Rhodopsin Kinase: Substrate Specificity and Factors That Influence Activity†

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ABSTRACT: Rhodopsin kinase was prepared from bovine retinas by the method of Sitaramayya [Sitaramayya, A. (1986) *Biochemsitry 25*, 5460] with some minor modifications. The enzyme is able to phosphorylate bovine rhodopsin in the disk membrane, rhodopsin from other species, and rhodopsin solubilized in mild detergent (dodecyl maltoside). Rhodopsin kinase can phosphorylate synthetic peptides containing the appropriate sequences from bovine rhodopsin; however, the K_m values for these peptides are about 3 orders of magnitude higher than that for rhodopsin or ATP. Some peptides from the cytosolic surface of rhodopsin inhibit the phosphorylation. These results suggest that more than one region of rhodopsin is involved in the interaction of rhodopsin of the kinase. Mg^{2+} is required for the Mg-ATP complex as shown by the observation that (ethylenedinitrilo)tetraacetic acid inhibits kinase activity. Second, free Mg^{2+} above the concentration required to complex all of the ATP present activates the kinase. Third, higher concentrations of Mg^{2+} yield Mg-ATP-Mg instead of Mg-ATP and therefore inhibit the kinase activity. Other physiologically important cations such as Ca^{2+}, Na^+, and K^+ reduce the activity of the kinase, probably by forming a metal ion–ATP complex, thereby reducing the concentration of Mg-ATP. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSO_2BzAdo), an inhibitor of kinases and ATPases, inhibits rhodopsin kinase according to pseudo-first-order kinetics. The relationship between the first-order constant and the concentration of FSO_2BzAdo is hyperbolic. This indicates that a reversible complex between the ATP analogue and the enzyme is formed prior to the covalent attachment of the analogue to rhodopsin kinase. Mg-ATP and ATP almost completely protect the kinase against inactivation when used at saturating concentrations. Mg^{2+} and rhodopsin have only a minor protective effect. These results suggest that modification occurs at the kinase active site.

Rhodopsin is the major protein in vertebrate rod outer segments (Smith et al., 1975; Krebs & Kühn, 1977). The absorption of light by rhodopsin triggers a series of events that ultimately results in visual excitation (Stryer, 1986). Rhodopsin[1] was found to be phosphorylated following light absorption, but on a time scale that was too long to be associated with visual excitation (Kühn et al., 1973). The most recent models of the visual process propose that phosphorylation is one mechanism of shutting off the excitation process [e.g., Sitaramayya & Liebman (1983)]. Recently, a number of other receptor proteins that operate via guanine nucleotide binding proteins have been found to be homologous to vertebrate rhodopsin (Kubo et al., 1986a,b; Dixon et al., 1986; Kobilka, 1987), suggesting a common origin for the family of receptors and a common mechanism of action. The availability of rhodopsin and the other components of the bovine visual system make it an ideal model for the family of receptors. Understanding the interaction of rhodopsin and its kinase may be directly applicable to understanding the interaction of these other receptors and their kinases, providing insights into their interactions. The kinase that phosphorylates β-adrenergic receptor appears to belong to this class of receptor kinases and shares many properties with rhodopsin kinase (Benovic et al., 1987).

Study of the interaction of rhodopsin with its kinase has been hampered by several difficulties. Several laboratories have been able to separate the soluble kinase activity from the membrane-bound rhodopsin fraction, although until recently (Sitaramayya, 1986) these preparations have been unstable. Such kinase extracts contain many proteins in addition to the M_r ~67 000 kinase, and it is not certain whether these other components are involved in or interfere with the phosphorylation reaction. Transducin, for example, can compete with rhodopsin kinase under certain circumstances (Kühn, 1984). When partially purified rhodopsin kinase has been reconstituted with washed rhodopsin-containing membranes, the extent of phosphorylation has been less than that obtainable directly with rod outer segment preparations [as many as seven to nine phosphoryl groups have been incorporated per rhodopsin in the best preparations (Wilden & Kühn, 1982)]. Similarly, detergents have been reported to inhibit the phosphorylation reaction (Shichi & Somers, 1978). This would prevent the use of solubilized purified rhodopsin as a substrate. We report on an assay system that yields improved levels of phosphorylation of rhodopsin in membranes (1.7 phosphoryl groups/rhodopsin). We use this assay to determine the enzymatic properties of rhodopsin kinase and compare these properties with those of other kinases. We show that rhodopsin kinase phosphorylates rhodopsin in mild detergent solution as well as it does in the membrane. We report that the kinase can phosphorylate synthetic peptides from the rhodopsin sequence and develop two assays in order to measure this phosphorylation. Finally, we begin testing the features of rhodopsin that are necessary for recognition by the kinase.

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[1] *Photolyzed* rhodopsin is the substrate of the kinase, not *rhodopsin* (the protein containing bound 11-*cis*-retinal) or *opsin* (the apoprotein). However, for simplicity we will refer to the kinase as *rhodopsin kinase* and to its substrate as *rhodopsin* rather than *photolyzed rhodopsin*.

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Materials and Methods

Materials and Concentrations of Reagents. The concentration of rhodopsin was measured in the presence of hydroxylamine by assuming a molar extinction coefficient of 40 600 at 498 nm (Wald & Brown, 1953). Rhodopsin's molecular weight was taken as 40 000 (Hargrave et al., 1983). The concentration of ATP was determined by using the absorbance at 259 nm and assuming a molar extinction coefficient of 15 400 (Bock et al., 1956). The concentration of 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSO_2BzAdo)[2] was measured according to the method of Hixson and Krebs (1979) by assuming a molar extinction coefficient of 15 700.

Isolation of Rod Outer Segments. Cattle eyes were collected at a local slaughterhouse and placed on ice in a light-tight container. Rod outer segments (ROS) were usually prepared as described by Wilden and Kühn (1982), but the methods of Sitaramayya (1986) or Schnetkamp et al. (1979) were also employed. ROS prepared by Ficoll flotation were judged to be intact due to their inability to use external [^{32}P]ATP to phosphorylate rhodopsin (Schnetkamp et al., 1979).

Preparation of Rhodopsin Kinase. Rhodopsin kinase was prepared essentially according to the method of Sitaramayya (1986) from the different ROS preparations described above. In the final KCl extraction, 10 mL of KCl buffer was used instead of 40 mL.

Assay for Rhodopsin Kinase. ROS washed with 5 M urea, prepared according to the method of Shichi and Somers (1978), were used as a substrate for rhodopsin kinase. The kinase activity was measured at 25 °C during illumination in a total volume of 400 μL. The standard reaction mixture contained 5 μM urea-washed ROS, 1 mM $MgCl_2$ [or $Mg(OAc)_2$], 100 μM [γ-^{32}P]ATP (1.2×10^4 cpm/nmol), 50 μL kinase extract, and 2 mM dithioerythritol in 70 mM potassium phosphate buffer (pH 7.5). Phosphorylation was initiated by adding the [γ-^{32}P]ATP, and the samples were irradiated for 5 or 10 min. The reaction was terminated by addition of 1 mL of cold 10% TCA containing 10 mM H_3PO_4. ROS were collected by centrifugation, and the pellet was washed repeatedly with 10% TCA and 10 mM H_3PO_4 until the counts in the wash were less than 300 cpm/mL. The pellet was then dissolved in 200 μL of 100% formic acid, diluted with 200 μL of water, and mixed with 5 mL of scintillation cocktail (Scinti Verse II, Fisher). Other conditions were occasionally used as noted in the text. When other buffers were used, the kinase extract buffer was exchanged by using a PD-10 column (Pharmacia) equilibrated with the desired buffer.

For the rhodopsin K_m determination, the rhodopsin concentration was varied from 0.2 to 7.5 μM at an ATP concentration of 100 μM. The reaction time (with illumination) was 7.5 min. For the ATP K_m determination, the ATP concentration was varied from 0.3 to 5 μM at a rhodopsin concentration of 7.5 μM. The reaction time was 7.5 min.

Phosphorylation of Synthetic Peptides from the Cytosolic Surface of Rhodopsin. Kinase extract (500 μL) was incubated with 300 μL of peptide dissolved in 100 mM Tris-HCl buffer (pH 7.5) containing 1.5 mM [γ-^{32}P]ATP (1.2×10^4 cpm/nmol), 5 mM DTE, and 2 mM $MgCl_2$. The mixture was incubated at 25 °C for several hours. At timed intervals, 50- or 100-μL samples were withdrawn, and the peptides were separated from radioactive ATP by one of two methods. In the first method [based on the procedure of Kemp et al. (1976)], 50 μL of the above mixture was mixed with 150 μL of 40% acetic acid. After 5–10 min, the sample was applied to the top of a 2-mL column containing AG 1X8 (Bio-Rad) ion-exchange resin equilibrated with 30% acetic acid. The unbound peptide fractions were collected directly into scintillation vials, and the radioactivity was measured.

A second method of analysis [based on the procedure of Kunzel and Krebs (1985)] employed reverse-phase HPLC on a C18 column (Whatman Partisil PXS 5/25 ODS-3) to separate radioactive ATP from phosphorylated peptides. Buffer A was 0.1 M sodium phosphate (pH 6.5) containing 0.1 M NaCl, and buffer B was 100% CH_3CN. The sample was injected and washed at a flow rate of 1 mL/min with buffer A for 30 min in order to remove the radioactive ATP. The peptides were then eluted with a linear gradient over 30 min from 0% to 15–40% B (the final concentration of B depended on the size of the peptide, with higher concentrations of B for longer peptides). The peptide fractions were collected and counted. Both methods gave equivalent results. For K_m determination the following conditions were used: peptide 327–347, concentration from 2 to 7 mM, reaction time 3 h; peptide 332–345, concentration from 2 to 10 mM.

Reductive Methylation of ROS. ROS (40 mg) were suspended at a rhodopsin concentration of 1 mg/mL in 100 mM sodium HEPES buffer, pH 7.5. [^{14}C]Formaldehyde (50 μCi) was added to 2.2% final concentration. $NaCNBH_4$ was added to 19 mM final concentration. The reaction mixture was kept in the dark at 4 °C and maintained at pH 7.5 with NaOH overnight. The ROS were collected by centrifugation and washed several times with 70 mM sodium phosphate buffer (pH 7.5) containing 5 mM DTE and finally suspended in this buffer. The stoichiometry of the reaction was determined from the radioactivity and absorbance at 498 nm after purification of rhodopsin by ConA–Sepharose 4B chromatography.

Acetylation of Rhodopsin. ROS were suspended to a rhodopsin concentration of 1.5 mg/mL in 10 mM sodium HEPES buffer, pH 7.5. An equal volume of saturated sodium acetate was then added, and the suspension was cooled on ice. Acetic anhydride was added at the rate of 10 μL/mL of suspension 5 times at 10-min intervals. The ROS were collected by centrifugation and washed several times with 70 mM sodium phosphate buffer (pH 7.5) containing 5 mM DTE. The extent of acetylation was determined by measuring the remaining free amino groups by reductive methylation using [^{14}C]formaldehyde as described above.

Succinylation of Rhodopsin. ROS were suspended at room temperature to a rhodopsin concentration of 0.4 mg/mL of 0.1 M borate buffer, pH 9.3. Succinic anhydride (20 mg/mL in dioxane) was added 6 times in the amount 10 μL/mL of suspension at 10-min intervals. The ROS were collected and washed, and the extent of reaction was measured by reductive methylation as described above.

Modification of Rhodopsin with N-Ethylmaleimide. [^{14}C]NEM was used to modify rhodopsin in ROS essentially as described by McDowell et al. (1979).

Production of Thermolytic F1–F2 Complex. Extensive thermolysin digestion of rhodopsin in ROS was performed according to the method of Hargrave et al. (1982). Briefly, digestion proceeded overnight at 7% w/w thermolysin to rhodopsin, yielding homogeneous molecular weight species of

[2] Abbreviations: ConA–Sepharose, concanavalin A–Sepharose; DTE, dithioerythritol; FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; ROS, rod outer segments; TCA, trichloroacetic acid; TrTAB, tridecyltrimethylammonium bromide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, *N*-ethylmaleimide; DMF, dimethylformamide; P_i, inorganic phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.

fragments F1 and F2 (Hargrave et al., 1987).

Regeneration of Modified Rhodopsins. In order to test the effect of the various modifications on rhodopsin, the ability of rhodopsin to regenerate in outer segments was measured. The modified rhodopsin was exposed to white light ("bleached") while suspended in 70 mM sodium phosphate buffer and 5 mM DTE (pH 7.5) at a rhodopsin concentration of about 1 mg/mL. A 3-fold molar excess of 11-*cis*-retinal was added in the dark followed by incubation for 3 h at room temperature. The amount of regeneration was determined from the light-sensitive absorbance at 498 nm in the presence of 10 mM hydroxylamine.

Purification of Rhodopsin. Rhodopsin was purified by chromatography on hydroxyapatite gel using the detergent tridecyltrimethylammonium bromide (Hong & Hubbell, 1973). After purification, the detergent was exchanged for dodecyl maltoside by using a ConA–Sepharose 4B column.

Modification of Rhodopsin Kinase by FSO_2BzAdo. The kinase extract in 20 mM Tris-HCl buffer, pH 7.5, containing 2.5% DMF with various concentrations of FSO_2BzAdo was incubated at 25 °C for 0, 10, 20, 30, or 40 min. The mixture was diluted with an equal amount of the above buffer containing 10 mM DTE, 2 mM $MgCl_2$, 500 μM [^{32}P]ATP, and 10 μM rhodopsin in urea-washed ROS. The sample was illuminated for 10 min, and the phosphate incorporated into rhodopsin was used as a measure of the extent of modification. The observed pseudo-first-order rate constant (k_{obsd}) for the modification was determined for various concentrations of the ATP analogue from the slope of $\ln (E/E_0) = k_{obsd}t$, where E and E_0 represent the activity at time t and zero time, respectively.

Results and Discussion

Rhodopsin Kinase Preparation. The method of Sitaramayya (1986) was used to prepare rhodopsin kinase from rod cell outer segments. The highest kinase activity was obtained from intact rod outer segments (ROS) prepared according to the method of Schnetkamp et al. (1979), yielding more than twice the activity obtained from the other ROS preparations.

Development of an Initial Velocity Assay. Our assay system uses rhodopsin in ROS membranes that have been treated with 5 M urea to inactivate the associated kinase activity (Shichi & Somers, 1979). Brief urea washes (5–10 min) remove essentially all kinase activity, but longer exposure to urea results in reducing the effectiveness of the membranes to serve as a phosphoryl acceptor. These membranes can incorporate up to 1.7 mol of phosphoryl group/mol of opsin. During the first 30 min of the assay, phosphoryl incorporation is linear with respect to time. Doubling the kinase concentration in this system results in doubling the rate of phosphoryl incorporation, indicating linearity of the reaction with respect to kinase concentration. Our standard kinase assay measures picomoles of phosphoryl incorporated per minute into the urea-washed ROS membranes incubated with kinase and [γ-^{32}P]ATP for 10 min. Typical activities of the kinase preparations (see Materials and Methods) were 100–220 pmol of P_i incorporated min^{-1} (50 μL of extract)$^{-1}$ (where 100 μL contains the kinase from one retina).

Some enzymatic properties of the kinase are fairly typical for protein kinases. Like many protein kinases (Miyamoto et al., 1969; Lerch et al., 1975; Glass & Krebs, 1979), the enzyme appears to be relatively insensitive to pH toward its natural substrate, showing a broad peak of activity centered at neutral pH (Figure 1). When a synthetic peptide containing most of rhodopsin's phosphorylation sites was employed as a substrate (peptide 327–347), a different activity–pH profile that continues to rise as pH is increased (Figure 1) was obtained. This suggests that the interaction between the peptide and the kinase is different from the interaction between rhodopsin and the kinase. Rhodopsin is a substrate for the kinase only after bleaching, and therefore some particular conformation of photolyzed rhodopsin is required for recognition by the kinase in addition to the appropriate amino acid sequence. Other protein kinases tested, such as cAMP-dependent protein kinase, are unable to phosphorylate either bleached rhodopsin or its carboxyl-terminal peptide 337–348 (data not shown). β-Adrenergic receptor kinase, which phosphorylates a homologous receptor protein (the β-adrenergic receptor), also phosphorylates bleached rhodopsin (Benovic et al., 1986). It has been reported that both opsin and rhodopsin are phosphorylated by protein kinase C (Kelleher & Johnson, 1986), but other investigators find unbleached rhodopsin is not a substrate for protein kinase C in vivo (Kapoor & Chader, 1984). All other kinases tested to date have been found to phosphorylate glycogen synthetase (Roach, 1986), but in our hands rhodopsin kinase does not phosphorylate glycogen synthetase (data not shown).

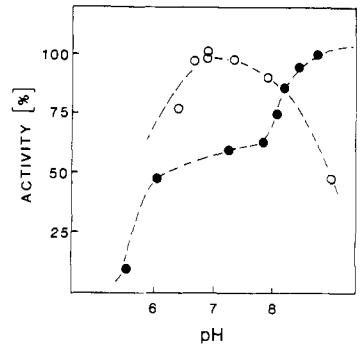

FIGURE 1: Effect of pH on rhodopsin kinase activity. The pH of the kinase assay mixture was varied by adding either 10% Tris base to increase the pH or 4% KH_2PO_4 to decrease the pH. The activity of rhodopsin kinase was then measured by the assay described under Materials and Methods with either urea-washed ROS (○) or peptide 327–347 (●) as substrate.

Effects of Magnesium and Other Cations on Rhodopsin Kinase Activity. Wilden and Kühn (1982) found that opsin kinase phosphorylated bleached rhodopsin to the highest extent when the concentrations of ATP and $MgCl_2$ were 3 and 1 mM, respectively. Higher and lower concentrations of Mg^{2+} (10 and 0.1 mM) resulted in lower extents of phosphorylation. We have extended the characterization of Mg^{2+} effects on rhodopsin kinase activity. When the Mg^{2+} concentration is increased above about 10 mM, kinase activity decreased (Figure 2). Presumably, this can result from a reduction in the concentration of Mg-ATP caused by the formation of Mg-ATP-Mg, but we cannot rule out other effects. In support of this interpretation, reducing the Mg-ATP concentration by complexing Mg^{2+} with EDTA also reduces the kinase activity. The optimum kinase activity is obtained with a 10 to 1 ratio of Mg^{2+} (1 mM) to ATP (0.1 mM), considerably more Mg^{2+} than is required to form the Mg-ATP complex with all of the ATP present (Figure 2A). This indicates that free Mg^{2+} stimulates rhodopsin kinase. Mg^{2+} stimulates kinase activity more at lower Mg-ATP concentrations than at higher concentrations (Figure 2B). This provides additional evidence that Mg^{2+} stimulates rhodopsin kinase activity and suggests that Mg^{2+} decreases the K_m for Mg-ATP, or increases the V_{max}, or both.

The effects of magnesium on the kinase are similar to that of Mg^{2+} on other protein kinases (Miyamoto et al., 1969; Granot et al., 1981; Cook et al., 1982; Braun et al., 1986). All

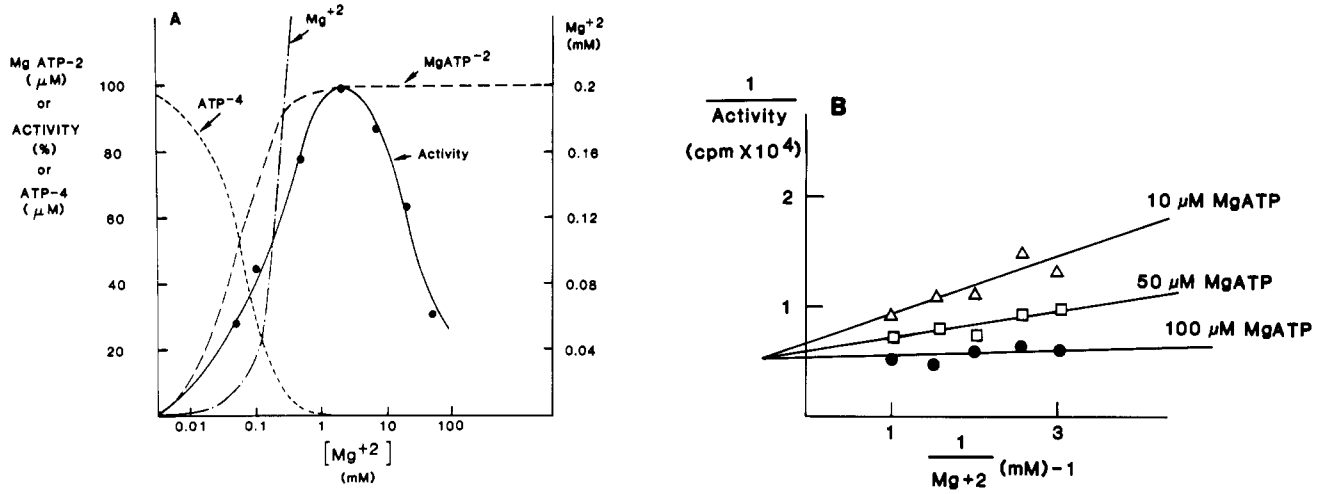

FIGURE 2: Effect of magnesium on rhodopsin kinase activity. Magnesium effects were measured in a buffer containing *N*-ethylmorpholine, 100 μM [^{32}P]ATP, and 5 mM 2-mercaptoethanol, pH 8.0, with an illumination time of 20 min. (A) Rhodopsin kinase activity was measured at various Mg^{2+} concentrations (●), taking the maximum activity as 100%. The concentrations of ATP^{-4} (---), Mg^{2+}_{free} (-·-), and $Mg\text{-}ATP^{2-}$ (– – –) were calculated by assuming a K_D of the Mg-ATP complex of 73 000 (O'Sullivan & Smithers, 1979). (B) The inverse of the activity is plotted as a function of the inverse of the Mg^{2+} concentration. Concentrations were adjusted so that the [$Mg\text{-}ATP^{2-}$] remained at 10 (△), 50 (□), or 100 μM (●) with the various Mg^{2+} concentrations calculated as in (A).

kinases that catalyze nucleotide-dependent transphosphorylation reactions require a divalent metal ion for activity (Knowles, 1980). The divalent metal ion is bound to the phosphate moiety of the nucleotide, and this complex serves as the substrate for the kinase. For many kinases, the maximum activity is obtained at a higher concentration of metal ion (usually Mg^{2+}) than is necessary to convert all of the ATP present to the Mg-ATP complex. This also minimizes the inhibitory effect of free ATP (Glass & Krebs, 1979; Takai et al., 1976; Kuo et al., 1976; Braun et al., 1986). Evidence has been presented that there are two types of divalent metal binding sites in cAMP-dependent protein kinase (Armstrong et al., 1980). High concentrations of Mg^{2+} are inhibitory, probably due to the formation of the unproductive Mg-ATP-Mg complex that binds to the kinase. Such a complex was detected by using NMR spectroscopy for cAMP-dependent kinase (Granot et al., 1981). Kinetically, for cAMP-dependent protein kinase an increase in the Mg^{2+} concentration results in a 5–6-fold decrease in V_{max} and a decrease in K_m for the Mg-ATP complex. The dissociation constant for the second Mg^{2+} of the unproductive Mg-ATP-Mg complex was 2–3 mM (Cook et al., 1982).

The effects of Ca^{2+} on the kinase activity were tested. Ca^{2+} inhibits the kinase activity at concentrations equivalent to the Mg^{2+} present (Figure 3A). However, increasing the Mg^{2+} concentration at least partially reverses the inhibition by Ca^{2+}, suggesting that the effect of Ca^{2+} is to reduce the Mg-ATP concentration by forming an unproductive Ca-ATP complex.

In earlier studies, Na^+ has been reported to stimulate rhodopsin kinase activity (Frank et al., 1973), inhibit the kinase activity (Shichi & Somers, 1978), or have no effect on the kinase activity (Chader et al., 1976). We find only minor inhibition of the kinase activity with either Na^+ or K^+ and then only at high concentrations. Even at 1 M concentrations, these ions reduce kinase activity by only 25% (Figure 3B). It is likely that this effect is due to a decrease in the concentration of Mg-ATP caused by the formation of a monovalent metal ion complex with ATP.

5′-[p-(Fluorosulfonyl)benzoyl]adenosine Inhibition of Rhodopsin Kinase Activity. The ATP analogue FSO_2BzAdo can inhibit kinase and ATPase activities by irreversibly binding at the active sites of these enzymes. When rhodopsin kinase was incubated with FSO_2BzAdo, it too was inhibited. A plot

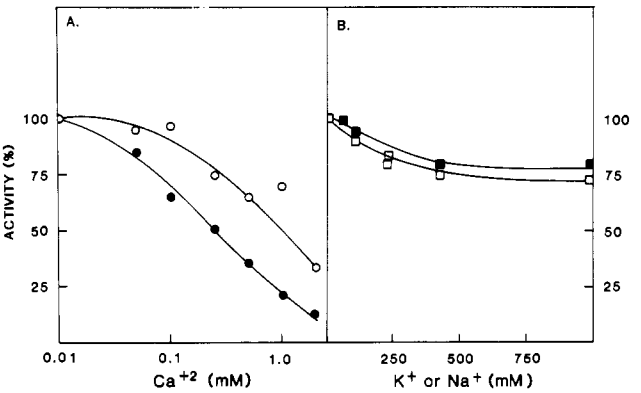

FIGURE 3: Effect of cations on rhodopsin kinase activity. The rhodopsin kinase activities were measured by using the rhodopsin kinase assay described under Materials and Methods. (A) Kinase activity plotted as a function of [Ca^{2+}] in the presence of 100 μM $MgCl_2$ (●) or 250 μM $MgCl_2$ (○). (B) Kinase activity plotted versus [Na^+] (□) or [K^+] (■) with [$MgCl_2$] = 1 mM.

of the logarithm of the kinase activity versus time of incubation with FSO_2BzAdo yields a straight line (data not shown) from which a pseudo-first-order rate constant can be calculated. A plot of this observed pseudo-first-order rate constant versus the concentration of FSO_2BzAdo yields a hyperbolic function (not shown). This indicates that the covalent reaction of FSO_2BzAdo is preceded by a noncovalent association of enzyme and inhibitor (Hixson & Krebs, 1979), where

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E - I$$

The inhibition constant, K_i, is k_{-1}/k_1, the dissociation constant for rhodopsin kinase and FSO_2BzAdo. The other constant, k_2, is the rate constant for the production of covalently modified and therefore inactive kinase. The data are plotted in Figure 4, yielding values for K_i of 0.9 mM and for k_2 of 0.043/min.

Rhodopsin kinase was reacted with FSO_2BzAdo in the presence of various compounds in order to assess their protective effect on kinase activity. The kinase also becomes inactivated when whole rod cell outer segments are treated with FSO_2BzAdo (unpublished results), but these experiments apply to the partially purified kinase preparation. When kinase

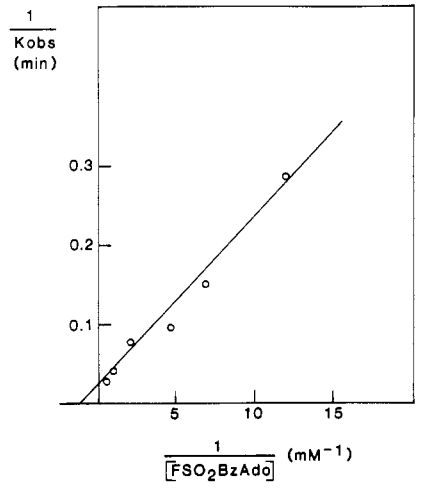

FIGURE 4: Inhibition of rhodopsin kinase by FSO_2BzAdo. Rhodopsin kinase is inhibited by FSO_2BzAdo following pseudo-first-order kinetics. The pseudo-first-order rate constants were determined as described under Materials and Methods. The inverse of the pseudo-first-order rate constant is plotted versus the inverse of the [FSO_2BzAdo] from which the inhibition constant, K_i, was determined to be 0.9 mM.

Table I: Effect of Protecting Agents on the Inactivation of Rhodopsin Kinase by FSO_2BzAdo[a]

sample	residual kinase act. (%)
control	38
ATP (0.5 mM)	86
Mg-ATP (1 mM Mg^{2+}, 0.5 mM ATP)	87
opsin (0.5 M)	61
Mg^{2+} (1 mM)	40

[a] The modification was carried out for 30 min using 1.5 mM FSO_2BzAdo. Kinase activity was measured as described under Materials and Methods.

is reacted with FSO_2BzAdo in the absence of protecting agents, kinase activity is reduced to 38% of its original value (Table I). In the presence of either ATP or Mg-ATP, the kinase is largely protected from inactivation, in a manner expected for substrate protection against active-site modification. Mg^{2+} alone exerts no protective effect. The presence of opsin in the reaction mixture provides limited protection, presumably due to the binding of kinase by opsin. Direct evidence for modification of the kinase was obtained by modification of the kinase preparation with [^{3}H]FSO_2BzAdo, following which we have identified a M_r ~67 000 radiolabeled protein by SDS–polyacrylamide gel electrophoresis (data not shown).

Rhodopsin Kinase Phosphorylation of Solubilized Opsin. Rhodopsin phosphorylation has routinely been performed using membrane suspensions. To answer several questions, we have examined the ability of rhodopsin kinase to phosphorylate rhodopsin solubilized in the mild detergent dodecyl maltoside. First, is there anything unique about the membrane structure that is necessary for the phosphorylation reaction? Urea-washed ROS were solubilized in dodecyl maltoside, kinase extract was added, and the light-induced phosphoryl incorporation was compared to that of an unsolubilized sample. During the first 15 min there is only a slight reduction in phosphoryl incorporation by the solubilized sample as compared to the membrane sample. This shows that the membrane structure is not necessary for the phosphorylation reaction. The kinase is quite stable in 6 mM dodecyl maltoside for at least 15 min.

Second, are there any membrane components besides rhodopsin that are necessary for phosphorylation? To answer this question, rhodopsin was chromatographically purified by using a hydroxyapatite column in TrTAB, and then the detergent was exchanged by using ConA–Sepharose in dodecyl maltoside. After adding kinase extract and bleaching, we observed essentially the same incorporation, indicating that no other membrane components are necessary for the phosphorylation reaction. Finally, when rod outer segments are incubated in the dark, there is usually a small amount of phosphoryl incorporated (e.g., from about 0.1 to 0.25 phosphates per rhodopsin). This could be due to the presence of some bleached rhodopsin in the ROS, or the kinase could be phosphorylating some unbleached rhodopsin. When kinase extract was added to the chromatographically purified rhodopsin and incubated in the dark, essentially no phosphoryl groups were incorporated. This supports the hypothesis that the low levels of phosphoryl incorporation into "rhodopsin" in dark-incubated ROS represents incorporation into residual small amounts of opsin rather than into (unbleached) rhodopsin.

Table II: Effect of Chemical Modification on the Phosphorylation and Regeneration Ability of Rhodopsin[a]

sample	phosphorylation act. (%)	regeneration ability (%)	mol of reagent incorpd/mol of rhodopsin
control	100	91	
NEM rhodopsin	96	97	2.3
reductively methylated rhodopsin	98	97	18.6[b]
acetylated rhodopsin	90	88	8.1
succinylated rhodopsin	3	0	7.7

[a] The modifications and regeneration were performed as described under Materials and Methods. Phosphorylation was carried out with 100 μM [^{32}P]ATP and 1 mM Mg^{2+} under illumination for 10 min. [b] Each lysine residue can incorporate two methyl groups.

Rhodopsin Kinase Phosphorylation of Rhodopsin with Blocked Sulfhydryl or Amino Groups. In the first attempts to find the features of rhodopsin that are necessary for recognition by the kinase, the effect of blocking the sulfhydryl and amino groups of rhodopsin was tested. Blocking the two free sulfhydryls or the amino groups of rhodopsin does not affect its ability to undergo phosphorylation (Table II). The amino groups were blocked with groups that maintained the positive charge of the side chain (reductive methylation), replaced the positive charge with a neutral group (acetylation), or replaced the positive charge with a negative charge (succinylation). Of these modifications, only succinylation had an effect on the phosphorylation reaction. However, succinylation was also the only modification of those tried that significantly reduced the regenerability of rhodopsin. Thus succinylopsin was unable to bind kinase or interact with retinal to re-form a native conformation, in contrast to the other lysine-modified rhodopsins. The lack of effect of modifying the sulfhydryl groups or the amino groups on rhodopsin's ability to be phosphorylated suggests that these groups are not important in the recognition by the kinase.

Bovine Rhodopsin Kinase Phosphorylation of Rhodopsin from Other Species. In a second approach to testing the primary structural requirements for recognition by bovine rhodopsin kinase, rhodopsins from other species were tested for their ability to be phosphorylated by bovine rhodopsin kinase. The rhodopsins of cattle, rabbit, pig, and alligator were purified by affinity chromatography on ConA–Sepharose in dodecyl maltoside, bovine rhodopsin kinase and ATP were added, and phosphoryl incorporation was measured as a function of time of illumination. Bovine rhodopsin is the best phosphoryl acceptor, followed closely by rabbit rhodopsin, then pig rhodopsin, and finally alligator rhodopsin. The ability of

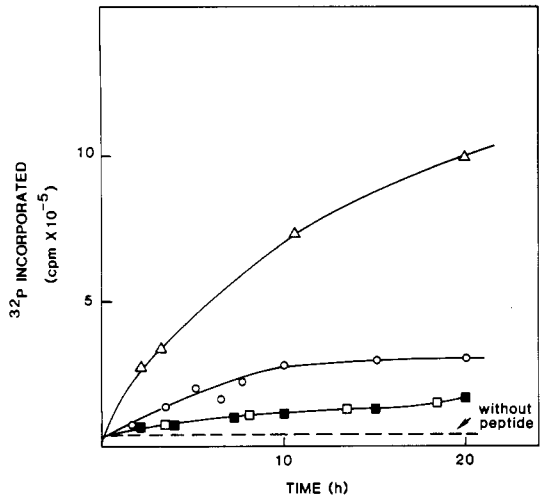

FIGURE 5: Phosphorylation of synthetic peptides by rhodopsin kinase. Bovine rhodopsin kinase was incubated with [^{32}P]ATP and synthetic peptides. The peptides from the bovine rhodopsin sequence contained serine and threonine residues known to become phosphorylated by rhodopsin kinase in intact rhodopsin [231–252 (□); 327–347 (△); 332–345 (○), and 337–348 (■)]. Phosphoryl incorporation into these peptides was measured at the times indicated by using the Dowex column procedure, reverse-phase HPLC, or both (see Materials and Methods).

Table III: Estimated K_m's for Kinase Substrates[a]

substrate	K_m
peptide 337–348	>30 mM
peptide 332–345	18 mM
peptide 327–347	8 mM
peptide 231–252	>30 mM
rhodopsin	3 μM
ATP	3 μM

[a] The phosphorylation reactions were performed as described under Materials and Methods. Peptides were separated from unreacted [^{32}P]ATP by either reverse-phase HPLC or chromatography on Bio-Rad AG 1X8 acetate (see Materials and Methods). The K_m's were calculated by nonlinear regression analysis.

these rhodopsins to be phosphorylated by bovine rhodopsin kinase paralleled the ability of these rhodopsins to be recognized by monoclonal antibodies directed against the carboxyl-terminal phosphorylation region of bovine rhodopsin (Adamus et al., 1987). This suggests that primary structure differences influence the interaction of the kinase with rhodopsin, giving us a tool to use to test what features of rhodopsin are necessary for recognition by the kinase.

Synthetic Peptides Serve as Substrates for Bovine Rhodopsin Kinase. As an additional way to test the structural features required for kinase recognition, we have tested the ability of synthetic peptides from the phosphorylation site of bovine rhodopsin to serve as substrates for the kinase. These peptides clearly serve as substrates for the kinase (Figure 5), but the amount of phosphoryl group incorporation is less than that for rhodopsin. The estimated K_m values of the various substrates are shown in Table III. Even the best peptide substrate, containing the entire caboxyl-terminal phosphorylation site, is still less than 0.1% as effective as rhodopsin as a substrate as judged by their relative K_m's. These data suggest that more than a simple primary structure is required for optimal recognition by rhodopsin kinase.

This is consistent with an earlier report that more than one region of rhodopsin is phosphorylated (McDowell et al., 1985). While the presence of two phosphorylation regions could be explained simply by the presence of multiple substrate sequences in the rhodopsin, the approximately 1000-fold difference in the K_m's of the peptides and rhodopsin suggests that the binding or recognition site of the kinase contains multiple regions of rhodopsin.

Table IV: Competitive Inhibition of the Phosphorylation of Rhodopsin by Synthetic Peptides from the Cytosolic Regions of Rhodopsin[a]

peptide	% phosphorylation of rhodopsin
control (without competitors)	100
insulin	107
albumin	88
casein (partially hydrolyzed)	118
loop I–II (65–75)	90
loop II–III (101–114)	86
loop III–IV (141–153)	80
loop IV–V (188–203)	53
loop V–VI (231–252)	22
loop VI–VII (273–285)	50
C-terminal peptide (337–348)	22
C-terminal peptide (332–345)	65
C-terminal peptide (327–347)	61
C-terminal peptide (310–321)	80
N-terminal peptide (2–32)	104
F1–F2 complex (thermolytic)	81

[a] Phosphorylation was carried out using our standard assay conditions that contained 5 μM rhodopsin (as urea-washed ROS) in the presence of 3 mM peptide (except for peptides 101–114 and 310–321, which were 0.8 mM). Insulin and albumin were 5 mg/mL, whereas casein was 2 mg/mL.

Synthetic Peptides Compete with Rhodopsin for Rhodopsin Kinase. We also investigated the effect of synthetic peptides on the phosphorylation of rhodopsin. In these experiments, the phosphorylation of rhodopsin by kinase was tested in the presence of synthetic peptides or other proteins to see if they would compete with rhodopsin for the kinase. The most effective competitors were the carboxyl-terminal peptide 337–348 and the loop V–VI peptide (231–252), both of which contain sites phosphorylated in rhodopsin (Table IV). Surprisingly, some inhibition was observed with peptides comprising the intradiskal loops IV–V and VI–VII. Since the assignment of these loops to the intradiskal region is secure (Ovchinnikov, 1982; Hargrave et al., 1983; Pappin et al., 1984), this cannot be a physiologically important finding. However, in loop IV–V the sequence Asn^{199}-Asn-Glu-Ser^{202} is similar to the cytoplasmic surface sequence Gln^{237}-Gln-Glu-Ser^{240} in which Ser^{240} is a site of phosphorylation. Such simulation of a phosphorylation site region by a sequence in the IV–V loop could explain its success as a competitor. Sequence similarities between the VI–VII loop and other phosphorylation sites is, however, not as obvious. Comparing inhibition data and phosphorylation data for the synthetic peptides offers other insights into the interaction of rhodopsin and its kinase. For example, peptide 337–348 almost completely inhibits the phosphorylation of rhodopsin; however, it is a poor substrate for the kinase. This suggests that binding to the kinase and phosphorylation by the kinase are two separate reactions that can be manipulated individually. Using different peptides from the phosphorylation region and using analogues of these regions should help us elucidate the features recognized by the kinase. For rhodopsin to be a substrate for rhodopsin kinase, it must assume very specific conformations during its bleaching process. The data presented here support this hypothesis.

CONCLUSION

It is clear from the present work that no elements of the lipid bilayer structure are necessary for the transphosphorylation reaction. It is likely that more than one portion of the poly-

peptide chain of rhodopsin is recognized by the kinase and that these elements must be present in the proper conformation for full phosphorylation activity. The binding of the kinase to rhodopsin and the phosphorylation of rhodopsin by the kinase appear to be two separate reactions. Primary structure plays a role in the recognition of rhodopsin by opsin kinase, with some changes having more effect than others. For example, modifying the sulfhydryl groups or the amino groups of rhodopsin has little effect on the phosphorylation, while using bovine rhodopsin kinase to phosphorylate rhodopsins from other species is less effective as the sequences become more divergent (as judged by immunological data). These data indicate some differences between rhodopsin kinase and most other protein kinases (cGMP- or cAMP-dependent protein kinases, casein kinase II, etc.), while more similarities exist between rhodopsin kinase and other kinases whose substrates are receptors that interact with G-proteins (Sibley et al., 1987).

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Influence of the Structure of the Lipid–Water Interface on the Activity of Hepatic Lipase†

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ABSTRACT: Factors affecting the hydrolytic activity of purified rat hepatic lipase have been examined in mixed-monolayer systems. When nonsubstrate lipids [either egg sphingomyelin or β-*O*-hexadecyl-γ-*O*-(1-octadec-9-enyl)-DL-phosphatidylcholine (OPPC-ether)] were used as inert matrices, hydrolytic activity for both triolein and dioleoylphosphatidylethanolamine was shown to decrease with increasing surface pressure (π); negligible activity occurred at $\pi \geq 30$ mN/m. Examination of the effect of introduction of cholesterol into either matrix containing 2 mol % triolein indicated that the mean molecular area decreased with increasing cholesterol and that, at $\pi = 24$ mN/m, triolein was fully miscible in the sphingomyelin matrix at cholesterol concentrations ≤32.5 mol % and in the OPPC-ether matrix at cholesterol concentrations ≤49 mol %. Above these critical concentrations of cholesterol, the phase diagrams indicate transitions that suggest that triolein is forced out of the monolayer. Introduction of increasing amounts of cholesterol into either inert matrix increased the rate of hydrolysis of triolein by hepatic lipase, although by different degrees. There are at least two factors contributing to these effects: (1) condensation of the monolayer by cholesterol, thus increasing the total surface concentration of triolein at $\pi = 24$ mN/m in the constant area surface balance, and (2) some change in triolein conformation and/or accessibility since at identical surface concentrations of triolein (8.7 ± 0.1 pmol/cm^2) and π (24 mN/m) the rate of hydrolysis of triolein by hepatic lipase is 1.5-fold higher in the OPPC-ether matrix than in the egg sphingomyelin matrix. A number of human apolipoproteins [A-I, A-II, C-II, and C-III(1,2)] were observed to inhibit the hydrolysis by hepatic lipase of 2 mol % triolein in an OPPC-ether matrix at subphase concentrations of apolipoprotein ≥0.1 nM.

The processing of lipoproteins in the plasma compartment is mediated by two enzymes, lipoprotein lipase (LPL)[1] and hepatic triglyceride lipase (HL) (Kinnunen, 1984; Jackson, 1983). Both enzymes have the capacity to hydrolyze triglycerides as well as phospholipids; however, they have different preferences for lipoprotein substrates. LPL, which is found in extrahepatic tissues, utilizes chylomicrons and very low density lipoproteins (VLDL) as its physiologic substrates and requires the participation of apolipoprotein C-II as an activator. Although LPL can hydrolyze both phosphatidylcholine and phosphatidylethanolamine, this phospholipase A_1 activity is low relative to its triglyceridase activity (Jackson, 1983). As its name suggests, HL is found in the liver, although a similar if not identical activity has been found in steroidogenic tissues (Persoon et al., 1986). HL is thought to play a role in processing chylomicron remnants, intermediate-density lipoproteins, and high-density lipoproteins (Daggy & Bensadoun, 1986). Like LPL, HL is an active triglyceridase but also has phospholipase A_1 activity (Laboda et al., 1986).

A difficulty in characterizing and comparing substrate specificities of lipases arises in the presentation of the lipid substrate. For studies on LPL and HL, many investigators have used lipoproteins as substrates, which present a complex and often uncontrollable mixture of lipids and apolipoproteins. Even less complex substrates, such as emulsions or liposomes, are often difficult to prepare in a reproducible manner. A particularly useful system for the study of the substrate specificity of lipases has been a lipid monolayer spread at the air–water interface. In this system, the composition and concentrations of the lipids in the monolayer, as well as surface pressure, can be varied in a controlled manner. Also, the effects of the addition of individual apolipoproteins can be assessed. Previous studies on the behavior of both LPL and HL in monolayer systems indicated that the effects of monolayer composition and surface pressure on the two enzymes are quite different (Demel et al., 1982, 1984; Jackson et al., 1986; Laboda et al., 1986). In the present study, we have used the monolayer system to examine the effects of surface charge and monolayer composition on the activity of HL. In particular, we have focused on factors that might influence the activity of HL at the surface of lipoproteins, namely, the

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[1] Abbreviations: HL, hepatic lipase; DOPE, 1,2-dioleoyl-L-3-phosphatidylethanolamine; DOPC, 1,2-dioleoyl-L-3-phosphatidylcholine; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; OPPC-ether, β-*O*-hexadecyl-γ-*O*-(1-octadec-9-enyl)-DL-phosphatidylcholine; π, surface pressure; *A*, mean molecular area (angstroms squared per molecule); LPL, lipoprotein lipase; VLDL, very low density lipoprotein(s); apo, apolipoprotein; HDL, high-density lipoprotein(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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