Rhodopsin Kinase Prepared from Bovine Rod Disk Membranes Quenches Light Activation of cGMP Phosphodiesterase in a Reconstituted System†

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ABSTRACT: Rhodopsin kinase was extracted into a buffer containing 200 mM KCl and no $MgCl_2$. The activity of the enzyme was stabilized with the use of a mixture of protease inhibitors, aprotinin, benzamidine, leupeptin, and pepstatin. The extract consisted of three major proteins of molecular weight (M_r) 65 000, 56 000, and 37 000, of which the M_r 65 000 protein was identified with the kinase activity since preparations containing the other proteins had no kinase activity and the M_r 65 000 protein was phosphorylated when the extract was incubated with ATP. A reconstituted cGMP phosphodiesterase (PDE) system consisting of peripheral protein-depleted rod disk membranes (RDM), GTP binding protein (G-protein), and PDE was used to test the effectiveness of the rhodopsin kinase preparation in mediating the ATP-dependent quench of light activation of PDE. In the absence of kinase, light-activated PDE activity lasted several minutes. In its presence, ATP and to a lesser extent GTP quenched the activation about as rapidly as in rod disk membranes. The influence of kinase was unaffected by increasing G-protein or PDE content of the reconstituted system but was slowed down by brighter flashes, showing that quench was caused by the inactivation of bleached rhodopsin and not of PDE or G-protein.

In the presence of GTP, photolyzed rhodopsin (R*) in rod disk membranes (RDM)[1] activates GTP binding protein (G-protein) (Godchaux & Zimmerman, 1979; Fung & Stryer, 1980; Liebman & Pugh, 1982) which in turn activates cGMP phosphodiesterase (PDE) (Uchida et al., 1981; Fung et al., 1981; Cook et al., 1985). After a dim light flash that bleached a small fraction (10^{-5}) of rhodopsin in the RDM, PDE activity peaks within a second and decays with a τ of about 45 s (Liebman & Pugh, 1980; Sitaramayya & Liebman, 1983a). In the presence of ATP the decay is accelerated (Liebman & Pugh, 1980). Bleached rhodopsin was reported to be phosphorylated by rhodopsin kinase with either GTP or ATP as phosphate donor (Frank et al., 1973; Kuhn & Dreyer, 1972; Bownds et al., 1972; Chader et al., 1980), and phosphorylated rhodopsin was shown to be much less effective in activating PDE (Sitaramayya et al., 1977). On the basis of these reports and their observation that AMP PNP, an analogue of ATP but not a substrate for kinase, failed to cause a rapid decay of activation, Liebman and Pugh (1980) hypothesized that rhodopsin phosphorylation by ATP and rhodopsin kinase cause rapid inactivation of R*. In support of this hypothesis, phosphorylation of R* was found to be rapid enough to be casual in quenching the PDE activity (Sitaramayya & Liebman, 1983b). Proteolytic removal of various lengths of C-terminal end of rhodopsin, where most of the phosphorylation occurs, greatly reduced the effectiveness of ATP in quenching the activation (Sitaramayya & Liebman, 1983a; Miller & Dratz, 1984). Phosphorylated rhodopsin was not as effective in activating G-protein and PDE as R* (Sitaramayya et al., 1977; Aton & Litman, 1984; Shichi et al., 1984; Arshavsky et al., 1985). Partially purified rhodopsin kinase restored the ATP effects to a kinase-free reconstitution system that was not influenced by ATP in its absence (Sitaramayya & Liebman, 1983a). It was, however, possible that proteins other than kinase in the kinase preparation cause the quench. There have been reports that one such protein of molecular weight (M_r) 48 000–50 000 (S-antigen, arrestin) participates in the PDE quench (Kuhn et al., 1984; Pfister et al., 1985; Wilden et al., 1986; Zuckerman et al., 1984, 1985; Zuckerman & Cheasty, 1986). Shichi and Somers (1978) reported that the rhodopsin kinase they isolated has a molecular weight of 53 000, close to that of the 48–50K protein, and Shichi (1981) and Nussenblatt et al. (1981) also reported that rhodopsin kinase and S-antigen may be one and the same protein. But reports from other laboratories showing that rhodopsin kinase is a bigger protein of M_r 68 000 (Kuhn, 1978; Lee et al., 1982) and the fact that rhodopsin kinase activity is very unstable (Shichi & Somers, 1978; Sitaramayya & Liebman, 1983a) appeared to suggest that the kinase isolated by Shichi and Somers (1978) was a product of partial proteolysis of the higher molecular weight kinase.

This report describes a procedure to extract rhodopsin kinase from bovine RDM. Several lines of evidence are presented to identify rhodopsin kinase activity with the M_r 65 000 protein in the extract. The activity of kinase has been stabilized with the use of a cocktail of protease inhibitors. A reconstitution system consisting of washed RDM, purified PDE, and G-protein was used to test the ability of the kinase extract in mediating the ATP- and GTP-dependent quench of light activation of PDE.

Materials and Methods

Isolation of RDM. All procedures were carried out at 0–4 °C in room light or under infrared illumination with the aid of an image converter. Fresh bovine eyes packed in ice in a light-tight container were processed after 2–3 h of dark ad-

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[1] Abbreviations: G-protein, GTP binding protein; PDE, cGMP phosphodiesterase; RDM, rod disk membranes; sRDM, RDM stripped of peripheral and soluble proteins; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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aptation. For RDM made in room light, the eyes were processed without dark adaptation. The rod outer segments were broken off the retinas by vortexing in 0.5 mL/retina of 45% (w/v) sucrose in isolation buffer A or B (buffer A consisted of 20 mM MOPS, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF, pH 8.0; buffer B was the same as buffer A except that the 100 mM KCl was excluded), diluted in the same medium to about 2.5 mL/retina, and vortexed again. About 2 mL of isolation buffer was layered on top of sucrose, and the tubes were centrifuged in a Sorvall refrigerated centrifuge (rotor SS 34) at 27000*g* for 30 min. The RDM collected from the sucrose–buffer interface and from the side of the tube were suspended in 2 volumes of isolation buffer and centrifuged at 27000*g* for 15 min. When isolation buffer A was used, the pellet contained three layers, a pigmented zone at the bottom followed by RDM and then a layer of white material [protein composition of the white material was reported by Virmaux et al. (1971)]. When isolation buffer B was used, the pellet had very little of the pigmented material. The pellet was suspended in about 0.5 mL/retina of 45% (w/v) sucrose in isolation buffer and vortexed for 1 min at maximum speed. The suspension was diluted in the same medium to about 2.5 mL/retina, overlaid with about 2 mL of isolation buffer, and centrifuged at 27000*g* for 15 min. RDM were collected from the sucrose–buffer interface, suspended in 2 volumes of isolation buffer, and centrifuged at 48000*g* for 15 min to sediment the RDM.

Two simple modifications were introduced in later experiments in the isolation and washing of RDM. One was to use 38% (w/v) sucrose instead of the 45% (w/v) in buffer B for the second floatation. This resulted in the separation of a large white pellet (Virmaux et al., 1971) from the rod disk membranes that floated. This was a critical step in that at lower than 37% (w/v) sucrose RDM did not separate as a clear carpet and were distributed throughout the centrifuge tube; with sucrose concentrations significantly above 38% (w/v), the contamination by the white material increased and some proteins from this material later appeared in the kinase preparation. The second modification was to make sure that whenever the rod disk membrane pellet was suspended, it was done with initial vortexing followed by repeated suspension through a blunt needle as described by Baehr et al. (1979). It was essential to suspend the material well enough that chunks of membrane pellet did not settle to the bottom of the tube within 2–3 min. If this was not done with each pellet, the rhodopsin kinase preparation extracted from these membranes showed significant contamination.

Preparation of Kinase. RDM prepared and washed in room light in buffer B were suspended in 40 mL/100 retina of buffer C (20 mM Tris, 10 mM DTT, and 1 mM PMSF, pH 7.5) containing 50 μg/mL protease inhibitors, aprotinin, benzamidine, leupeptin, and pepstatin and mixed with 40 mL of chilled deionized water and 160 μL of 1 M $MgCl_2$. The suspension was left overnight at 0–4 °C under illumination. A small aliquot was saved, and the rest was centrifuged for 30 min at 48000*g*. In later experiments GTP was added to the suspension to a final concentration of 40 μM before centrifugation. The supernatant was centrifuged once again to sediment any residual membranes and was designated Mg^{2+} extract. The pellet was washed once in 20 mL of buffer D (10 mM Tris, 5 mM DTT, and 0.5 mM PMSF, pH 7.5) containing 25 μg/mL of the protease inhibitors.

The resulting pellet was suspended in a mixture of 40 mL of buffer C with 50 μg/mL of protease inhibitors, 32 mL of chilled deionized water, and 8 mL of 2 M KCl. The suspension was left overnight at 0–4 °C under illumination and centrifuged at 48000*g* for 20 min. The supernatant was centrifuged once again to sediment any residual membranes and was designated KCl extract.

The KCl extract contained a M_r 65 000 protein, which is identified with kinase activity, and two other major proteins of M_r 56 000 and 37 000. Several attempts to separate the 65 000-Da protein and rhodopsin kinase activity from the other proteins by salt fractionation, ion exchange, gel filtration, and dye–ligand chromatography have been unsuccessful.

Preparation of sRDM. Dark RDM made as described earlier in isolation buffer A were suspended in hypotonic buffer (10 mM Tris-HCl, 1 mM DTT, and 1 mM EDTA, pH 7.5) at 5 mL/retina, and centrifuged at 48000*g* for 40 min. The pellet was put through the washing procedure three more times and suspended in buffer A at 0.5 mL/10 retina. These membranes, designated sRDM, were used in reconstitution experiments and as substrate in rhodopsin kinase assays. In some experiments sRDM washed with 5 M urea according to Schichi et al. (1983) were used instead of sRDM. While urea-washed membranes were generally found to be comparable to sRDM as substrate in rhodopsin kinase assays, some batches were found to produce only a fifth to a tenth as much activity as sRDM when tested against the same preparation of rhodopsin kinase. Only those urea-washed sRDM preparations comparable to sRDM as substrate for kinase were used in the reconstitution experiments discussed here. Protein composition of these two membrane preparations is shown in Figure 3. Urea-washed membranes had no measurable rhodopsin phosphorylating or PDE activity. Activation of sRDM by a saturating light flash elicited about 1–2% of the RDM PDE activity (the activity in RDM was about 8 μM cGMP hydrolyzed s^{-1} (μM rhodopsin)$^{-1}$) but no measurable activity with light flashes that bleached 10^{-5}–10^{-4} fraction of rhodopsin as used in the reconstitution studies. The kinase activity in sRDM was less than 0.5% of that in RDM. When these membranes were used in reconstitution experiments, addition of ATP alone (no kinase) caused a marginal reduction in turnoff time (Figure 6) which was probably due to the residual kinase activity on these membranes. The sRDM used in these experiments differed from those employed in earlier experiments (Sitaramayya & Liebman, 1983a) in that the wash buffer was supplemented with 1 mM EDTA to further reduce the kinase contamination. Reconstitution experiments with sRDM and urea-washed sRDM provided similar results.

Preparation of PDE and G-Protein. PDE and G-protein were prepared according to the procedure of Baehr et al. (1979, 1982).

Reconstitution of Rhodopsin Kinase with the PDE System. In order to test the effect of rhodopsin kinase on the light activation of PDE, rhodopsin kinase preparation (Figure 4C) was reconstituted with purified PDE and G-protein and sRDM or urea-washed sRDM as the source of rhodopsin. The rhodopsin:G-protein:PDE proportion in the reconstitution was 100:7:1.5, the same as their ratio in isolated RDM (Sitaramayya et al., 1986). Except when its concentration was varied, rhodopsin kinase was added to the reconstitutions in amounts to elicit as much kinase activity as was found in RDM normally (82.34 ± 30.09 pmol of phosphate incorporated min^{-1} (nmol of rhodopsin)$^{-1}$ in room light, $n = 7$). Generally, the amount of kinase preparation added was 2.5–3.0 μg of protein (about 0.4 μg of M_r 65 000 protein) to 80 μg of rhodopsin, 11.2 μg of G-protein and 5.6 μg of PDE.

Assay for Rhodopsin Phosphorylation. Rhodopsin kinase activity was measured in a total volume of 300 μL in a buffer

consisting of 25 mM potassium phosphate, 2 mM $MgSO_4$, and 2 mM DTT, pH 7.5. The tubes were held in an ice bath in room light. sRDM or urea-washed sRDM were added to the assays to a final concentration of 8 μM rhodopsin. RDM or any preparation in which rhodopsin kinase activity was measured was added to the tubes, and the tubes were then transferred to a water bath at 23 °C. Two minutes later, (γ-^{32}P)ATP was added to the tubes to a final concentration of 50 μM and the reaction allowed to continue for 10 min. Controls run simultaneously consisted of buffer, sRDM, and ATP. Two methods were used to measure the incorporated radioactivity. In one case reaction was terminated with 5% (w/v) trichloroacetic acid, the precipitated protein washed 4 times in the same acid, the pellet dissolved in 0.1 N NaOH, and the incorporated ^{32}P measured by liquid scintillation counting. In the other method, a 25-μL aliquot was removed from the assay and spotted on a 2 × 2 cm strip of Whatman 3MM chromatographic paper (Corbin & Reimann, 1974). The strips were immersed in cold 10% (w/v) trichloroacetic acid (25 mL/strip) and stirred gently. The acid was changed every 15 min, and after three changes ^{32}P on the strips was measured by liquid scintillation counting. Kinase activities measured by both methods were comparable. By either method, increasing the amount of a kinase preparation in assay by 2-fold resulted in a 2-fold increase in measured activity.

Autoradiography. In experiments designed to identify the phosphorylated proteins, the protein preparation was incubated with 50 μM (γ-^{32}P)ATP in the same buffer used in rhodopsin kinase assays. After a 10-min incubation at 23 °C, an aliquot of the assay mixture was mixed with an equal volume of electrophoresis sample buffer and electrophoresed. The gel was stained with Coomassie Brilliant Blue, destained, and dried onto a filter paper. Kodak X-Omat AR film was exposed to the dry gel for about 2–3 days. The molecular weights of radioactive protein bands were calculated from standard curves obtained with molecular weight standards electrophoresed simultaneously with the test samples.

Phosphodiesterase Assay. cGMP phosphodiesterase activity was measured according to Liebman and Evanczuk (1982) at 5 mM substrate concentration in 0.5 mL of buffer A held at 23 or 37 °C. Assays were done on rod disk membranes or reconstituted RDM at a rhodopsin concentration of 4 μM and G-protein and PDE at 0.3 and 0.06 μM, respectively. Unless otherwise specified, GTP and ATP were added at a final concentration of 500 μM, and the reaction was initiated with a light flash that bleached a 4.1×10^{-5} fraction of rhodopsin in the assays. The initial velocity (V_0) was the slope of the light-activated pH drop at its peak. Turnoff time (τ_{off}) in seconds was obtained by dividing the μM cGMP hydrolyzed by the time PDE activity returned to base line by initial velocity in μM/s.

Other Assays. Protein was measured by the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard. Rhodopsin concentration in the membrane preparations was measured from the absorbance difference at 500 nm before and after bleaching in 50 mM NH_2OH by using a molar extinction coefficient for rhodopsin of 40 000. SDS–polyacrylamide gel electrophoresis was done in 15% (w/v) polyacrylamide slab gels according to the procedure of Baehr et al. (1979).

Results

Isolation of a Stable and Highly Enriched Rhodopsin Kinase Preparation. Correlation of rhodopsin kinase activity with a protein extracted from rod disk membranes has been difficult because of the large number of protiens in these extracts (Sitaramayya & Liebman, 1983a). Given the instability of rhodopsin kinase, separating the kinase from the numerous contaminants proved difficult. We therefore investigated conditions for selective retention and elution of kinase from RDM.

Table I: Influence of 200 mM KCl on the Binding of Rhodopsin Kinase to RDM under Various Conditions[a]

	activity in supernatant as % of activity in RDM			
	4 °C		23 °C	
	without KCl	with KCl	without KCl	with KCl
dark	17.9	62.0	11.1	51.9
room Light	26.3	64.0	11.4	70.3

[a] Rod disk membranes isolated in the dark in buffer A were suspended in buffer C (0.4 mL/retina) and divided into several portions. To the test samples was added salt to a desired final concentration, and controls received distilled water in the same volume. Pairs of treated and control tubes were left overnight either in room light or in the dark, at room temperature (23 °C) or at 4 °C as desired. The tubes were then centrifuged at 48000g for 30 min. The supernatants were spun once again to sediment any residual membranes and assayed for rhodopsin kinase activity. Untreated membranes left under identical conditions served as controls for total kinase activity. In this experiment a 100-μL aliquot of the RDM left at 4 °C had 36 pmol of phosphotransferase activity/min while the RDM left at 23 °C had 12 pmol of activity/min.

Table II: Influence of KCl and Mg^{2+} on Extraction of Kinase Activity[a]

treatment	kinase activity in supernatant as % of activity in RDM
water (control)	4.8
2 mM $MgCl_2$	2.5
100 mM KCl	28.3
200 mM KCl	60.2
200 mM KCl + 2 mM $MgCl_2$	55.5

[a] RDM were isolated in the dark in buffer B. The protocol was the same as in Table I except that all the tubes in this experiment were left at 4 °C in room light. The kinase activity in the RDM was 63 pmol of phosphate incorporated/min for a 100-μL aliquot.

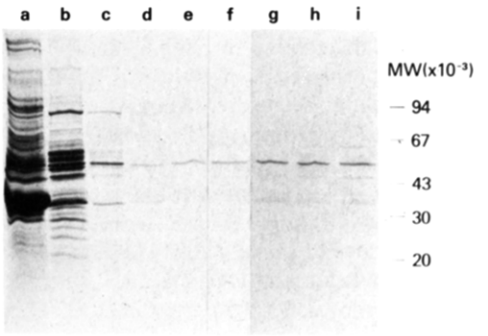

FIGURE 1: RDM (a), first wash (b), second wash (c), control extract of washed RDM (d), Mg^{2+} extract (e), and KCl extract (f) were subjected to SDS–PAGE and Coomassie Blue staining. Lanes g–i show KCl extracts from another experiment obtained at 50 (g), 100 (h), and 200 mM (i) KCl. The amount of material electrophoresed in each lane corresponded to about 8% of one retina.

Incubation in 200 mM KCl strongly favored release of rhodopsin kinase activity from RDM isolated in 100 mM KCl (Table I). Under the various conditions tested, at 4 and 23 °C, in the dark or in room light, the majority of the kinase activity was extracted by 200 mM KCl. Table II shows that in RDM isolated in 2 mM $MgCl_2$ without KCl (buffer B) virtually all the kinase activity was retained on the membranes. Again, a majority of the kinase activity was released from these membranes in the presence of 200 mM KCl. Repeated washing of membranes in 2 mM Mg^{2+} removed the majority

Table III: Extraction of Kinase Activity[a]

	kinase activity as % of activity in RDM
first wash	2.2 ± 0.8 (n = 3)
second wash	2.7 ± 1.5 (n = 3)
third wash	3.4 ± 1.8 (n = 3)
KCl extract	55.9 ± 17.3 (n = 6)

[a] RDM from 100 retinas were suspended in isolation buffer B (0.4 mL/retina) and vortexed for about a minute at maximum speed. The suspension was passed several times through a blunt 17-gauge needle. A small aliquot of the suspension was saved and the rest were centrifuged at 48000g for 20 min. The supernatant was spun once again to sediment any residual membranes. The pellet was washed two more times, and the washes were saved. The washed RDM were then extracted with 200 mM KCl as in Table I. The kinase activity in the RDM was 82.34 ± 30.09 (n = 7) pmol of phosphate incorporated min^{-1} (nmol of rhodopsin)$^{-1}$ in room light at 37 °C.

of the soluble proteins (Figure 1) but not kinase activity (Table III). These observations provided a strategy to retain kinase activity on the membranes while eliminating most of contaminating proteins seen in the earlier preparations. Furthermore, these results provide an explanation for the observations of Kuhn (1978) and Sitaramayya and Liebman (1983b) that RDM isolated in moderate ionic strength buffers like buffer A lost the majority of the kinase activity during isolation.

Figure 1 shows the protein composition of RDM (lane a), the first two washes (lanes b and c), and the KCl extract (lane f). For comparison, a control extract (lane d) and one obtained with 2 mM Mg^{2+} (lane e) were also shown. As seen in lanes b and c, a large amount of protein was removed from RDM in the washes, and the subsequent extraction with KCl resulted in a relatively cleaner preparation (lanes f–i) of kinase activity. A prominent protein band of molecular weight 65 000 was found in KCl extract but not in control or Mg^{2+} extracts. On visual inspection the amount of M_r 65 000 protein in extracts obtained with 50, 100, and 200 mM KCl (Figure 1, lanes g, h, i) appeared to correlate with the kinase activity (about 225, 500, and 1000 pmol of phosphate incorporated into 2.4 nmol of rhodopsin by a 100-μL aliquot of each of these extracts) and was tentatively identified with rhodopsin kinase. Two other proteins, the slowest moving of the triplet of proteins above 30 000 Da and a 47 000–48 000-Da protein, were also found in increasing amounts as the KCl concentration for extraction was increased (Figure 1, lanes g, h, i), but these proteins were also present in washes (lanes b and c) that had very little kinase activity and were not found in cleaner KCl extracts obtained in subsequent experiments using RDM isolated with 38% (w/v) sucrose for the second floatation (see Materials and Methods for details) (compare Figure 1, lane i, with Figure 2). The 65 000-Da protein in the cleaner extracts was about 5% (4.72 ± 0.13; n = 3) of the total protein in the extract as determined from the densitometric traces. In a typical experiment starting with 50 mg of washed RDM protein, the KCl extract contained 2700 μg of protein and about 60% of the kinase activity of RDM. On the basis of the estimate that only 5% of this extracted protein is kinase and assuming that most of the protein in RDM was rhodopsin, a rhodopsin-to-kinase ratio of 360 was calculated (Sitaramayya et al., 1985).

The M_r 39 000 and 37 000 proteins in KCl extract (Figure 2) run parallel to the 39 000 and 37 000 subunits of G-protein when electrophoresed simultaneously (not shown), suggesting that some G-protein was extracted by KCl. Since these two proteins constitute the majority of the protein in the KCl extract, removal of these proteins from the extract would improve the specific activity of kinase in the extract. To accomplish this, the washed RDM were extracted in buffer

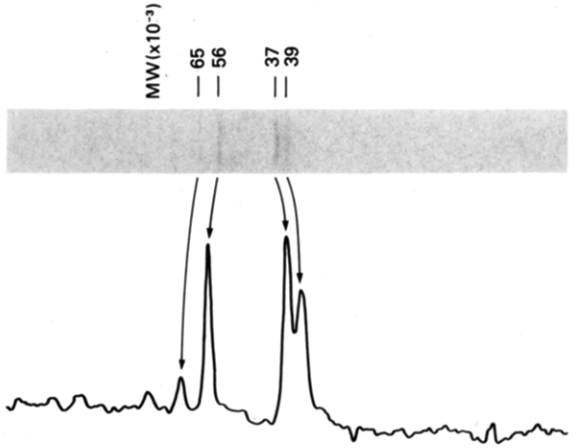

FIGURE 2: KCl extract from RDM isolated with 38% (w/v) sucrose in the second floatation was electrophoresed and stained with Coomassie Blue. The densitometric trace shows that the 65 000-Da protein (rhodopsin kinase) was only about 5% of the total protein in the extract.

C first with 2 mM Mg^{2+} and 40 μM GTP (Kuhn, 1980) followed by 200 mM KCl. Figure 4 shows these two extracts resolved by SDS–PAGE and stained by Coomassie Brilliant Blue. G-Protein with the α subunit far in excess over the β subunit [note that in the gels run according to Baehr et al. (1979) the 39 000-Da α subunit migrates faster than the 37 000-Da β subunit] and the 56 000-Da protein appear in Mg^{2+} extract; the 65 000-Da rhodopsin kinase, the 56 000-Da protein, and a large amount of the 37 000-Da G-protein β subunit appear in KCl extract. The identity of the M_r 37 000-Da protein with the β subunit of G-protein was confirmed by western blots using antibodies raised against the β subunit of N_s. A small amount of M_r 39 000 G-protein α subunit was also present in KCl extract. Densitometric traces of Mg^{2+} and KCl extracts were also shown in Figure 4. The amount of protein in the KCl extracts varied from 850 to 1000 μg when the starting material was 50 mg of washed RDM protein. The 65 000-Da protein contributed about 16% of the total protein in KCl extract, about 3–4-fold greater than in the KCl extracts before this modification. With the total kinase activity in the extract remaining about the same as before and the total protein about 3–4-fold less, the specific activity of this KCl extract was about 3–4-fold higher than that of the preparation shown in Figure 2. The KCl extract was free of contamination by proteins of molecular weight of about 48 000–50 000. Under the assay conditions described here, the specific activity of KCl extracts was about 30-fold greater than that of washed RDM (about 2 nmol of phosphate incorporated min^{-1} mg^{-1} by RDM and about 62 nmol min^{-1} mg^{-1} by KCl extract).

Shichi and Somers (1978) reported that isolated kinase activity was unstable. The KCl extracts in our experiments also lost their activity within 4–5 days, making further purification of kinase difficult. There were reports in the literature which suggested that instability could be due to proteolysis and could be prevented by inclusion of protease inhibitors in all buffers used in purification (Peart et al., 1966; Tucker et al., 1981; Adelson & Klee, 1981). In preliminary experiments, adding a mixture of protease inhibitors, aprotinine, leupeptin, benzamidine, and pepstatin at 25 μg/mL each to all buffers resulted in a KCl extract that had stable kinase activity. In subsequent experiments, however, the protease inhibitors were not included in buffers used in the isolation and washing of RDM. Inclusion of the inhibitors in buffers used for extraction was sufficient to stabilize the kinase activity, and this was done in all further experiments. While

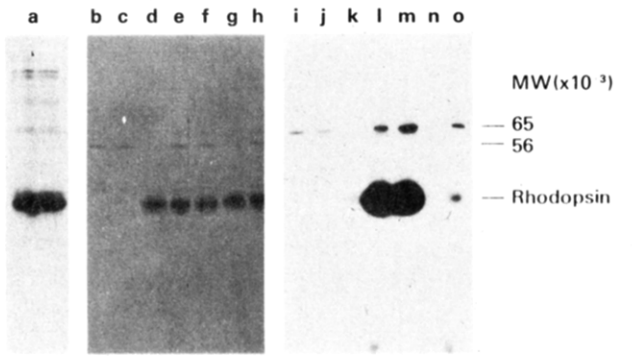

FIGURE 3: Protein composition of sRDM (a) and urea-washed sRDM (d) as seen after SDS–PAGE and Coomassie Blue staining. Lanes b–h are Coomassie Blue stained protein patterns of various preparations incubated with (γ-^{32}P)ATP for 10 min at 22 °C, and lanes i–o show their respective autoradiograms. Preparations b–f were incubated in room light, and g and h in the dark. The composition of various lanes is (b) KCl extract; (c) KCl extract with 0.2 M NaCl; (d) urea-washed membranes; (e) urea-washed membranes with KCl extract; (f) urea-washed membranes and KCl extract with 0.2 M NaCl; (g) urea-washed membranes; (h) urea-washed membranes and KCl extract. Rhodopsin content of urea-washed membranes per lane was about 16 μg. The KCl extract added to the assays was 4.6 μg of protein. The concentration of rhodopsin in the assays was 8 μM. The portion of the figure showing protein pattern was printed at lower contrast in order to show the M_r 65 000 protein.

a typical KCl extract without protease inhibitors lost about 90% of its activity in 4–5 days after isolation, the preparation in protease inhibitors retained activity for several weeks. One preparation that was monitored for stability showed 76% of original activity 37 days after isolation and 46% after 76 days.

Phosphorylation of Rhodopsin Kinase. Incubation of KCl extract with (γ-^{32}P)ATP followed by SDS–PAGE and autoradiography revealed that one protein in the extract was phosphorylated (Figure 3). The molecular weight of this protein was 65 000, which agreed with the observation of Lee et al. (1982) that rhodopsin kinase was "autophosphorylated" and also supported the tentative identification of kinase activity with this protein. Rhodopsin was not required for the phosphorylation of kinase. Incubation of the extract with urea-washed RDM in the dark resulted in phosphorylation of kinase with a very small fraction of rhodopsin also phosphorylated. This small amount of rhodopsin phosphorylated in the dark probably represents the fraction bleached unintentionally. In room light, the kinase was phosphorylated, but the majority of phosphorylated protein was rhodopsin. No other proteins besides rhodopsin and rhodopsin kinase were phosphorylated. The identification of the heavily phosphorylated band with rhodopsin was based upon three observations: the phosphorylated band matched with the Coomassie Blue stained rhodopsin band; phosphorylation was light-sensitive; and polymers of the phosphorylated band appeared when the samples were warmed before electrophoresis (not shown), a property typical of rhodopsin. In view of an earlier report that high concentrations of NaCl inhibited rhodopsin kinase activity (Shichi & Somers, 1978), 200 mM NaCl was tested for its influence on the phosphorylation of kinase in KCl extract and on the phosphorylation of rhodopsin by the extract. Figure 3 shows that neither phosphorylation was inhibited by NaCl. Incubation of urea-washed membranes alone with (γ-^{32}P)ATP did not result in phosphorylation of rhodopsin.

PDE Activation Quenched by Rhodopsin Kinase in Reconstitution System. The kinase preparation (Figure 4C) was reconstituted with purified PDE, G-protein, and sRDM in order to test its ability to mediate nucleotide-dependent quench of PDE activation.

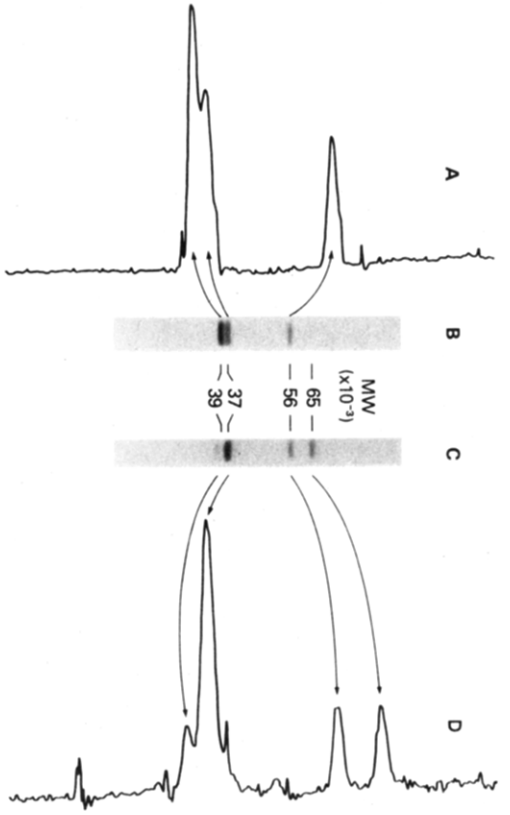

FIGURE 4: Most of the G-protein α subunit and part of the M_r 56 000 protein were solubilized on extraction with GTP and Mg^{2+} (B and densitometric trace A). The subsequent extraction of RDM with KCl solubilized mostly the β subunit of G-protein, some 56 000-Da protein, and about 60% of the 65 000-Da rhodopsin kinase (C and densitometric trace D).

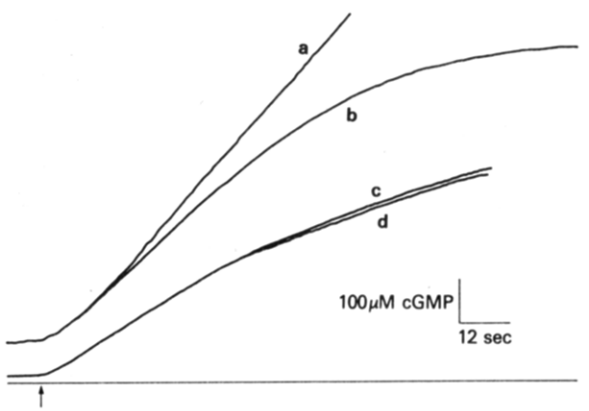

FIGURE 5: PDE activity of a reconstituted preparation activated by a light flash (marked by ↑) that bleached a 4.1 × 10^{-5} fraction of rhodopsin is shown. GTP concentration in assays was 50 μM for trace c and 500 μM for trace a; traces d and b show the respective preparations supplemented with 2.5 μg of protein of rhodopsin kinase preparation. T = 37 °C. The ordinate in this and following figures shows the μM cGMP hydrolyzed. Reconstitutions contained 4 μM rhodopsin, 0.3 μM G-protein, and 0.06 μM PDE. Assays were done in 0.5 mL of buffer A, and the activity was followed by monitoring the drop in pH caused by cGMP hydrolysis.

(1) GTP-Dependent Quench of PDE Activation. GTP is an essential constituent in the light activation of PDE. However, GTP was also reported to serve as a substrate for the mechanism that quenches the activation of PDE (Liebman & Pugh, 1980). The influence of GTP was minimal at concentrations <50 μM. The concentration of GTP for half-maximal quench was 1400 μM (Liebman & Pugh, 1980). If rhodopsin kinase mediated the effect of GTP on quench, then

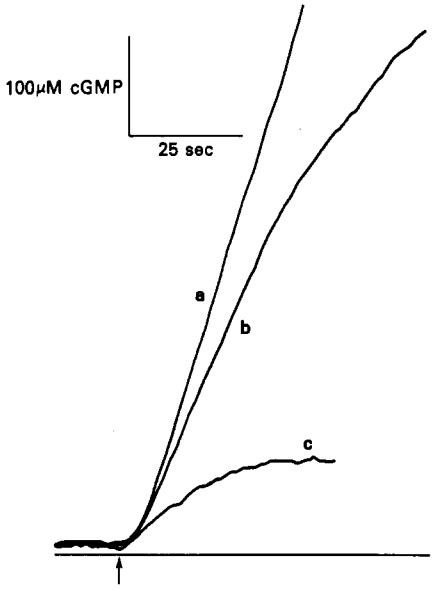

FIGURE 6: Influence of rhodopsin kinase on the reconstituted PDE system in the presence of ATP and GTP. Trace a shows the preparation with 500 μM GTP alone, b with GTP and ATP both at 500 μM, and c with both the nucleotides and 2.5 μg of protein of rhodopsin kinase preparation. Light flash (marked by ↑) used for activation was similar to that in Figure 5. T = 37 °C. Reconstitutions contained 4 μM rhodopsin, 0.3 μM G-protein, and 0.06 μM PDE. Assays were done in 0.5 mL of buffer A, and the activity was followed by monitoring the drop in pH caused by cGMP hydrolysis.

addition of kinase to a reconstituted PDE system with 50 μM GTP should not quench light activation of PDE, while at higher GTP concentrations it should. Figure 5 shows the effect of kinase on the light activation of PDE at 50 and 500 μM GTP. At the lower concentration, there was no noticeable influence of kinase on the activation (Figure 5, traces c and d), but at 500 μM, kinase caused a significant decrease in τ_{off} from more than 5 min to about 80 s. The V_0 was only reduced by about 17% (Figure 5, traces a and b). Here and in the following experiments when the τ_{off} is stated as >5 min, it indicates that the PDE activity was monitored for at least 5–6 min after the light flash and the activity at that time had not returned to preflash level.

(*2*) *ATP-Dependent Quench of PDE Activation.* In RDM ATP caused a rapid quench of PDE activation as well as a reduction in initial velocity of PDE (Sitaramayya & Liebman, 1983a). In the reconstitution experiments done at 37 °C and shown in Figures 6 and 8, kinase reduced τ_{off} from more than 5 min in GTP controls to about 16–17 s. The initial velocity was reduced 3–4-fold. In an experiment done at 22 °C and shown in Figure 7, τ_{off} in the presence of kinase was about 52 s. This and other experiments on the temperature dependence of quench times (not shown) showed that Q_{10} for the kinase-mediated quench was about 2.

(*3*) *Quench Independent of the Concentration of Active G-Protein and PDE.* The reconstitution system provides the possibility to test whether ATP-dependent quench was due to the influence of kinase on bleached rhodopsin, G-protein, or PDE. In the experiment shown in Figure 7, at 4.1 × 10^{-5} fraction rhodopsin bleached, the τ_{off} was 52 s. With all other conditions remaining constant, increasing the concentration of G-protein by 3-fold showed the expected increase in initial velocity by 3-fold but had no significant effect on the τ_{off}, which was 47 s. Increasing the PDE concentration by 3-fold had no effect at all on either V_0 or τ_{off} (49 s). However, increasing the concentration of bleached rhodopsin by 3-fold by using a brighter activating flash increased V_0 and prolonged the τ_{off}

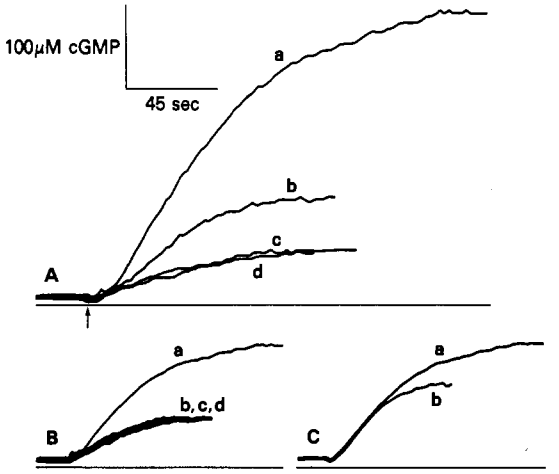

FIGURE 7: Influence of an increase in G-protein, PDE, or R* on rhodopsin kinase mediated PDE quench is shown. The standard reconstitution system contained 4 μM rhodopsin, 0.3 μM G-protein, 0.06 μM PDE, and 2.5 μg of protein of the kinase preparation in 0.5 mL of buffer A. Trace d in A shows activity of a reconstituted preparation with 500 μM GTP and 500 μM ATP activated by a light flash (marked by ↑) that bleached a 4.1 × 10^{-5} fraction of rhodopsin. Trace c is same as d except that the preparation had 3 times more PDE added (0.18 μM instead of 0.06 μM). Trace b is same as d except that the preparation was supplemented with 3 times more G-protein (0.9 μM instead of 0.3 μM). Trace a shows a preparation same as the one in d except that the light flash was 3 times brighter (a 1.3 × 10^{-4} fraction of rhodopsin bleached instead of 4.1 × 10^{-5}). In B traces b–d were normalized to the same initial velocity to show that the PDE activity in all decayed with about the same τ, while trace a on top shows a slower decay. In C, the initial velocity of traces a and b were normalized to show that the activity of these two decayed at different rates. T = 22 °C. PDE activity was followed by monitoring the drop in pH caused by cGMP hydrolysis.

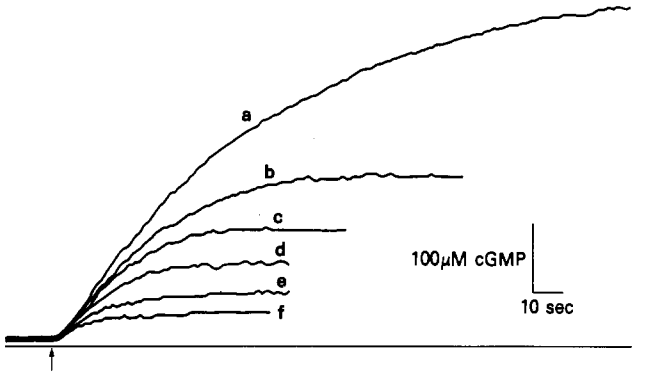

FIGURE 8: Effect of various concentrations of rhodopsin kinase on the quench of PDE activation is shown. Trace a shows a reconstituted PDE preparation activated by a light flash (marked by ↑) similar to that in Figures 5 and 6, with GTP and ATP present at 500 μM each. The amount of kinase added was (b) 1/66, (c) 1/33, (d) 1/10, (e) 1/2, and (f) 1 equiv (2.5 μg of protein of the kinase preparation) of the kinase activity present in RDM measured as described under Materials and Methods. T = 37 °C. The reconstitutions contained 4 μM rhodopsin, 0.3 μM G-protein, and 0.06 μM PDE in 0.5 mL of buffer A. PDE activity was followed by monitoring the drop in pH caused by cGMP hydrolysis.

to 80 s, suggesting that the effect of kinase was only on R* and not on PDE or G-protein.

(*4*) *Turnoff Sensitive to Low Concentrations of Kinase.* Figure 8 shows the influence of a range of kinase concentrations on the quench of PDE activation. Even at 1/66 its normal concentration, kinase has a distinct influence on the τ_{off}. Raising the concentration to 1/10 the normal level caused further reduction in τ_{off}, and at its normal concentration the decrease in τ_{off} was more than 5-fold compared against ATP + GTP control without kinase (Figure 8, trace a) and more

than 10-fold compared with GTP control (not shown); the reduction in V_0 was about 3–4-fold when compared with either of them.

Discussion

Prior to the discovery of G-protein's role in the activation of PDE, ATP was used as a cofactor in the activation of PDE (Bitensky et al., 1973). The reasons for the requirement of ATP were unknown, but it appeared that phosphorylation of rhodopsin or PDE could be essential in the activation of PDE. However, quite unexpectedly, reconstitution experiments showed that PDE was not phosphorylated and that phosphorylated rhodopsin was much less efficient than the nonphosphorylated control in activating PDE (Sitaramayya et al., 1977). Liebman and Pugh (1980) found that ATP rapidly turned off the GTP-supported light activation of PDE at physiological bleach levels. These authors suggested that the ATP-dependent mechanism was mediated by rhodopsin kinase. Since then several lines of evidence have suggested that rhodopsin phosphorylation by ATP and rhodopsin kinase was indeed the mechanism of quench of PDE activation. The phosphorylation of rhodopsin was demonstrated under conditions of low bleach where PDE quench was also shown (Sitaramayya & Liebman, 1983b); the phosphorylation was rapid and preceded the quench (Sitaramayya & Liebman, 1983b). In addition, phosphorylated rhodopsin was less effective in activating G-protein, which is essential for PDE activation (Shichi et al., 1984), and removal of c-terminal end of rhodopsin, which is the major phosphorylation site on rhodopsin, prevented ATP from quenching PDE activation (Miller & Dratz, 1984). However, a direct demonstration that rhodopsin kinase quenches PDE activation requires a reconstitution study with purified components, rhodopsin kinase, PDE, G-protein, and stripped RDM.

Stable Rhodopsin Kinase Preparation. The preparation of rhodopsin kinase described here (Figure 4C) has properties different from those reported for purified kinase by Shichi and Somers (1978). The most significant of them is the increased stability of kinase activity achieved here with the use of protease inhibitors. Unfortunately, the stabilized and apparently unproteolyzed enzyme could not be purified further by using procedures such as salt fractionation and blue agarose chromatography that were successfully used by Shichi and Somers (1978). Of the three major proteins in our kinase preparation (M_r 65K, 56K, and 37K), the M_r 65 000 protein was identified as rhodopsin kinase since the protein was "autophosphorylated" as reported for rhodopsin kinase by Lee et al. (1982), protein fractions not containing this protein had no kinase activity, and the molecular weight agrees with that estimated for kinase by several investigators (Kuhn, 1978; Lee et al., 1982; Pfister et al., 1985). However, we cannot be certain that the M_r 65 000 protein is not a proteolytic fragment of a higher molecular weight precursor. Since Feraudi (1983) reported a molecular weight of 75 000 for kinase, it is possible that this precursor was rapidly reduced to a 65 000-Da protein during the extraction of retina from the eye cup and the subsequent vortexing. If so, inclusion of this cocktail of protease inhibitors in the isolation buffer did not prevent the proteolysis.

Kinase Mediates Quench Supported by GTP or ATP. Several observations made on RDM were reproduced by using the rhodopsin kinase preparation in the reconstituted system. ATP has two distinct effects on the PDE activation in RDM. It quenches the activation rapidly (Liebman & Pugh, 1980) and also reduces the initial velocity (Sitaramayya & Liebman, 1983a; Kawamura, 1983). As seen in Figures 6 and 8, at 500 μM ATP, kinase brings about both a reduction in the τ_{off} from more than 5 min to about 16–17 s and a reduction in the initial velocity by about 3–4-fold [a 3–4-fold reduction in τ_{off} and a 2-fold reduction in V_0 were observed in RDM by Sitaramayya and Liebman (1983a,b)].

Liebman and Pugh (1980) showed that while PDE activation was rapidly quenched by ATP at very low concentrations (half-maximal quench at 4 μM), GTP was also effective in quenching at concentrations greater than 50 μM (K_m = 1400 μM). The reconstitution experiments showed that at 50 μM GTP, kinase had no effect on the activation, but at 500 μM, kinase reduced the τ_{off} significantly. In studies with RDM it was not possible to suggest whether the effects of ATP and GTP were mediated by two different kinases. The present reconstitution experiments show that the same kinase preparation mediates both effects. In addition, it is noteworthy that the kinase had little effect on the V_0 of PDE at either 50 or 500 μM GTP, which again is consistent with the observations made in studies with RDM.

Quench Proportional to Kinase Concentration. Another observation made on RDM was explained by these results. RDM depleted of 85% of kinase activity still exhibited ATP-dependent quench of the light activation of PDE (Sitaramayya & Liebman, 1983a). Donner and Hemila (1985) reported from their study of rod photocurrents that with 60–80% of kinase activity inhibited by adenosine they found no change in the time course of decay of excitation. The present observation that kinase, even at 1/10 of its normal concentration, has a strong influence on the τ_{off} explains these observations.

Inactivated Component Is Bleached Rhodopsin, Not G-Protein or PDE. When the intensity of the activating flash is increased, the τ_{off} increases, apparently, because the quench mechanism has to act against a greater amount of target component generated by the higher bleach. Since higher intensity flashes generate more R* and consequently more activated G-protein and PDE, the target component could be any one or more of these three. In the reconstitution system, both G-protein and PDE concentrations can be raised independently of the other to much higher levels and thus afford a test whether these proteins are the target for the quench mechanism. Apparently, if they are, rhodopsin kinase mediated quench will be swamped and the τ_{off} will be increased greatly. Figure 7 shows this not to be true. When the concentration of G-protein was increased by 3-fold, the initial velocity of the activated PDE increased about 3-fold but the τ_{off} was unaffected, showing that the quench mechanism was not directly influencing the active G-proteins or PDEs. At weak flashes the PDE activity is limited by the amount of activated G-protein, and therefore raising the PDE concentration alone made no significant impact on the activity expressed or τ_{off}. Raising the intensity of flash 3-fold increased R*, activated both G-protein and PDE, and also increased the τ_{off} significantly. Taken together, these observations suggest that the quench mechanism inactivated only the R* and had no influence on the PDE and G-protein.

Isolation of a pure M_r 65 000 protein has not yet been achieved. However, the kinase preparation described here meets several requirements for use in reconstitution experiments since (1) the preparation has stable kinase activity; (2) several observations suggest the identity of kinase with the 65 000-Da protein; (3) the time course of ATP-dependent quench of PDE activation mediated by this preparation is nearly the same as in RDM; and (4) though several proteins are phosphorylated in RDM, most of them in the dark and some in response to light (Szuts, 1985), the preparation described here phosphorylated only rhodopsin, permitting an

unambiguous correlation of kinase effects with rhodopsin phosphorylation.

There have been reports that a M_r 48 000 protein (48K) participates in the ATP-mediated quench of the PDE activation (Zuckerman et al., 1984, 1985; Zuckerman & Cheasty, 1986; Kuhn et al., 1984; Pfister et al., 1985; Wilden et al., 1986). However, there are significant differences in the proposed role of this protein. Zuckerman et al. (1984) show that the 48K protein binds ATP, a reaction catalyzed by R*. Zuckerman and Cheasty (1986) suggest that phosphorylation of R* by kinase causes the reduction in the V_0 of PDE, while the turnoff time reduction results from the competition between activated G- and 48K proteins for sites on PDE. Wilden et al. (1986) report that phosphorylation of R* is the only ATP-dependent step and that it partially reduces the ability of R* in activating the PDE. 48K protein binds to phosphorylated R*, further reducing its activity. At its normal concentration in the RDM, the contribution of the 48K protein to the PDE quench does not appear to be significant since Wilden et al. (1986) found that excessive amounts of the protein had to be used to elicit quench comparable to that in experiments reported here. However, according to Zuckerman (personal communication), significant effects of the proein on turnoff time are observed at its normal concentration in RDM. While our study does not exclude a role for the 48K protein in the regulation of PDE in RDM, GTP- and ATP-dependent quench of PDE is demonstrated here in the reconstitution system mediated by rhodopsin kinase without the 48 000–50 000-Da protein. Since attempts to prepare acvtive kinase free from the 56- and 37-kDa proteins have not been successful, a role for these proteins in the quench mechanism cannot yet be excluded.

Vertebrate rods hyperpolarize transiently in response to dim light flashes and return to base line within 2–4 s (Baylor et al., 1979; Detwiler et al., 1980). Since cGMP controls the cation conductance into the rod outer segment and its light-activated hydrolysis apparently leads to rod hyperpolarization (Fesenko et al., 1985; Cobbs & Pugh, 1985; Zimmerman et al., 1985), the reversal to prebleach level requires rapid termination of light-activated PDE activity. The turnoff times measured in the reconstitution system described here or by Wilden et al. (1986) are slower by about an order of magnitude compared to the declining phase of the rod photoresponse. They are also significantly slower than the turnoff times (about 4 s) measured in suspensions of RDM isolated with minimal loss of soluble components (Sitaramayya & Liebman, 1983b), suggesting that other soluble components besides rhodopsin kinase and 48K protein may be essential to terminate the light-activated PDE activity in a time compatible with the physiological response of the rod.

Whether R* is inactivated by rhodopsin kinase alone or by kinase and 48K protein, the PDE activity continues until the activated G-proteins become inactive. The current notion is that G-protein remains active until the bound GTP is hydrolyzed to GDP. The reported GTPase rates for G-protein are about 1–2 per minute (Godchaux & Zimmerman, 1979; Kuhn, 1980; Yamanaka et al., 1985), which is far too slow compared to the declining phase of the rod photoresponse. Here also it is likely that a soluble protein in rod outer segments may enhance this rate by 10–20-fold or there should be a rapid alternative to GTP hydrolysis to inactivate GTP-bound G-proteins.

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Registry No. PDE, 9068-52-4; rhodopsin kinase, 54004-64-7.

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Formation of Mixed Disulfide Adducts at Cysteine-281 of the Lactose Repressor Protein Affects Operator and Inducer Binding Parameters†

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ABSTRACT: The lactose repressor protein has been modified with three sulfhydryl-specific reagents which form mixed disulfide adducts. Methyl methanethiosulfonate (MMTS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) completely reacted with all three cysteine residues, whereas only partial reaction was observed with didansylcystine. Cysteines-107 and -140 reacted stoichiometrically with MMTS and DTNB, while Cys-281 was modified only at higher molar ratios. Didansylcystine reacted primarily with cysteines-107 and -140. Affinity of MMTS-modified repressor for 40 base pair operator DNA was decreased 30-fold compared to unmodified repressor, and this decrease correlated with modification of cysteine-281. DTNB-modified repressor bound operator DNA with a 50-fold weaker affinity than unmodified repressor. Modification of the *lac* repressor with didansylcystine decreased operator binding only 4-fold, and nonspecific DNA binding increased 3-fold compared to unmodified repressor. No change in the inducer equilibrium binding constant was observed following modification with any of these reagents. In contrast, inducer association and dissociation rate constants were decreased ~50-fold for repressor completely modified with MMTS or DTNB, while didansylcystine had minimal effect on inducer binding kinetics. Correlation between modification of Cys-281 and the observed decrease in rate constants indicates that this region of the protein regulates the accessibility of the sugar binding site. The parallel between the increase in the K_d for repressor binding to operator, the altered rate constant for inducer binding, and modification of cysteine-281 suggests that this region of the protein is crucially involved in the function of the repressor protein.

Synthesis of lactose metabolic enzymes in *Escherichia coli* is regulated by the interaction of the lactose repressor with operator DNA (Miller & Reznikoff, 1980). The affinity of the repressor for operator is modulated by the binding of small sugar molecules. These inducers elicit a conformational change in the repressor protein to a form with decreased affinity for operator DNA (Lin & Riggs, 1975). Because the affinity of the protein for nonspecific DNA is not affected by inducer binding, the remainder of the genomic DNA competes ef-

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