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The kinetics and thermodynamics of bleaching of rhodopsin in dimyristoylphosphatidylcholine

Identification of meta-I, meta-II, and meta-III intermediates

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ABSTRACT The effects of light on rhodopsin reconstituted into dimyristoylphosphatidylcholine at a molar ratio of 1:70 have been studied as a function of temperature and time. The lipid phase behavior and thermal stability of rhodopsin in the system used to measure the photolytic reactions were also determined. Thus, it was shown that the gel-to-fluid phase transition of the reconstituted membrane had a marked influence on the bleaching kinetics and thermodynamics of rhodopsin-bleaching equilibria, whereas lipid–protein interactions were also directly involved. Rhodopsin photolysis resulted in temperature-sensitive equilibria between three main photoproducts, with absorption maxima of ~480, 380, and 465 nm. Below the lipid phase transition temperature, the main photoproduct had an absorption maximum at 480 nm. With increasing temperature progressively more of the 380 nm–absorbing species was formed. The photoproduct with a spectral-maximum at 465 nm absorption was formed more slowly. Increasing temperatures decreased the ratio of the 465:380 nm–absorbing species. The thermal reactions were reversible: on cooling the higher-temperature products were converted back to the lower-temperature products. The results indicate that rhodopsin has extensive photochemical activity when reconstituted in dimyristoylphosphatidylcholine. The equilibria that we have measured resemble those of rhodopsin in the disk membrane. However, the kinetics of meta-II and meta-III formation appear to be considerably faster in the reconstituted membranes and the meta-I-to-meta-II equilibrium is displaced in the direction of the meta-I state relative to native rod outer segment disk membranes. The displacement of the meta-rhodopsin equilibrium from its position in the rod outer segment is attributed mainly to the effects of lipid–lipid interactions in the membrane bilayer and correlates with the difference in gel-to-fluid phase transition temperature of the different lipids.

INTRODUCTION

Lipid–protein interactions have been studied extensively using techniques that provide structural, dynamic, and thermodynamic information about the interactions (1, 2). Far fewer studies have concentrated on the functional importance of these interactions due in no small part to difficulties in relating activity to the entire protein population of a sample. Functional changes that give rise to a spectroscopically detectable alteration in the protein provide an assay in which this problem is overcome.

Rhodopsin is an integral membrane protein that functions in vision as the primary light-absorbing pigment. This has led to a number of studies on reconstitution of this protein to ascertain the functional significance of lipid–protein interactions (3–9). After investigation of rhodopsin reconstituted into a variety of lipids it was proposed that meta-II formation is specifically prevented in short-chain saturated lipids (4, 6–8). This observation is potentially of great functional significance as GTP-dependent displacement of the meta-rhodopsin equilibrium by transducin has shown that meta-II rhodopsin is equivalent to R*, the active product of rhodopsin photolysis (10, 11). Moreover, since rhodopsin solubilized in a range of detergents does form a meta-II intermediate, and the rate of its formation appears to increase with the ability of the detergent to denature rhodopsin (3), the proposal that short-chain saturated lipids prevent rhodopsin from forming meta-II implies that a very significant and specific interaction occurs between these lipids and rhodopsin. However, in a recent study, where the photoproducts of rhodopsin reconstituted into dimyristoylphosphatidylcholine (DMPC)[1] were investigated at short times after flash photolysis, evidence of significant and rapid formation of meta-II was obtained both spectroscopically and biochemically (9). Therefore, though it is clear that the interaction of rhodopsin with short-chain saturated lipids significantly influences the conformational changes that result from isomerization of the retinal chromophore, the degree to which these interactions differ from those of other lipids remains controversial.

Many physical studies of proteins in reconstituted systems have made use of the saturated lipid DMPC (e.g., 12–14). This is due to the availability of the pure lipid, the stability of the saturated fatty acid chains, and the relatively low temperature of the gel-to-fluid bilayer phase transition. The observation that rhodopsin is prevented from undergoing normal photolysis in this lipid (4, 6) poses questions as to the exact bearing of the interactions measured in the reconstituted systems on the situ-

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[1] Abbreviations used in this paper: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid; MOPS, 3-(*N*-morpholino)propanesulphonic acid; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine; PFPA, polychromatic flash photolysis apparatus; ROS, rod outer segment.

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ation in the native membrane. Since the lipid-protein interactions seen in the reconstituted membranes (12–14) are not significantly different from those observed in the native membrane (15–17), it is probable that the effects of reconstitution are primarily due to the influence of the bulk lipid environment on the relative stabilities of the photolytic products rather than on rhodopsin itself.

Here we have reinvestigated the rhodopsin–DMPC system in some detail using rapid kinetic spectrophotometry, differential scanning calorimetry, and electron spin resonance (ESR) spectroscopy. Using rapid-recording, polychromatic flash photolysis, we have confirmed that rhodopsin undergoes photobleaching to the meta-II state when reconstituted with DMPC and have demonstrated unambiguously that a temperature-dependent equilibrium exists between meta-I and meta-II states in these membranes. In addition, we have shown that in this short-chain saturated lipid environment, the rate of formation of the meta-III state is much more rapid than it is in the rod outer segment (ROS) disk membrane. Thus, the functional effects of changes in lipid–lipid and lipid–protein interactions are manifested by a displacement of the meta-I to meta-II equilibrium and an increase in the rate of formation of both the meta-II and meta-III intermediates, relative to the situation in native ROS disk membranes. In addition, the stability of rhodopsin towards thermal denaturation was also demonstrated to be similar in the reconstituted DMPC system to that in the native disk membrane.

MATERIALS AND METHODS

Preparation

Bovine ROS were isolated as described previously (18) and were washed exhaustively in isotonic and then hypotonic buffer to remove peripheral proteins (19). All procedures were carried out in the dark or under dim red light (Osram 4563), unless otherwise stated. Rhodopsin was purified and delipidated by affinity chromatography over concanavalin-A Sepharose in 30 mM octyl glucoside (20). Rhodopsin was reconstituted in DMPC (Fluka AG, Buchs, Switzerland) by using exhaustive dialysis from a mixed cholate (15 mM)/octyl glucoside (15 mM) detergent system (13). The resulting preparation was purified by isopycnic sucrose density gradient centrifugation, and the sharp rhodopsin-containing band was harvested, washed twice to remove sucrose, and then briefly bath sonicated to break up vesicle aggregates formed on centrifugation.

Characterization of the reconstitution

Phospholipid was determined by phosphate analysis (21), and protein was determined according to Markwell et al. (22) using rhodopsin ($A_{280}/A_{500} < 1.7$) as a standard. Rhodopsin absorption spectra were measured in 0.05% deoxycholate. Differential scanning calorimetry was performed with a calorimeter (model DSC-2B; Perkin-Elmer Cetus Instruments, Norwalk, CT) using large, stainless steel sample pans. Lipid extraction was carried out on ice with all solutions argonated. Samples containing between 25 and 100 nmol rhodopsin were saturated with NaCl, and lipid was extracted using the Folch procedure (17). Lipid composition was analyzed by silicic acid thin-layer chromatography (1).

ESR

The reconstituted membranes were doped with spin-labeled phosphatidylcholine, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine (14-PCSL), by ethanol injection, to yield a spin-label concentration < 1% of total lipid (15). ESR spectra were recorded using a 9-GHz spectrometer (model E-12; Varian Analytical Instruments, Sunnyvale, CA) equipped with an automatic variable temperature unit capable of measuring the temperature to 0.1°C. The secondary modulation output was fed into the field-frequency lock to fix spectral recording on the central line peak (23).

Rhodopsin bleaching

Reconstituted vesicles were diluted to a rhodopsin concentration of 5 mM in 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid (Hepes), 10 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS) 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. All measurements were made using the polychromatic flash photolysis apparatus (PFPA) described previously (24). This spectrophotometer allowed recording of the full visible spectrum (the digitized output of the 32-channel diode array) in 128 μs or single wavelength recording with 4 μs time resolution. The temperature control thermostatted the whole of the large brass block holding the cuvette, and sample temperature was checked before and after the spectral measurement. Data processing used a suite of programs written for a dedicated computer (Digital Equipment Corp., Marlboro, MA) (24).

To measure both the equilibria resulting from bleaching and their kinetics, a flash of light was applied orthogonally to the measuring beam. The flash, of 10 μs duration, was delivered from a xenon flash (EG & G Inc., Salem, MA) via a light guide, passed through a yellow cutoff filter (model GG495; Schott Optical Glass Inc., Duryea, PA) and was reflected back through the cuvette to increase the amount of bleaching. The yellow cutoff was used to ensure homogeneous excitation (blue light was scattered strongly by the sample), to prevent photocycling of rhodopsin bleaching-intermediates produced by the measuring light before the flash, and because the logarithmic current-voltage converters used in the spectrophotometer are more susceptible to flash artifacts in the blue, where the basal photocurrent is much lower as a consequence of reduced lamp and detector efficiency. The flash was applied after a delay of 12.5 ms and bleached ~8% of the rhodopsin in the sample. The reproducibility of the sample light-exposure resulting from the flash was >99%. After photolysis, spectral recording was carried out repetitively for a total of 50 ms. To investigate the spectral characteristics and amounts of photoproducts produced as a function of time, full visible light spectra were measured at 128-μs intervals before and after flash photolysis. The measuring light beam bleached a total of ~0.1% ms^{-1} of the rhodopsin in the sample. Difference spectra were calculated by subtraction of the spectrum preceding the flash from that recorded at a given time point after the flash. These difference spectra are the digital equivalent of setting all channels of the PFPA to zero for the unbleached complex at the temperature of measurement. To correct for significant photolysis of rhodopsin by the measuring light (at time points of ≥10 ms after the flash), a second difference spectrum obtained from the sample in question for an identical period before the flash was also subtracted. Test subtractions of this second difference spectrum scaled by factors ranging from 90 to 110% resulted in only very small changes in the overall spectral shape (data not shown). This was expected as in all cases where the correction for measuring light bleaching during spectral recording was used it was only ~10% of the photolytic bleaching.

Thermal effects were measured in the absence of bleaching by introducing prewarmed samples (43°C) into the precooled cuvette (10°C) in the precooled, brass sample-holder block. A bleaching train as detailed above was measured, and after this the spectral changes with decreasing temperature, as the sample cooled, were followed by briefly (10 ms) opening the automatic shutter at 10-s intervals. The instantaneous temperature was measured and the sample was stirred for 6 s each time the shutter closed.

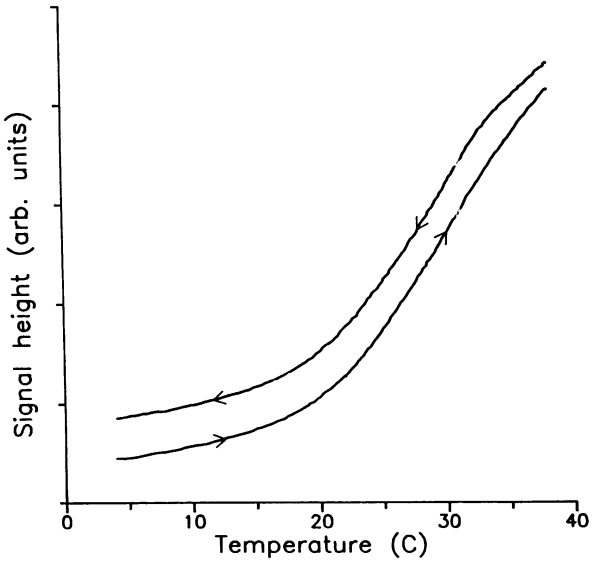

FIGURE 1 Gel-liquid crystalline phase transition of DMPC in rhodopsin recombinants used for flash photolysis. Change in the central ESR lineheight of 14-PCSL was monitored continuously as a function of temperature in an aliquot of the unbleached complex used in the photolysis experiments. The trace shows both a heating scan and a subsequent cooling scan measured at a rate of 1°C/min (the traces have been displaced vertically from one another to allow the heating and cooling scans to be clearly distinguished from each other).

The kinetics of bleaching were studied in the absence of photolysis induced by the measuring light, by filtering this source through a 1-cm pathlength cuvette containing a saturated solution of $CoSO_4$ and a blue filter (model UG5; Schott Optical Glass Inc.). Artifactual activation of many channels of the PFPA was reduced by a second filter (model UG5, Schott Optical Glass Inc.) placed between the sample and the diode array. The flash, filtered as described above, was applied orthogonally 5 ms after starting spectral recording using the 360-, 380-, and 400-nm channels of the PFPA, which were monitored quasisimultaneously before and after the flash with a time resolution of 30 or 300 μs between recorded data points.

RESULTS

Reconstitution

Rhodopsin was purified almost to homogeneity; SDS-PAGE revealed contamination of rhodopsin only by a small amount of a 220-kD protein. This contaminant copurified with rhodopsin during affinity chromatography in octyl-glucoside but amounted to <2.5% of the total intensity of gel-scans recorded on a laser-ultrascan (LKB, Turku, Finland) (data not shown). Rhodopsin was free of phospholipid and was not phosphorylated, since <1 mol phosphate was found per 100 mol rhodopsin. The absorption spectrum of purified rhodopsin had A_{280}/A_{500} and A_{400}/A_{500} absorbance ratios of 1.67 and 0.21, respectively. Organic extraction of large quantities of rhodopsin (>1 μmol) denatured the protein but revealed no remaining associated neutral lipid or phospholipid. The method of reconstitution did not change the absorption spectrum of rhodopsin dissolved in deoxycholate. The lipid/protein ratio was 70:1 mol/mol, and the extracted lipid contained no lysophosphatidylcholine or fatty acid that might have resulted from lipid degradation during preparation or measurement. Previously, it has been shown that this method of reconstitution reduces residual detergent to below a molar ratio of 1:1,000 lipids (13).

Thermal transitions

The change in central line height of the ESR spectrum from probe amounts of spin-labeled phosphatidylcholine, 14-PCSL, in the reconstituted complex was used to monitor the lipid phase behavior. It has been shown that this method is an accurate and sensitive indicator of gel-to-liquid crystalline phase transitions (23). The change in signal height with temperature was fully reversible, with no hysteresis between heating and cooling scans at rates of 1°C/min. The broad discontinuity observed in the temperature-dependent signal narrowing corresponded with the melting of the lipid chains (Fig. 1). The transition was centered around 25°C and extended over a 14–17°C range as compared with the sharp phase transition at 24°C for the pure lipid. In contrast to the situation in DMPC alone, no spectral narrowing associated with a lipid pretransition was observed.

Differential scanning calorimetry of several complexes with lipid/protein ratios from 60 to 100:1 mol/mol confirmed that a broadening of the lipid phase transition similar to that measured by ESR occurred (Fig. 2). In addition to this reversible process, two irreversible

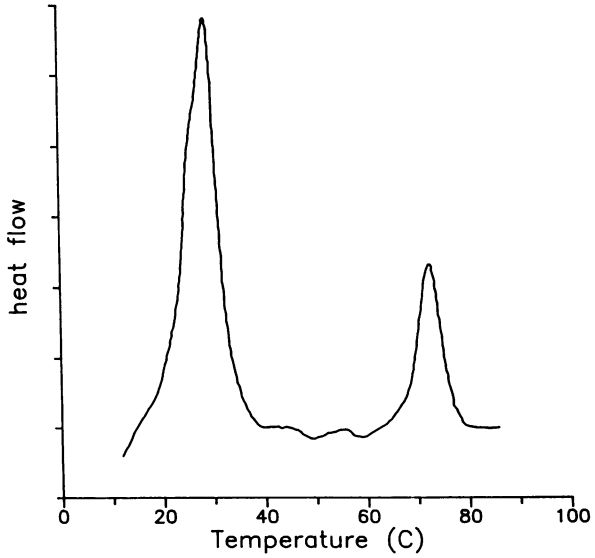

FIGURE 2 Reversible and irreversible thermal transitions of rhodopsin–DMPC recombinant membranes. DSC traces of a rhodopsin reconstitution in DMPC at a lipid/protein ratio of 64:1 mol/mol. First heating scan of the unbleached complex (5°C/min) showing the endotherm from the reversible transition of the membrane lipid and the irreversible endothermic denaturation of the membrane protein.

endotherms were measured at higher temperature. The endothermic peak observed over the temperature range from 65 to 75°C in the first scan only (Fig. 2) resulted from the thermal denaturation of rhodopsin. In samples that also contained opsin another irreversible endotherm was recorded between 50 and 60°C (data not shown).

Rhodopsin photolysis

Measurement of ROS disks

To demonstrate that it was possible to measure the light-independent equilibrium of photolysed rhodopsin (the thermal reactions) accurately with the PFPA, the temperature dependence of the meta-rhodopsin equilibrium in ROS disks was recorded. The spectrum shown in Fig. 3 *a* represents the temperature-dependent conversion of meta-I to meta-II in the disk membrane and was derived by intersubtraction of difference spectra recorded at 13 and 33°C. The difference spectra were derived by subtraction of the spectrum (the digitized output of the diode array) recorded before the light flash from the spectrum recorded 50 ms after exposure of disk membranes to a bleaching flash of light. The intersubtraction of the difference spectra eliminated rhodopsin bleaching since the concentration of rhodopsin in the samples measured at the different temperatures was identical and the light exposure of the two samples was >99% identical.

Photoproducts formed within 1 ms

A similar analysis of the thermal reaction of rhodopsin reconstituted with DMPC was undertaken (Fig. 3, *b* and *c*). The difference spectra obtained by subtraction of the spectra recorded immediately before the bleaching flash from those recorded 0.75 ms after it (Fig. 3 *b*) demonstrate that there is a change in the spectral characteristics of the major photoproduct with temperature. At lower temperatures, bleaching was arrested at a meta-I like intermediate with an absorption maximum at 480 nm. Increasing temperature caused a progressive increase in the amount of meta-II–like photoproduct with an absorption maximum at 380 nm. The isosbestic point recorded at ~410 nm indicates that over the relatively narrow temperature range of this study the relative changes in bandwidth of both photoproducts were small and similar. By intersubtraction of the difference spectra in Fig. 3 *b*, rhodopsin bleaching could be eliminated, since the sample concentration was identical and the flash exposure within 1% between the samples recorded at different temperatures. These intersubtractions (the digital equivalent of carrying out photolysis in a double-beam spectrophotometer on identical samples at two temperatures, Fig. 3 *c*) clearly demonstrate the temperature dependence of the very rapid (<1 ms) meta-rhodopsin–like equilibrium that occurs after photolysis of rhodopsin reconstituted in DMPC membranes. The main criterion that was used to judge that equilibrium had been established was examination of sequential spectra recorded at 128-μs intervals. To do this, comparison of the shape of difference spectra derived as for Fig. 3 *b* was made for consecutive postflash time points. The time course of the rapid phase of production of the meta-II–like photoproduct recorded at 380 nm (Fig. 4) was also consistent the equilibrium being established within 1 ms. At longer times, comparable with those required to establish equilibrium between the meta-I and meta-II states in disk membranes, the meta-rhodopsin equilibrium in the reconstituted rhodopsin–DMPC system was complicated by the formation of an additional spectral component attributed to the meta-III intermediate (see below).

Bleaching kinetics and later photoproducts

Bleaching kinetics were measured by recording the absorbance at 380 nm in the absence of significant photolysis by the measuring light. The increase in signal amplitude at 380 nm with increasing temperature (Fig. 4) corresponds with the change in the 480:380-nm absorption ratio described above for rhodopsin in DMPC (Fig. 3). However, the kinetics revealed that the thermal reaction was biphasic. The fast phase that was complete within 0.75 ms corresponds to the spectral equilibrium depicted in Fig. 3 *b* and *c*. In ROS disks, the rate of this meta-I to meta-II reaction was considerably slower, particularly at lower temperatures (as judged by examining the shape of difference spectra obtained by sequential subtraction of the preflash spectrum from those recorded at 128-μs intervals after it) and required up to 50 ms to be established completely at 13°C (Fig. 3 *a*).

Difference spectra corresponding to the slower phase of the thermal reaction of rhodopsin in DMPC (0.75–10 ms after the flash) are given in Fig. 5 *a* and show that during this phase different reactions were occurring. Intersubtraction of the difference spectra shown in Fig. 5 *a* demonstrate that more than two intermediates contribute to the spectral changes (Fig. 5 *b*). By subtraction of weighted amounts of the difference spectrum representing the thermal reactions of rhodpsin bleaching-intermediates at 25°C from its counterpart at 35°C, three spectral components that were involved in the reactions of the slow phase could be distinguished. The difference spectra obtained from these subtractions are shown in Fig. 6.

Reproducibility

The spectra presented show a consistent trend in their changes with temperature (Figs. 3 *b* and 4); the spectra presented here represent only a small fraction of the spectra recorded from the sample for which the phase behavior and composition was accurately determined. Spectra recorded at identical temperatures were superimposable, spectra recorded at other temperatures, using other bleaching protocols, at a lower pH, and from a second DMPC rhodopsin recombinant of approximately the same lipid/protein composition were all consistent with the results presented here (data not shown).

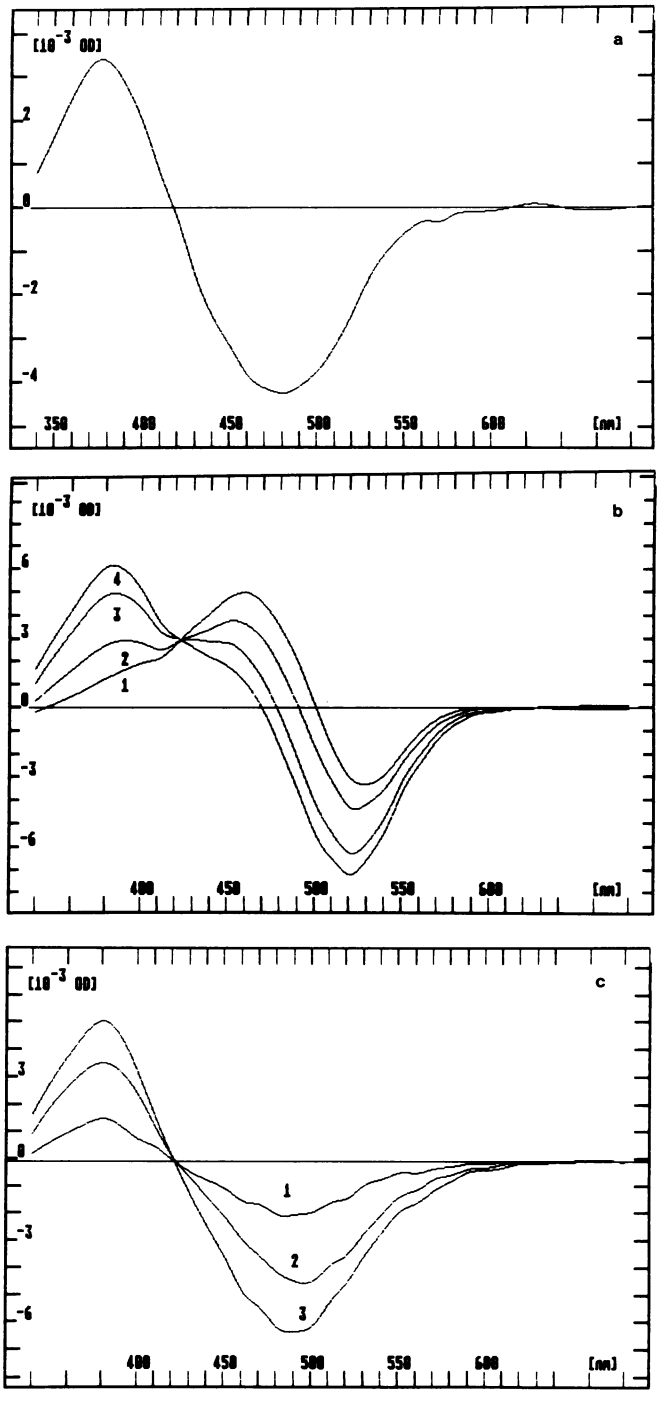

FIGURE 3 Temperature dependence of the meta-I, meta-II equilibrium in ROS disks and DMPC recombinants. (*a*) Temperature dependence of the equilibrium between meta-I and meta-II in ROS disks. The absorption spectrum recorded before exposure of ROS disks equilibrated at 13 or 33°C to a photolytic light flash that bleached ~8% of the total rhodopsin in the sample was subtracted from that recorded 50 ms after it. Before the zero-time spectrum and light flash, the sample was exposed to the measuring light for 12.5 ms. The spectrum shown was derived by subtraction of the 13°C difference spectrum from the 33°C difference spectrum and therefore depicts the temperature dependent shift in the equilibrium concentrations of meta-I and meta-II. (*b*) Rhodopsin photolysis and the temperature-dependent meta-I, meta-II equilibrium in DMPC-recombinant membranes. The absorption spectrum recorded immediately before exposure of DMPC recombinants to a photolytic flash that bleached ~8% of the rhodopsin in the sample was subtracted from the spectrum recorded 0.76 ms after the flash. The sample was exposed to measuring light for 12.5 ms before application of the flash. The samples were equilibrated at 25°C (*1*), 31°C (*2*), 35°C (*3*), and 39°C (*4*). (*c*) The temperature dependence of the meta-I, meta-II equilibrium in rhodopsin DMPC recombinant membranes. The difference spectrum recorded at 25°C in *b* was subtracted from the difference spectrum recorded at 31°C (*1*), 35°C (*2*), and 39°C (*3*) to demonstrate the temperature-dependent increase in the amount of meta-II present at equilibrium.

Reversibility

Rhodopsin photolysis was carried out at an elevated temperature and a series of spectra were measured at 35°C, the sample was then cooled in the dark to 26°C within a period of 30 s and a second set of spectra recorded. This allowed thermally induced changes in the meta-rhodopsin equilibrium position to be studied in the absence of bleaching (Fig. 7 *a*). The rapid lowering of the temperature substantially decreased the amount of meta-II–like product and this was linked with a similar rise in the higher wavelength absorption around 480 nm. Correction, by subtraction, for the change in light scattering background between the two temperatures (which was linearly dependent on wavelength, see Fig. 7 *a* at wavelengths longer than 600 nm, where no absorption from rhodopsin or its photoproducts occurs) was made. The similarity between the spectral change obtained on temperature reversal and the difference between the (difference) spectra recorded separately at these two temperatures is shown (Fig. 7 *b*).

DISCUSSION

Thermal transitions

In aqueous dispersions of pure DMPC, the gel-to-fluid bilayer phase transition is centered at 24°C and occurs over a temperature interval of <1°C (e.g., ref. 25). When rhodopsin was reconstituted into this lipid at a molar ratio of 1:70 mol/mol, little change in the transition midpoint was observed, but there was a large broadening of the temperature range of chain melting (Fig. 1). The transition was fully reversible with no hysteresis between heating and cooling scans. Taken together, these data are consistent with a reduction in lipid cooperativity, without extensive demixing of the membrane components. A lipid/protein ratio of 70:1 mol/mol was therefore also used for the functional studies, as it matches that measured for ROS disks, and it has been reported that progressive delipidation of disks influences rhodopsin photolysis (5). In the native membrane, the ROS disk, the extracted lipid undergoes a chain-melting phase transition at ~ −6°C (26). Thus, differences in the photolysis of rhodopsin in the two environments may arise both from alterations in lipid–protein interactions and from changes in the properties of the lipid itself. There was no evidence for phase separation of rho-

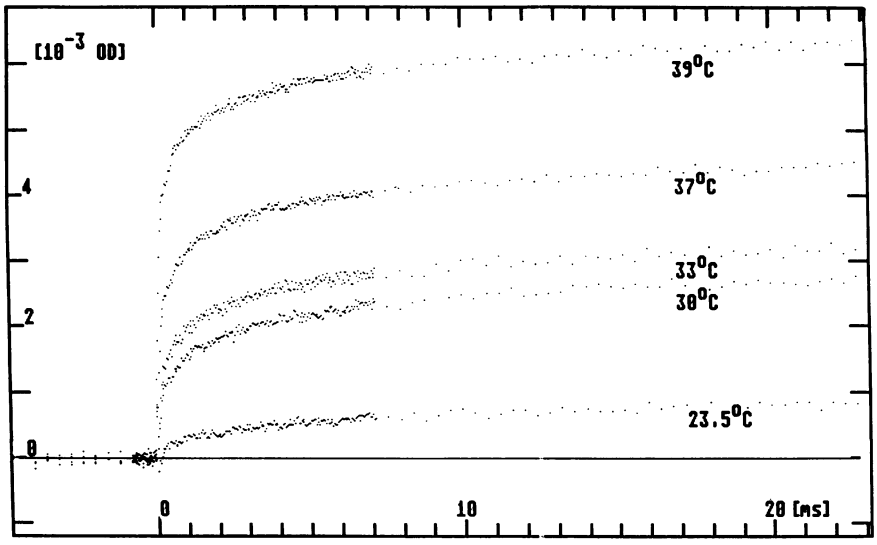

FIGURE 4 Kinetics of formation of the 380 nm spectral component. Variation in the rate of rhodopsin photolysis to the meta-II state in DMPC vesicles, with change in temperature. At time zero, the samples equilibrated at the temperatures indicated were exposed to a photolytic flash that bleached ~8% of the rhodopsin in the sample. Spectral recording, using a measuring light source attenuated at wavelengths longer than 410 nm (as described in the text) was started 5 ms before the flash. Digital resolution was 32 μs for the first 7 ms followed by 300 μs for the remainder of the trace. Traces correspond to the absorption maximum at 380 nm and no correction of the recorded data was made.

dopsin in that the midpoint of the phase transition was unaltered, and the broadening of the transition was symmetric and was dependent on protein/lipid ratio over a relatively wide range (data not shown). Therefore, over the temperature range used for functional studies, changes in the interactions between components remained small.

The thermal denaturations of opsin and of rhodopsin in the ROS disk membrane have been reported to take place at 58 and 72°C, respectively (26). The present work demonstrates that reconstitution of rhodopsin with DMPC has a minimal effect on the thermal stability of either form of the protein (c.f., Fig. 2). The thermal stability of rhodopsin is sensitive to its environment, as is clearly shown by studies using different detergents (3, 27–29). Therefore this implies that there is preservation of the structure of both the unbleached and fully bleached protein structure on reconstitution in DMPC. In addition, this suggests that DMPC–(rhod)opsin interactions contribute to the stability of the protein to the same extent as the interactions between rhodopsin and the native lipid, at these elevated temperatures.

Rhodopsin photolysis in ROS disk membranes

Photolysis of rhodopsin in ROS disks has been studied extensively (30–33). Therefore, to demonstrate the validity of the approach used here to study rhodopsin photolysis in reconstitutions with DMPC, absorption spectra from ROS disks were recorded as a function of time after a bleaching light flash. The protocol for flash photolysis was identical for spectra shown in Figs. 3, 5, and 6. However, because we were recording spectra at 128-μs intervals, the actual exposure of sample to the measuring light varied between the different data sets shown. Because of the relatively slow equilibrium of meta-rhodopsin in ROS disks, this control experiment includes substantially more exposure of the sample to the measuring light than was the case for the majority of the experiments reported in which flash photolysis of rhodopsin recombinants in DMPC is described (see below). One concern is that secondary photolysis by exposure of partially bleached samples to the measuring light may result in generation of significant levels of isorhodopsin and of rhodopsin. The data shown from this control experiment represent the worst case because the exposure of the sample at the spectral time points chosen for subtraction was its maximum. Whenever significant exposure to measuring light occurred, the difference spectra were corrected for rhodopsin bleaching by subtraction of preflash bleaching totaling that postflash (the correction was made for the spectra shown in Figs. 5 *a*, 6, and 7). Alternatively, the spectra shown were derived by intersubtraction of difference spectra (Figs. 3, *a* and *c* and 5 *b*). In these figures components resulting from rhodopsin bleaching both by the photolytic flash and by the measuring light source are canceled because the intensity of both light sources, the time of light exposure, and the concentrations of rhodopsin in the samples were maintained constant within 1%. This also cancels any secondary photolysis of rhodopsin bleached by the measuring light. Therefore only secondary photolysis of products of the flash need be considered: 0.08 (the fraction bleached by the flash) × 50 (time; ms) × 0.1 (% rhodopsin bleached by the measuring light; ms^{-1}) × relative quantum yield percent. The relative quantum yields of rho-

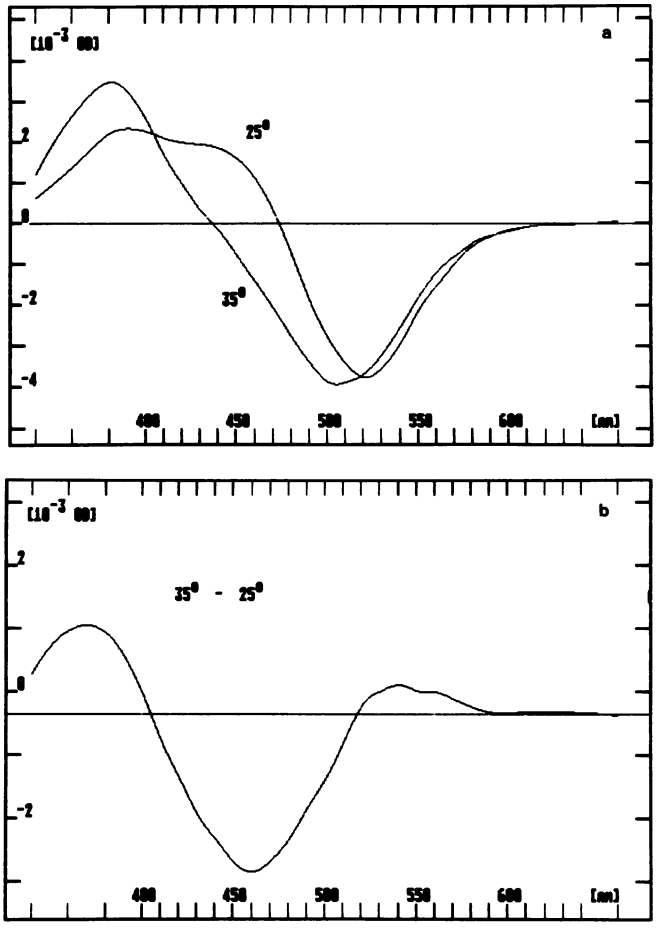

FIGURE 5 Spectral changes associated with the slow phase of the DMPC recombinant meta-rhodopsin equilibrium. (*a*) Difference spectra calculated by subtraction of the absorption spectrum recorded before illumination of DMPC recombinant vesicles equilibrated at the temperature shown from that recorded 10 ms after exposure to the photolytic flash. The samples were exposed to measuring light for 12.5 ms before application of the light flash. Spectra have been corrected for bleaching by the measuring light during data acquisition by subtraction of a component representing an equivalent length (10 ms) of preflash bleaching recorded for each sample during this 12.5-ms preflash time period. (*b*) Difference spectrum calculated by subtraction of the spectrum recorded at 25°C as in *a* without correction for measuring light exposure from the equivalent spectrum recorded at 35°C. Components due to rhodopsin bleaching by the photolytic flash and measuring light cancel because the sample concentration was identical and light exposure of the samples at the different temperatures was within 1%. The difference spectrum demonstrates that more than two spectral components are involved.

dopsin to meta-rhodopsin and of meta-rhodopsin to rhodopsin and isorhodopsin have been reported to be 1:0.5:0.09, respectively, and to follow the quantum yields for the solution state isomerization of the 11-*cis*, 9-*cis*, and all-*trans* retinal chromophore (34). Therefore, secondary photolysis would be expected to result in regeneration of ~0.2% rhodopsin of the sample and formation of <0.04% isorhodopsin. Therefore, errors of <5% are expected to be present in the spectra shown resulting from the longest exposure of samples to the measuring light due to secondary absorption. In accordance with this estimate, the spectrum shown in Fig. 3 *a* represents the temperature-dependent equilibrium of only two components; with increasing temperature, 50 ms after flash-photolysis of ROS-disks, there is less 480-nm absorption maximum component and more 380-nm absorption maximum component. Thus, increasing the temperature from 13 to 33°C results in a reduction in the equilibrium concentration meta-I with an absorption maximum of 480 nm and its conversion to meta-II with an absorption maximum of 380 nm. The meta-rhodopsin equilibrium measured here has an isosbestic point of ~420 nm. In all the above, the result is consistent with what is predicted from previous studies (30–33) demonstrating the validity of this method of analysis.

The meta-I to meta-II transition in DMPC

We have identified photoproducts only by their absorption spectrum, and analogy with spectral intermediates formed in ROS disks could conceivably be misleading. However, because of their spectral similarity, the sequence in which they are formed, and the reversible equilibrium between them (c.f., Fig. 7), the photoproducts with absorption maxima at 480, 380, and 465 nm will be referred to as meta-I, meta-II, and meta-III rhodopsin, respectively. The spectra shown in Fig. 3 *b* represent the changes caused by rhodopsin bleaching and the formation, within 0.76 ms of the photolytic flash, of the meta-I and meta-II intermediates at the temperatures shown. These spectra are the raw data obtained by direct subtraction of the signal recorded in each channel of the PFPA immediately before the photolytic flash from those recorded 0.76 ms later. This method of spectral recording, making use of the spectrum before flash photolysis as the reference spectrum, obviates the need for correction for errors such as the light scattering by the sample. The isosbestic point of the superimposed spectra (Fig. 3 *b*) and comparison with difference spectra recorded at subsequent time points (full spectra were recorded every 0.128 ms) demonstrated that at all temperatures investigated the meta-I, meta-II equilibrium had been established within the first 0.76 ms after the flash. Intersubtraction of these spectra, as indicated in Fig. 3 *c*, shows that there is a progressive shift towards the meta-II state in the position of equilibrium with increasing temperature. Comparison with the results from native disks and reconstitutions in unsaturated lipids (3, 8, 30) shows that DMPC has a general influence on the meta-I to meta-II equilibrium, which essentially correlates with the difference in phase transition temperatures of the various lipids.

Recently, a qualitatively similar behavior of the rhodopsin bleaching intermediates has been reported at times up to 3 s after the bleaching flash (9). However, quantitative differences exist between the present study

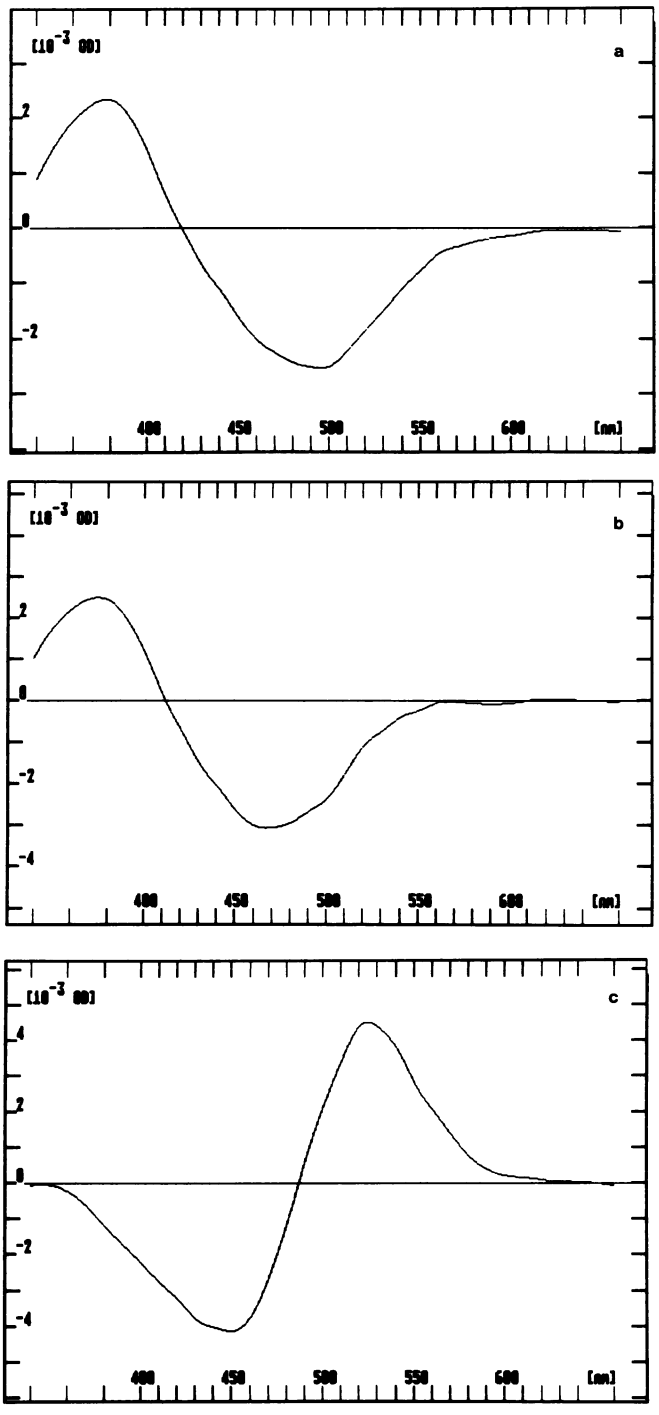

FIGURE 6 Resolution of the temperature-dependent slow phase of the DMPC recombinant meta-rhodopsin equilibrium. The multicomponent slow-phase spectrum was resolved by subtraction of weighted amounts of the difference spectrum derived by subtraction of the spectrum recorded immediately after flash photolysis from that recorded 10 ms later at 25°C from the equivalent recorded at 35°C. Before the flash that bleached ~8% of rhodopsin in the sample, the samples were exposed to the measuring light for 12.5-ms and, before the weighted subtraction, spectra were corrected for rhodopsin exposure to the measuring light by subtraction of a spectrum derived from 10 ms of preflash exposure of that particular sample. The subtraction of various weighted amounts of the spectrum recorded at 25°C containing different proportions of the three components also present in the spectrum at 35°C was carried out until, by trial and error, each one of the three components in turn was matched between the spectra recorded at different temperatures. The main criteria for subtraction end points were that there should only be a single baseline crossing and that at least one of the resulting spectral components should have a spectrum that closely resembled the meta-I or meta-II spectrum. The resolved spectra represent temperature dependent increases in meta-II relative to meta-I (*a*); in meta-II relative to meta-III (*b*); and in meta-I relative to meta-III (*c*).

and that of Mitchell et al. (9). This may in part result from an instability of the meta-I, meta-II equilibrium in DMPC that is discussed further below.

The cooperative lipid–lipid interactions appear to influence both the position and the temperature dependence of the equilibrium. We postulate that these interactions restrict the membrane expansion required for the meta-I to meta-II transition (32, 33) and that this is a major determinant of the differences observed between rhodopsin photolysis in ROS disks and reconstituted DMPC membranes. A similar correlation of the meta-rhodopsin equilibrium with a lipid-free volume parameter has been observed for rhodopsin reconstituted in different lipid systems (9, 35). Although it was found that the shift in phase transition temperature between the native lipid and DMPC largely correlates with the effect on the bleaching of rhodopsin, there is evidence that other factors may also play a role. The bulk DMPC lipid is already in a liquid-crystalline state at 33°C, whereas the meta-I to meta-II equilibrium is still well shifted from its position in ROS disks, up to the highest temperature measured (39°C). Thus the lipid–rhodopsin interface probably also plays a role in determining the relative stability of the different rhodopsin bleaching intermediates. In line with this, rather different properties have been reported for the interfacial lipid molecules from those measured in the bulk lipid (14, 15, 17).

The kinetics of meta-II formation in DMPC were measured in the absence of bleaching by the measuring light and are clearly biphasic at the resolution of our spectral recording (Fig. 4). The final amplitude of the fast phase matches that of the meta-II absorption shown in Fig. 3, ruling out significant absorption at 380 nm due to the meta-I formation. The fast phase of the kinetics is significantly more rapid than the rate of meta-II formation in ROS disk membranes (3, 9) and was too rapid for quantitative analysis of its rate. However, the equilibrium was effectively established within ~1 ms at 25°C in DMPC, whereas in ROS disks at this temperature the time required for the 380-nm absorption to increase to $1 - 1/e$ of the steady state has been reported to be 4 ms (9). In general, detergents that destabilize rhodopsin structure tend to increase the rate of meta-II production (3). The similarity in denaturation temperatures of rhodopsin and of opsin in DMPC to those in disk membranes indicates that the effects of the lipid may be more complex than simply the stability to thermal denaturation suggests. To form meta-II rhodopsin from meta-I,

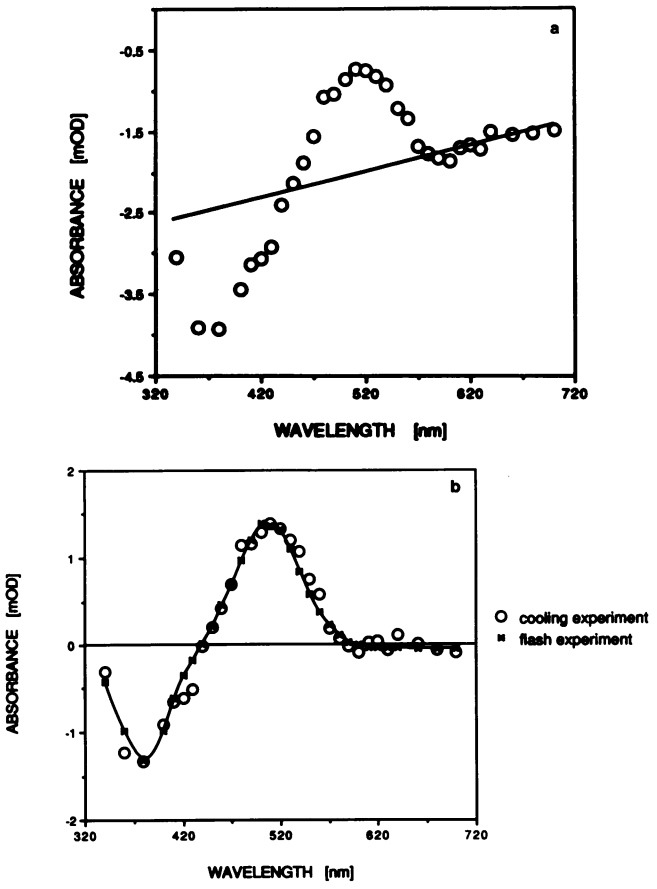

FIGURE 7 Temperature-dependent reversal of the formation of meta-II in rhodopsin-DMPC recombinants. Thermal reversibility of the meta-I to meta-II transition of the rhodopsin–DMPC complex at pH 7.0. Before measurement the sample was warmed by incubation at 43°C. The warmed sample was added to the precooled measuring cuvette and spectral recording and photolysis was started within 5 s. The sample temperature was measured immediately after each period of spectral recording and the sample was stirred vigorously between spectral measurements to ensure that the sample temperature was uniform. Automatic gating of the shutter cut off illumination between measurements. (*a*) The difference spectrum shown was generated by subtraction of the spectrum recorded at 35°C from the spectrum measured 30 s later at 26°C. The sample was exposed to the measuring light for a total of 62.5 ms (12.5 ms before the flash that bleached ~8% rhodopsin and 50 ms after it) at the end of which the 35°C spectrum was measured and for a further 20 ms before recording of the 26°C spectrum. Rhodopsin photolysis by the measuring light was corrected for by subtraction of preflash bleaching (2 × 10 ms) corresponding to the 20-ms exposure during cooling. An underlying linear light scattering change resulting from the change in sample temperature is shown. (*b*) After subtraction of the light-scattering component shown in *a*, the temperature-dependent conversion of meta-II to meta-I has been compared with a difference spectrum constructed by intersubtraction of the photolytic changes recorded separately at 25 and 35°C.

the retinal Schiff's base must be deprotonated (36). In the native environment, this is a complex process that actually results in proton uptake (6, 37). It may be that reconstitution of rhodopsin with short-chain lipids specifically alters the protein conformation towards the ends of the membrane-spanning helices and thereby makes the retinal Schiff's base more acidic in the meta-I state. This in turn may explain the observation that the meta-I, meta-II equilibrium of rhodopsin in DMPC bilayers appeared relatively insensitive to changes in pH when compared with native membranes (6) and this study (data not shown).

Formation of meta-III in rhodopsin-DMPC

Recent investigations on the formation of the meta-II intermediate in native and a variety of reconstituted membrane systems (9, 35, 38) have demonstrated that the absorption changes at 380 nm cannot be described by a single exponential process. As a result, Mitchell et al. (9) proposed the existence of two kinetically distinct forms of meta-II rhodopsin. In the present experiments with polychromatic flash photolysis, it was possible to obtain spectral resolution in addition to kinetic resolution of the two phases. From the time-resolved spectra, it is possible to ascribe the slower phase of the kinetics to an equilibrium between the meta-II and meta-III states, on the basis of the relatively slow appearance of a photoproduct absorbing at 465 nm (see Figs. 5 and 6). The possibility that the extra intermediate results from the formation of isorhodopsin can be ruled out for several reasons. The absorption maximum of isorhodopsin is at 490 nm (a longer wavelength than the absorption maximum of meta-I) whereas the absorption maximum of the slowly appearing photoproduct is at 465 nm (a shorter wavelength than that of meta-I; Figs. 5 and 6), and exactly that reported for meta-III. Moreover, as discussed above for the control experiment (Fig. 3 *a*), there was no evidence in the spectrum for significant production of isorhodopsin nor would any be predicted from the actual sample exposure to measuring light. At the time points shown in Figs. 5 and 6, the actual postflash exposure of sample to measuring light was only 20% that of the control experiment (Fig. 3 *a*), making secondary photolysis even less likely than in the data recorded for ROS disks. The kinetics assessed by comparison of successive difference spectra at shorter and longer time points than shown in Fig. 5 also suggest that the production of the 465-nm component approached equilibrium, rather than that it continued to increase with increased exposure to the measuring light; this is consistent with the slow phase of the biphasic kinetics (Fig. 4).

Difference spectra that have been corrected for postflash bleaching by the measuring light are given in Fig. 5 *a* and show the result of rhodopsin photolysis 10 ms after the bleaching flash at both 25° and 35°C. These spectra can be compared directly with those recorded at short times after the flash (Fig. 3 *b*) and show that the slow phase of the increase in absorption at 380 nm corresponds to a different spectral process from that of the fast phase. Subtraction of the spectrum recorded at 35°C from that recorded at the lower temperature does not produce a simple two-component difference spectrum,

as it did for the corresponding spectra recorded at earlier times (Fig. 3 *c*) but clearly shows the presence of one or more additional components (Fig. 5 *b*). Weighted subtractions (Fig. 6) generated three two-component difference spectra that correspond to meta-I with meta-II (Fig. 6 a), meta-III with meta-II (Fig. 6 *b*), and meta-III with meta-I (Fig. 6 *c*) components, respectively. It appears from this that the position of the meta-II, meta-III equilibrium was shifted towards meta-II by increased temperature, indicating that free energy of the meta-II intermediate in the DMPC recombinants decreased relative to that of the meta-III intermediate with increasing temperature.

Temperature-dependent reversibility of the meta-rhodopsin equilibria in DMPC

The observations discussed above suggest that the same photointermediates were observed for rhodopsin in DMPC as in the native membrane, albeit at different concentrations and with different rates of formation. This is substantiated by reversal of the thermal transitions by cooling a mixture of the photoproducts (Fig. 7). Cooling the equilibrium mixture of photoproducts from 35 to 25°C resulted in spectral changes that, after correction for bleaching by the measuring light and for light-scattering changes, was similar to the subtraction of the spectrum recorded at 25°C, which is dominated by the meta-I intermediate, from the spectrum recorded at 35°C, which is dominated by the meta-II intermediate (see Fig. 7 *b*).

Comparison with previous results

First it should be mentioned that in previous reports saturated short-chain phospholipids have been suggested to affect the photolysis of rhodopsin to a greater extent than do either other phospholipids or detergents. Such lipids have been suggested to retard photolysis greatly (4), to give rise to somewhat different mechanisms for hydrolysis of the retinal Schiff's base (6, 7), or to prevent formation of the meta-II intermediate (8). However, the most recent study on reconstitution of rhodopsin in a short-chain saturated lipid (9) has come to rather different conclusions regarding the photochemical functionality of rhodopsin in DMPC, which also are consistent with the results presented here. This latter work used a reconstitution method somewhat different from those used in the previous studies but that is rather similar to that described here.

In the work by Mitchell et al. (9) it was demonstrated that a significant shift in the meta-rhodopsin equilibrium toward the meta-II state was induced by addition of transducin to the bleached sample reconstituted in DMPC. In addition, the photolysed rhodopsin in DMPC was found to be capable of activating guanine nucleotide phosphate exchange by transducin. Though these results do not demonstrate unequivocally that the meta-II state is stable in DMPC membranes in the absence of transducin, it is extremely likely that this is the case. Moreover, it was also demonstrated that a spectral component from rhodopsin in DMPC that absorbed at 380 nm, which was indistinguishable from that induced by transducin, could form rapidly after illumination.

The present results essentially confirm the findings of Mitchell et al. (9) but also extend them in very important ways: by demonstrating the existence of a meta-rhodopsin equilibrium that can be reversed by changing the temperature and by showing the rapid formation of the meta-III intermediate for rhodopsin in DMPC. These new measurements only were made possible by exploiting the combined spectral and time resolution of the polychromatic flash photolysis method (24). It is found that, although there are specific effects of reconstitution with DMPC on the rate and equilibrium positions of the photolytic reactions, the differences between reconstitution of rhodopsin into DMPC and other lipids (3–5, 7–9) are quantitative rather than representing fundamental changes in the photolytic pathway.

Previous studies of rhodopsin DMPC recombinants that have seen no or only very limited formation of meta-II have relied either on detection of very small (because of the position of the meta-I/meta-II equilibrium at the temperature chosen) rapid transients at 480 nm or on less direct measurements at much longer times (4, 6, 7); these differences are likely to be sufficient to account for the differences reported. Baldwin and Hubbell (6) used a long bleach of rhodopsin (10–30 s), followed by spectral recording (1-min scans) at periods occurring minutes after photolysis. Thus, their results cannot be directly compared with our data, which were recorded in the period 100 μs to 10 ms after photolysis. The time period of the much faster meta-I to meta-II and meta-III equilibria in DMPC, however, corresponds directly with that of our measurements. Thus, the detailed studies by Baldwin and Hubbell (6, 7) on the influence of lipid environment pertain to the slower thermal reactions of bleached rhodopsin and demonstrate very profound effects on the reactions subsequent to the meta-rhodopsin equilibrium in DMPC that is reported here. These results at later times may also be related to the much more rapid formation of the meta-III intermediate in DMPC than in ROS disks. Finally, it should be pointed out that Wiedmann et al. (8) have also reported no formation of meta-II rhodopsin in DMPC but this work refers to a different reconstitution procedure from that used by Mitchell et al. (9) and from that used here.

CONCLUSION

We have shown that photolysis of rhodopsin yields spectral components that resemble meta-I, meta-II, and meta-III in DMPC recombinant membranes, and that, in the dark, lowering the temperature appears to reverse

the meta-I to meta-II reaction. However, this is not to say that the lipid environment does not profoundly alter the temperature dependence of the meta-rhodopsin equilibrium. Indeed, the influence of DMPC on the meta-rhodopsin equilibrium is more marked than that found when rhodopsin is reconstituted into a range of other lipids that resemble more closely those of the native membrane (4, 7, 8). In DMPC reconstitutions, far higher equilibrium ratios of meta-I rhodopsin to meta-II are found than in ROS disks or in reconstitutions with longer-chain unsaturated lipids, at corresponding reduced temperatures. At low temperatures, the rate of formation of both the meta-II and meta-III intermediates from meta-I intermediate was much faster than the corresponding reactions either in ROS disks or in other reconstituted membranes. Subsequent to the meta-rhodopsin equilibria, there also may be more rapid hydrolysis of the chromophore to free retinal than occurs at a similar temperature in ROS disks (6). We have demonstrated that the thermal stability of rhodopsin was not changed by reconstitution with DMPC. The phase transition of the lipid was broadened by inclusion of the protein but remained centered about the transition temperature of the pure lipid, implying that rhodopsin interacted with lipid irrespective of the phase state of the bulk DMPC. There are clear indications that interactions between rhodopsin and DMPC do affect the function of the protein, while nevertheless preserving the crucial initial steps of the normal photocycle. However, the general properties of the bulk lipid environment of rhodopsin appear to be a far more critical functional determinant than are specific lipid–protein interactions. Finally, since both the kinetics and thermodynamics of bleaching are very sensitive to the lipid environment, it seems probable that a relatively large reorganization of the membrane domain of rhodopsin is required at the stage of meta-II production.

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