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Structure and function in rhodopsin: The fate of opsin formed upon the decay of light-activated metarhodopsin II *in vitro*

(opsin unfolding/folding/denaturation/11-*cis*-retinal/chromophore/regeneration)

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ABSTRACT **We report that the light-activated bovine metarhodopsin II, upon decay, first forms opsin in the correctly folded form. The latter binds 11-*cis*-retinal and regenerates the native rhodopsin chromophore. However, when the opsin formed upon metarhodopsin II decay is kept in 0.1% dodecyl maltoside, it converts in a time-dependent manner to a form(s) that does not bind 11-*cis*-retinal. On subsequent addition of 11-*cis*-retinal, slow reversal of the non-retinal-binding forms to the correctly folded retinal-binding form has been demonstrated. We have studied the influence, on the above interconversions, of pH, phospholipids (rod outer segment and soybean), dithiothreitol, and a mixture of reduced and oxidized glutathione. Chromophore regeneration in the presence of 11-*cis*-retinal was highest at pH 6.0–6.3. The addition of dithiothreitol just before bleaching gave back only a small amount (7%) of rhodopsin on the subsequent addition of 11-*cis*-retinal, whereas the slow phase(s) of chromophore formation was completely abolished. The presence of a mixture of reduced and oxidized glutathione did not significantly affect the results. Addition of phospholipids, either from soybean or rod outer segment, prior to bleaching stabilized the initially formed opsin, resulting in much higher chromophore regeneration. However, addition of the phospholipids after conversion of the opsin to non-retinal-binding form(s) arrested the subsequent reversal of the opsin to the retinal-binding form.**

Unfolding and refolding of the seven-helix integral membrane protein bacteriorhodopsin was studied in the early 1980s (1). Efficient refolding from a completely denatured state was demonstrated for this protein (2–4). To date, subsequent investigations to refold the dim-light photoreceptor bovine rhodopsin, which contains the specialized three domains, from unfolded states have, however, been uniformly unsuccessful. These attempts constituted a wide variety of conditions including the ideas developed for refolding of bacteriorhodopsin (A. Kronis and H.G.K., unpublished work). We now report on the development of a partially reversible denaturation–renaturation system for this sensory protein. Rhodopsin upon light activation undergoes 11-*cis* → all-*trans* isomerization of the retinal chromophore; the metarhodopsin II (Meta II) intermediate formed, after execution of its signal transduction function, discards the bound molecule of all-*trans*-retinal, and the resulting opsin is believed to recycle by re-forming the functional chromophore by capturing a new molecule of 11-*cis*-retinal (5). We have now investigated the nature and behavior of the opsin formed from light-activated Meta II in *in vitro* systems, particularly in the detergent *n*-dodecyl β-D-maltoside (DM).† We find that in the presence of 11-*cis*-retinal, added immediately after illumination of rhodopsin, the native rhodopsin chromophore regenerates and the rate of its formation parallels the rate of decay of the Meta II intermediate. Thus, the opsin formed upon decay of this intermediate regains the conformation of the native ground-state opsin. However, while being kept in the dark in DM, the opsin converts to one or more non-11-*cis*-retinal-binding forms in a time-dependent manner. Upon subsequent addition of 11-*cis*-retinal, a slow regeneration of the native rhodopsin chromophore is observed. Usually, three rate components are observed for this process: the first, a rapid one ($t_{1/2}$ = 15–60 sec) ascribed to the surviving correctly folded opsin, the second with a $t_{1/2}$ of some minutes, and the third with a $t_{1/2}$ of 6–10 hr. We document these findings and present additional observations on the influence of different conditions on the reversal to the correctly folded opsin. The system appears promising for further studies of unfolding and refolding of the photoreceptor.

MATERIALS AND METHODS

Materials. Bovine retinae were purchased from J. A. Lawson Co. (Lincoln, NE); DM was from Anatrace (Maumee, OH). 11-*cis*-Retinal was a gift of P. Sorter (Hoffmann–La Roche) and R. Crouch (Medical University of South Carolina and the National Eye Institute).

Preparation of Delipidated Rod Outer Segment (ROS) Rhodopsin. ROS membranes were prepared under dim red light (>650 nm) from frozen bovine retinae according to Wilden and Kuhn (7). Urea-washed ROS membranes were prepared by the method of Shichi and Somers (8). The membranes were suspended in 10% (wt/vol) sucrose containing 5 mM Tris·HCl (pH 7.5), quickly frozen in liquid N_2, and stored at −70°C. ROS rhodopsin was prepared from the urea-washed ROS membranes by solubilization in 1% DM (9) and was further purified (delipidated) by affinity chromatography on Con A-Sepharose (Pharmacia) (9, 10). The Sepharose (250 μl) was washed six times by using, each time, 12 ml of 100 mM sodium phosphate buffer (pH 6.3) containing 150 mM NaCl, 1 mM EDTA, and 0.1% DM, which is hereafter denoted buffer A. The rhodopsin that bound to the Sepharose was eluted with 0.3 M α-methylmannoside in buffer A (the column was washed three times with 300 μl of the eluant each time for a 250-μl column of Sepharose). Rhodopsin was stored in the dark at 4°C. The preparations showed UV/visible absorbance ratios (A_{280}/A_{500}) of 1.7–1.8.

Soybean and ROS Phospholipids. Soybean phospholipids (asolectin) were purified by the method of Kagawa and Racker (11). ROS phospholipids were prepared according to Wiegand and Anderson (12) except that prior to lipid extraction ROS membranes were bleached for 20 min at 4°C in the presence of 20 mM hydroxylamine with a 300-W projector lamp using a

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Abbreviations: Meta II, metarhodopsin II; DM, dodecyl maltoside; DTT, dithiothreitol; ROS, rod outer segment.

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long-pass filter with a cutoff at 495 nm (Melles Griot, Irvine, CA). Phosphorous was determined by the method of Fiske and SubbaRow as modified by Dittmer and Wells (13).

UV/Visible Spectroscopy. All UV/visible spectra were measured with a Perkin–Elmer λ6 or λ7 UV/visible spectrophotometer, equipped with water-jacketed cuvette holders connected to a circulating water bath (model RTE 5DD; Neslab Instruments, Portsmouth, NH). Matched quartz cuvettes (1-cm light path, 0.2-cm-wide black side walls and base) were purchased from Hellma (Forest Hills, NY) or Uvonic Instruments (Plainview, NY). The average concentration of rhodopsin samples in buffer A was 0.8 μM rhodopsin (additions were as indicated in the legends to the figures). They were illuminated in the quartz cuvettes with a 150-W fiber optic light (Fiber Lite A-200; Dolan–Jenner, Woburn, MA) equipped with a >495-nm long-pass filter.

Rate of Decay of Meta II. A rhodopsin sample prepared as above was illuminated for 15 sec, long enough to completely convert the initial 500-nm-absorbing species to the Meta II form. Aliquots of the irradiated sample were acidified at different time intervals to pH 1.9 with 2 M H_2SO_4, and the absorbance at 440 nm due to the remaining protonated Schiff base was recorded (14).

Hydroxylamine Treatment of 500-nm-Absorbing Rhodopsin Regenerated with 11-*cis*-Retinal After Decay of Meta II. Hydroxylamine hydrochloride (2 M, pH 7.0) was added in the dark to samples containing regenerated rhodopsin chromophore to a final concentration of 20 mM, and the UV/visible spectrum was measured after 1 hr at room temperature.

RESULTS

Regeneration of Rhodopsin Chromophore. Addition of 11-*cis*-retinal immediately after photolysis of rhodopsin regenerates rhodopsin chromophore at a rate that parallels the rate of Meta II decay. Delipidated rhodopsin in buffer A at pH 6.3 was photobleached for 15 sec, and 11-*cis*-retinal was immediately added. Fig. 1 shows the time course of chromophore regeneration (A_{500} increase). Also shown is the time course of decay of the Meta II intermediate as determined by the decrease in absorbance at 440 nm characteristic of the denatured protonated Schiff base. The recovery of the native rhodopsin chromophore is essentially quantitative. Both curves in Fig. 1 have a time constant (half-life) of about 18 min, which is consistent with the half-life of Meta II decay determined by transducin activity decay (14). We conclude that the light-activated conformation of rhodopsin, after release of the all-*trans*-retinal, reverts to the correctly folded ground (dark) state opsin, which binds 11-*cis*-retinal. This binding and the consequent regeneration of the A_{500} chromophore occur at a fast rate.

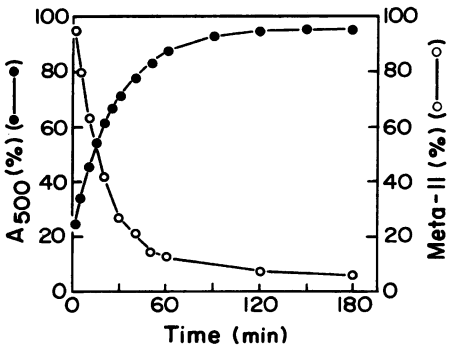

FIG. 1. Time course of decay of light-activated Meta II in the absence of 11-*cis*-retinal and of A_{500} chromophore formation upon addition of 11-*cis*-retinal immediately after illumination of the rhodopsin sample. The horizontal axis represents time after illumination.

Time-Dependent Conversion of the Opsin Initially Formed upon Meta II Decay to Non-Retinal-Binding Species. Rhodopsin was bleached at pH 6.3 by the standard protocol. The bleached sample was divided into aliquots, and the latter were kept in the dark for varying lengths of time (1–24 hr). To each sample, 11-*cis*-retinal (1.5 mole equivalents) was added. Chromophore (A_{500}) formation at room temperature was followed in the dark for up to 1 week. The results are shown in Fig. 2. The first striking observation was that the ability to regenerate the A_{500} chromophore rapidly upon addition of 11-*cis*-retinal decreases with a longer incubation in the dark after bleaching. Further, the extent of total regeneration also decreased with longer incubation before retinal addition. Thus, after 1 hr in the dark (Fig. 2, curve 1) without 11-*cis*-retinal, the extent of chromophore regeneration was 78%, whereas after a 24-hr incubation (Fig. 2, curve 4), the chromophore regeneration was only 35%. Nonlinear curve fitting (SIGMAPLOT; Jandel, Corte Madera, CA) showed three exponents in chromophore regeneration: the first with a $t_{1/2}$ of 15–60 sec, the second with a $t_{1/2}$ of 20–30 min, and the third (the slowest) with a $t_{1/2}$ of 6–10 hr. These exponents made different contributions to the total chromophore regeneration. The contribution of the first (fast) phase, ascribed to the chromophore regeneration from the surviving correctly folded opsin, decreased (curves 1–4, Fig. 2) while the contribution from the third (slow) phase became more prominent. Thus, of the total chromophore regenerated in curve 4, 77% (= 27/35; see Table 1) was by the slow phase.

Table 1 summarizes the contributions of the three kinetic phases to the regenerations in the four time points described in Fig. 2.

Characterization of Rhodopsin Formed from Opsin After Meta II Decay. The fact that only the 500-nm-absorbing chromophore characteristic of visual rhodopsin is formed throughout the experiment of Fig. 2 was proved as follows: delipidated rhodopsin (UV/visible spectrum in Fig. 3, curve 1) was bleached as in the experiment of Fig. 2. The bleached solution was kept in the dark for 3 hr, 11-*cis*-retinal (1.5 mole equivalents) was added, and 72 hr later the spectrum was taken (curve 2). Neutral hydroxylamine hydrochloride was then added in the dark to a final concentration of 20 mM. After 1 hr at room temperature (UV/visible spectrum in Fig. 3, curve 3), the sample showed no significant decrease in A_{500}. Finally, when the hydroxylamine sample was bleached, all the absorbance at 500 nm disappeared (curve 4).

Effect of pH on Opsin Interconversions. (*i*) *pH variation before bleaching.* The effect of pH in the range 5.0–7.0 was investigated in this experiment (Fig. 4). First, it was determined that this pH range did not significantly (<20%) affect the rate of Meta II decay (data not shown). Thirty minutes

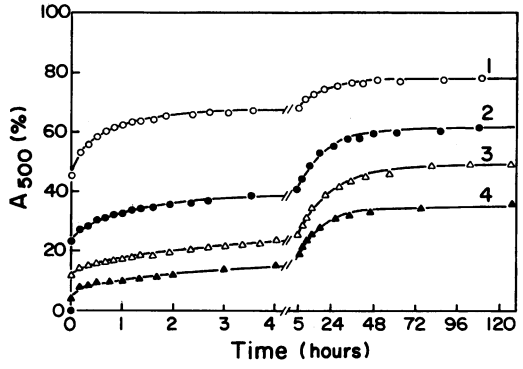

FIG. 2. Time course of the chromophore (A_{500}) formation after addition of 11-*cis*-retinal to bleached rhodopsin samples that had been kept in the dark for different lengths of time at room temperature. The curves are numbered according to the lengths of time that the bleached rhodopsin sample was kept in the dark before addition of 11-*cis*-retinal: curve 1, 1 hr; curve 2, 3 hr; curve 3, 8 hr; and curve 4, 24 hr.

Table 1. Chromophore formation from opsin formed after Meta II decay: Calculated contributions of three kinetic phases

pH	Time after Meta II formation,* hr	% chromophore formed in different kinetic phases†			% total chromophore formed‡
		Phase 1	Phase 2	Phase 3	
6.3	1	50	15	13	78
6.3	3	25	8	28	61
6.3	8	14	3	32	49
6.3	24	8	—	27	35
5.0	24	2	—	16	18
7.0	24	1	—	2	3

*Total time for which the Meta II was kept after illumination and before addition of 11-*cis*-retinal.
†Calculations based on parameters of triple exponential curve fitting. Phase 1, $t_{1/2}$ = 15–60 sec; phase 2, $t_{1/2}$ = 20–30 min; phase 3, $t_{1/2}$ = 6–10 hr.
‡Based on initial opsin content in illuminated rhodopsin.

before photobleaching, samples of delipidated rhodopsin in buffer A were adjusted to pH 5.0, 6.0, or 7.0 by diluting a rhodopsin stock solution (30 μM; pH 6.3) 40-fold with 100 mM sodium phosphate buffer at pH 4.9, 6.0, or 7.05 containing 150 mM NaCl, 1 mM EDTA, and 0.1% DM, respectively. The samples were photobleached and then kept for 24 hr at room temperature in the dark. 11-*cis*-Retinal (1.5 mole equivalents) was added, and the absorbance at 500 nm was recorded for up to 1 week. Fig. 4 shows the results. The sample at pH 6.0 achieved the highest chromophore regeneration (≈32%), and the slow phase ($t_{1/2} \approx$ 10 hr) made the main contribution. As shown, regeneration by the slow phase was also observed to some extent at pH 5.0. On the other hand, at pH 7.0, no significant chromophore regeneration was detected. The curve-fitting results for samples at pH 5.0 and 7.0 are included in Table 1. (*ii*) *pH variation after storage of bleached rhodopsin.* The effect of pH in the range pH 5.0–8.0 was investigated (Fig. 5). A delipidated rhodopsin stock solution (30 μM in buffer A, pH 6.3) was photobleached and then kept for 24 hr at room temperature in the dark. Just before 11-*cis*-retinal addition (1.5-fold), the sample was divided into aliquots, and the pH value was adjusted to 5.0, 6.0, 7.0, or 8.0 by diluting the aliquots 40-fold with buffer A at pH 4.9, 6.0, 7.05, or 8.1, respectively. After the addition of 11-*cis*-retinal, the absorbance at 500 nm was recorded for up to 1 week. As shown in Fig. 5, no significant difference in chromophore regeneration was observed in the pH range between 5.0 and 7.0 in the first 60 min. However, later, a significant amount of slow-phase regeneration (≈10 hr) was observed only at pH 6.0. At pH 8.0, the fast-phase regeneration is significantly inhibited as compared with that at other pH values, suggesting that the correctly folded opsin rapidly converts to non-retinal-binding forms when the pH is changed to 8.0.

Effect of Phospholipids on Chromophore Regeneration. Phospholipids (soybean, 0.3 mM; ROS, 0.1 mM based on phosphorous content) were added either immediately before illumination of delipidated rhodopsin in 0.1% DM or after the bleached rhodopsin had been stored in the dark for 24 hr at room temperature. In every case, 11-*cis*-retinal was added 24 hr after bleaching. The remaining operations were as in the standard experiment. Fig. 6*A* shows the results. Both sets of phospholipids, when added before bleaching, stabilized the initially formed correctly folded opsin as evidenced by the stronger rise of the rhodopsin chromophore. Clearly, the ROS lipids were much more effective in stabilizing the native opsin conformation. Addition of the phospholipids 24 hr after storage of the opsin abolished the reversal of the non-retinal-binding forms to the retinal-binding form. Thus, the binding of the phospholipids to the different species evidently "froze" these structures.

Effect of Dithiothreitol (DTT) and of Glutathione (Reduced and Oxidized) on Chromophore Regeneration. Native rhodopsin contains a disulfide bond between C-110 and C-187 and six non-disulfide-bonded cysteine residues (15). During the aboved-described opsin interconversions, the free cysteine residues as well as the disulfide bond may well be involved in disulfide exchange reactions. Therefore, it was of interest to study the effect of DTT and that of a redox mixture (reduced and oxidized glutathione). As shown in Fig. 6*B*, addition of DTT prior to bleaching drastically reduced the regeneration of

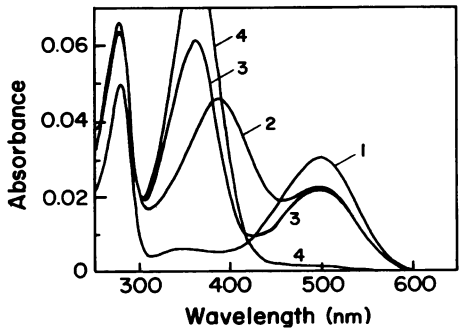

FIG. 3. Characterization of the rhodopsin (A_{500} chromophore) formed upon addition of 11-*cis*-retinal after Meta II decay. Curve 1, UV/visible spectrum of the dark-state rhodopsin sample (pH 6.3) before bleaching. Curve 2, after bleaching, the bleached rhodopsin sample was kept in the dark for 3 hr at room temperature, 11-*cis*-retinal was then added, and the UV/visible spectrum was taken after 72 hr in the dark. Curve 3, neutral hydroxylamine was added to the sample of curve 2, and the UV/visible spectrum was taken after 1 hr in the dark at room temperature. Curve 4, the hydroxylamine-containing sample of curve 3 was illuminated, and the UV/visible spectrum was then taken.

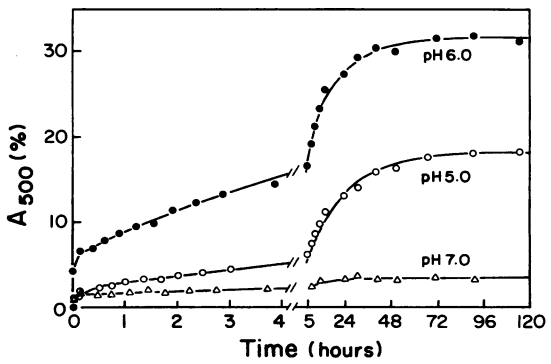

FIG. 4. Effect of pH on chromophore (A_{500}) regeneration from bleached rhodopsin samples. Rhodopsin samples were adjusted to pH 5.0, 6.0, and 7.0 with phosphate buffers, illuminated, and then kept in the dark for 24 hr at room temperature before addition of 11-*cis*-retinal. The increase in absorption at 500 nm was then recorded as a function of time.

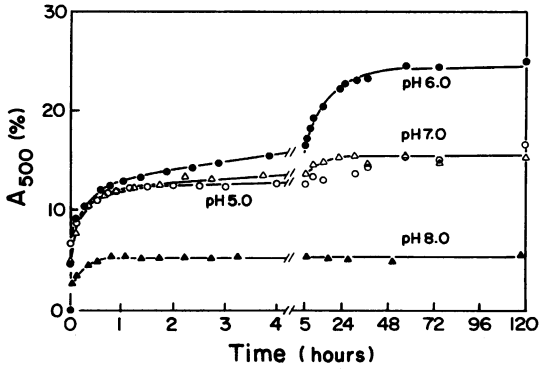

FIG. 5. Time coruse of A_{500} chromopore formation in the presence of 11-*cis*-retinal at different pH values. Delipidated rhodopsin in 0.1% DM at pH 6.3 was illuminated, and the sample was kept in the dark for 24 hr. The sample was then divided into aliquots, and the pH of each aliquot was adjusted to pH 5.0, 6.0, or 8.0. 11-*cis*- Retinal was then added, and the A_{500} values were recorded for up to 1 week.

the chromophore upon addition of 11-*cis*-retinal 24 hr after bleaching and abolished the slow phase reversals completely. The presence of the redox reagent did not have a dramatic effect, perhaps indicating that the disulfide bond present in the ground-state rhodopsin had been intact.

DISCUSSION

Light-catalyzed isomerization of 11-*cis*-retinal to all-*trans*-retinal in rhodopsin results in a series of transient structural changes that are manifested in spectroscopically distinct intermediates. One of these, Meta II, executes the primary signal transduction processes (5). The opsin formed upon Meta II decay is presumed to recycle by binding a new molecule of 11-*cis*-retinal. In the present work we have asked if we can generate a reversible unfolding–folding system for the opsin formed from Meta II. We have demonstrated that in DM micelles, the opsin formed upon Meta II decay indeed relaxes to the native ground-state opsin, which is fully capable of binding 11-*cis*-retinal to form the functional rhodopsin (Fig. 1). It is noteworthy that 11-*cis*-retinal does not displace all-*trans*-retinal if the latter is still linked to the opsin via a Schiff base. The correctly folded free opsin simply offers the binding pocket that 11-*cis*-retinal occupies.

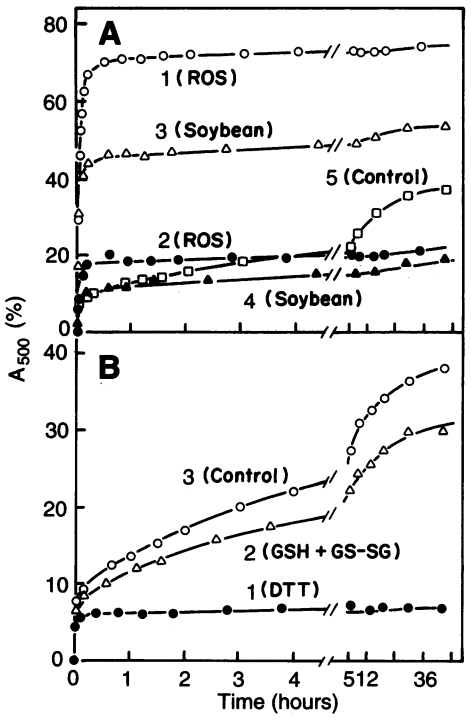

FIG. 6. Effects of exogenously added phospholipids. DTT, and redox reagents on chromopore regeneration. (*A*) ROS lipids (0.1 mM; curve 1) and soybean lipids (0.3 mM; curve 3) were added to delipidated rhodopsin in 0.1% DM at pH 6.3 immediately before photobleaching. The same lipids were added (curves 2 and 4, respectively) after storage of the bleached samples in the dark for 24 hr and just before 11-*cis*-retinal addition. Curve 5 is the control in which no phospholipids were added. (*B*) Dithiothreitol (1 mM; curve 1) or glutathione (1 mM reduced and 1 mM oxidized; curve 2) were added to delipidated rhodopsin samples in 0.1% DM at pH 6.3 immediately before bleaching. Curve 3 shows the control. Twenty-four hours after bleaching, 11-*cis*-retinal was added to each sample, and A_{500} was recorded as a function of time.

When the opsin formed on Meta II decay is kept in DM, it undergoes time-dependent changes to a form(s) that is misfolded in some way and has lost the retinal-binding pocket. However, this form(s) can revert to the retinal-binding form slowly when incubated in the presence of 11-*cis*-retinal (Fig. 2). As is clear from the previous experience with the bacterioopsin → bacteriorhodopsin system, the binding of 11-*cis*-retinal in the present case greatly stabilizes the protein in the native form. Thus, it appears that the binding of 11-*cis*-retinal simply influences the equilibrium between the differently folded opsin(s) in DM micelles by "withdrawing" the reconstituted rhodopsin. However, a less likely possibility must also be considered—that 11-*cis*-retinal influences the refolding of nonnative forms of opsin to the retinal-binding form. The re-gain of the correctly folded opsin from the non-retinal-binding forms is slow and occurs mainly by the slowest kinetic phase ($t_{1/2}$, 6–10 hr) observed. Hopefully, conditions can be found to accelerate this reversal, and this is one immediate goal of our current work. So far we have studied only the influence of pH on this reaction (Figs. 4 and 5). Other parameters remain to be investigated.

Rhodopsin regenerated under the conditions of our experiments, either from Meta II or from the misfolded forms, has been characterized by UV/visible absorption spectrum, by its inertness to hydroxylamine in the dark, and by the formation of the characteristic 380-nm-absorbing Meta II upon illumination (Fig. 3).

The effects of the addition of the phospholipid mixtures, especially the ROS phospholipids at the two stages in our standard protocols of Figs. 2, 4, and 5, were as expected (Fig. 6*A*). Since the correctly folded opsin is the primary product upon Meta II decay, its conformation would be stabilized by the preferential, relative to DM, binding of the phospholipids. By same line of reasoning, when the phospholipids are added to a preformed mixture of the different forms of opsin, all the species should bind the phospholipids, to the extents compatible with their structures. This would result in "freezing" of these structures. Hence, the opsin interconversions on the subsequent addition of 11-*cis*-retinal were not seen. It should be emphasized that these are only initial experiments, and further investigation of mixtures of different phospholipids and different detergents, in varying compositions, should, in fact, provide potentially useful avenues for unfolding–folding pathways.

The addition of DTT prior to illumination of rhodopsin essentially eliminated (Fig. 6*B*) the subsequent chromophore generation. Presumably, DTT reduced the C-110/C-187 disulfide bond in Meta II, and the reduced form did not subsequently return to the correctly folded ground-state opsin. This was somewhat surprising since, as shown previously using the C110A/C187A mutant, the disulfide bond is not required for *in vivo* folding of the opsin to the ground state (16). Evidently, the C-110/C-187 disulfide bond is required for conversion of the opsin in Meta II to the native ground-state form. An attempt to control the redox state by a mixture of reducing and oxidized glutathione did not show a profound influence on the folding.

The present results open a new door to studies of unfolding and refolding of the general class of G-coupled seven-helix integral membrane receptors. The overall problem of unfolding–folding in this family of three-domain integral membrane proteins is much more complicated than that in bacteriorhodopsin. In the latter, the main task seems to be only the cooperative formation of the correct cluster of helices in the membrane-embedded domain, whereas folding of rhodopsin and related receptors is a sequential domain-by-domain process (6).

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