is evident that the molecular events occurring in the lumi to meta I transition are opposite in nature to those in the meta I to meta II transition. Namely, the former transition results in the formation of the specific interaction(s) because both the enthalpy and entropy decrease, while the latter transition results in the formation of a flexible state at the expense of a loss of interaction(s). It is of interest to discuss next what kinds of molecular events are occurring during the transitions.

Recent spectroscopic studies have suggested that a specific interaction between the 9-methyl group of the chromophore and a nearby protein in rhodopsin disappears during the lumi to meta I transition (Shichida et al., 1991), while a strong hydrogen-bonding interaction is newly formed in the meta I state (Ganter et al., 1989). Since the former interaction is effective for the stabilization of the 11-cis form of the chromophore in rhodopsin, its disappearance could be due to the isomerization of the chromophore. The losses of interactions in other regions of the chromophore are also elucidated up to the formation of meta I (Shichida et al., 1987, 1991; Albeck et al., 1989; Ganter et al., 1989; Okada et al., 1991). All of these events are explained by changes in the chromophore−opsin interaction suitable for the stabilization of the all-trans chromophore. Since the thermodynamic parameters suggest a large conformational change in the total protein during the following meta I to meta II transition, we can speculate that meta I is in a state in which a thermodynamically stable conformation in the restricted region near the retinylidene chromophore is established. Therefore, it is interesting to speculate that the primary role of the light energy to produce a highly twisted chromophore eventually end in the meta I state, and the subsequent change is due to the relaxation of the whole protein into a thermodynamically stable state. In this sense, meta I could be in a state analogous to a precursor of the active state in other ligand-binding receptors.

In the transition from meta I to meta II, the incorporation of a proton into the protein moiety from the outer environment and the deprotonation of the retinylidene Schiff base were observed (Wald et al., 1950; Matthews et al., 1963; Ostroy, 1974; Parkes & Liebman, 1984). The incorporation of the proton may cause the breaking of the hydrogen-bonding (or salt bridge) system formerly present in the protein, and the deprotonation of the Schiff base results in the loss of the hydrogen-bonding network, including water molecules near the Schiff base (Maeda et al., 1993). Therefore, the increases in both entropy and enthalpy might be due to the breaking of the hydrogen-bonding system in the protein, which eventually causes the formation of the active state of rhodopsin. In fact, recent mutagenesis studies suggested that the breaking of the salt bridge between the Schiff base and its counterion E113 is essential for the activation of transducin (Cohen et al., 1992).

In the present analysis, we assumed that the equilibrium mixtures contain only three intermediates, lumi, meta I, and meta II. Since the transition from lumi to meta II should contain several molecular events, other intermediates might be present, in addition to the three intermediates in the equilibrium mixture (Straume et al., 1990; Thorgeirsson et al., 1993). Therefore, reaction kinetic analysis with incorporation of the thermal equilibrium between lumi and meta I would be indispensable for better understanding of the molecular events occurring in the formation process of the active intermediate, meta II.

ACKNOWLEDGMENT

We thank Dr. T. Okada, Prof. A. Maeda, and Prof. T. Yoshizawa for valuable discussions during the course of preparation of the manuscript.

REFERENCES

Albeck, A., Friedman, N., Ottolenghi, M., Sheves, M., Einterz, C. M., Hug, S. J., Lewis, J. W., & Kliger, D. S. (1989) *Biophys. J. 55*, 233−241.

Applebury, M. L. (1984) *Vision Res. 24*, 1445−1454.

Bennet, N., Michel-Villaz, M., & Kühn, H. (1982) *Eur. J. Biochem. 127*, 97−103.

Cohen, G. B., Oprian, D. D., & Robinson, P. R. (1992) *Biochemistry 31*, 12592−12601.

Cooper, A. (1981) *FEBS Lett. 123*, 324−326.

Cooper, A., & Converse, C. A. (1976) *Biochemistry 15*, 2970−2978.

DeGrip, W. J., Gray, D., Gillespie, J., Bovee, P. H. M., Van Den Berg, E. M. M., Lugtenburg, J., & Rothschild, K. J. (1988) *Photochem. Photobiol. 48*, 497−504.

Fukada, Y., & Yoshizawa, T. (1981) *Biochim. Biophys. Acta 675*, 195−200.

Fung, B. K.-K., Hurley, J. B., & Stryer, L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 152−156.

Ganter, U. M., Gartner, W., & Siebert, F. (1988) *Biochemistry 27*, 7480−7488.

Ganter, U. M., Schmid, E. D., Perez-Sala, D., Rando, R. R., & Siebert, F. (1989) *Biochemistry 28*, 5954−5962.

Hubbard, R., Brown, P. K., & Kropf, A. (1959) *Nature 183*, 442−446.

Imai, H., Imamoto, Y., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1992) *30th Ann. Meeting Biophys. Soc. Jpn. Suppl. 32*, 79.

Maeda, A., Ohkita, Y. J., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1993) *Biochemistry 32*, 12033−12038.

Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215−240.

Mizukami, T., Kandori, H., Shichida, Y., Cheng, A.-H., Derguini, F., Nakanishi, K., & Yoshizawa, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4072−4076.

Okada, T., Kandori, H., Shichida, Y., Yoshizawa, T., Denny, M., Zhang, B.-W., Asato, A. E., & Liu, R. S. H. (1991) *Biochemistry 30*, 4796−4802.

Okada, T., Matsuda, T., Kandori, H., Fukada, Y., Yoshizawa, T., & Shichida, Y. (1994) *Biochemistry 33*, 4940−4946.

Okano, T., Fukada, Y., Artamonov, I. D., & Yoshizawa, T. (1989) *Biochemistry 28*, 8848−8856.

Ostroy, S. E. (1974) *Arch. Biochem. Biophys. 164*, 275−284.

Parkes, J. H., & Liebman, P. A. (1984) *Biochemistry 23*, 5054−5061.

Schertler, G. F. X., Villa, C., & Henderson, R. (1993) *Nature 362*, 770−772.

Shichida, Y., Matuoka, S., & Yoshizawa, T. (1984) *Photochem. Photobiol. 7*, 221−228.

Shichida, Y., Ono, T., Yoshizawa, T., Matsumoto, H., Asato, A. E., Zingoni, J. P., & Liu, R. S. H. (1987) *Biochemistry 26*, 4422−4428.

Shichida, Y., Kandori, H., Okada, T., Yoshizawa, T., Nakashima, N., & Yoshihara, K. (1991) *Biochemistry 30*, 5918−5926.

Straume, M., Mitchell, D. C., Miller, J. L., & Litman, B. J. (1990) *Biochemistry 29*, 9135−9142.

Thorgeirsson, E. T., Lewis, J. W., Wallace-Williams, S. T., & Kliger, D. S. (1992) *Photochem. Photobiol. 56*, 1135−1144.

Thorgeirsson, E. T., Lewis, J. W., Wallace-Williams, S. T., & Kliger, D. S. (1993) *Biochemistry 32*, 13861−13872.

Wald, G., Durell, J., & St. George, R. C. C. (1950) *Science 111*, 179−181.

Yoshizawa, T., & Wald, G. (1963) *Nature 197*, 1279−1286.

Yoshizawa, T., & Shichida, Y. (1982) *Methods Enzymol. 81*, 333−354.