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Substrate Recognition Determinants for Rhodopsin Kinase: Studies with Synthetic Peptides, Polyanions, and Polycations†

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ABSTRACT: Rhodopsin kinase phosphorylates serine- and threonine-containing peptides from bovine rhodopsin's carboxyl-terminal sequence. K_m's for the peptides decrease as the length of the peptide is increased over the range 12–31 amino acids, reaching 1.7 mM for peptide 318–348 from the rhodopsin sequence. The K_m for phosphorylation of rhodopsin is about 10^3 lower than that for the peptides, which suggests that binding of rhodopsin kinase to its substrate, photolyzed rhodopsin, involves more than just binding to the carboxyl-terminal peptide region that is to be phosphorylated. A synthetic peptide from the rhodopsin sequence that contains both serines and threonines is improved as a substrate by substitution of serines for the threonines, suggesting that serine residues are preferred as substrates. Analogous 25 amino acid peptides from the human red or green cone visual pigment, a β-adrenergic receptor, or M_1 muscarinic acetylcholine receptors are better substrates for bovine rhodopsin kinase than is the peptide from bovine rhodopsin. An acidic serine-containing peptide from a non-receptor protein, α_{s1}B-casein, is also a good substrate for rhodopsin kinase. However, many basic peptides that are substrates for other protein kinases—histone IIA, histone IIS, clupeine, salmine, and a neurofilament peptide—are not phosphorylated by rhodopsin kinase. Polycations such as spermine or spermidine are nonessential activators of phosphorylation of rhodopsin or its synthetic peptide 324–348. Polyanions such as poly(aspartic acid), dextran sulfate, or poly(adenylic acid) inhibit the kinase. Poly(L-aspartic acid) is a competitive inhibitor with respect to rhodopsin (K_I = 300 μM) and shows mixed type inhibition with respect to ATP.

Rhodopsin is the photoreceptor protein of rod cells in the vertebrate retina. As part of the visual transduction process, rhodopsin becomes phosphorylated by a specific protein kinase, rhodopsin kinase. This phosphorylation following light absorption is one mechanism of terminating the excitation process [reviewed by Stryer (1986)]. Receptor phosphorylation may be a general process for receptor deactivation in other signal transduction systems. A number of other receptor proteins have been found to be homologous to rhodopsin and have been shown to undergo phosphorylation [reviewed by Sibley et al. (1987); Dohlman et al., 1987].

The phosphorylation of rhodopsin by rhodopsin kinase has been extensively studied [reviewed by Hargrave (1982) and Kühn (1984); Hargrave et al., 1988]. Prior to its exposure to light, rhodopsin is not a substrate for rhodopsin kinase. Following light exposure, rhodopsin undergoes a change in conformation to form metarhodopsin II, which is a substrate for the kinase. Phosphorylation occurs on serines and threonines located mostly in a compact region of rhodopsin's carboxyl-terminal sequence (Hargrave et al., 1980; Thompson & Findlay, 1984).

The sites of phosphorylation for many protein kinase substrates have been identified and their sequences determined. Such sequence information and the use of synthetic peptides as substrates have led to an understanding of the recognition determinants for many protein kinases. Extensive studies have been performed for determination of substrate specificity for cAMP and cGMP protein kinases, protein kinase C, and casein kinase II, among others (Kemp et al., 1975, 1976, 1977; Glass & Krebs, 1979; Kuenzel & Krebs, 1985; Kuenzel et al., 1987; House et al., 1987; Hassell et al., 1988). In preliminary experiments we showed that rhodopsin kinase could phosphorylate synthetic peptides containing the phosphorylation sites

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from bovine rhodopsin, although the K_m's for 12–22 amino acid long peptides were 3 orders of magnitude higher than that for rhodopsin (Arendt et al., 1981; Palczewski et al., 1988a).

Among the many cellular compounds that have been shown to have an effect on biological activity of various proteins, the most intriguing are the polyamines (Tabor & Tabor, 1976; Jänne et al., 1978; Theoharides, 1980). Although polyamines may not exert their effects on all kinases in vivo, spermine, spermidine, and polylysine-containing peptides have been observed to specifically stimulate phosphorylation of membrane proteins in nerve cells in culture (Gatica et al., 1987). Spermidine has been reported to activate rhodopsin kinase (Shichi & Somers, 1978), which prompted us to examine the effects of polyamines in more detail.

In the present paper we have systematically tested substrate determinants for rhodopsin kinase using synthetic peptides. Effects of activators and inhibitors of the kinase have been studied to better understand the properties of rhodopsin kinase in its phosphorylation of freshly photolyzed rhodopsin.

Materials and Methods

The following chemicals were purchased from Sigma Chemical Co.: spermine, spermidine, ATP disodium salt, histone IIA, histone IIS, heparin, heparin sulfate, chondroitin sulfate C, poly(adenylic acid), poly(A)–Sepharose, Tris; peptide homopolymers poly(L-Asp)·Na (M_r = 11 500), poly(L-Glu)·Na (M_r = 13 600), poly(L-Lys)·HBr (M_r = 3300), poly-Ala (M_r = 22 000), poly(L-Ser) (M_r = 5100); and random copolymer poly(Glu-Lys)·HBr 6:4 (M_r = 22 000). Dextran sulfate (M_r = 500 000) was purchased from Fluka. [γ-^{32}P]-ATP was obtained from New England Nuclear.

Solid-Phase Peptide Synthesis. Solid-phase peptide synthesis was performed at the 0.3 mmol level by using (hydroxymethyl)phenylacetamidomethyl (PAM) resin with an Applied Protein Technologies (Cambridge, MA) Model PSS-80 peptide synthesizer. *tert*-Butyloxycarbonyl (*t*-Boc) amino acids were coupled according to the diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) strategy with automated monitoring of the coupling efficiency. Coupling was repeated when the percent coupling was less than 99.5%. The side-chain functional groups were protected as follows: 2-chlorobenzoxycarbonyl for lysine; tosyl for arginine; benzyl for serine, threonine, glutamic acid, and aspartic acid; and dinitrophenyl for histidine. After synthesis, the peptides were cleaved from the resin by using liquid HF and purified by preparative HPLC on a Whatman Partisil 10 ODS-3 column (22 × 250 mm). Elution was performed by using an acetonitrile gradient in dilute trifluoroacetic acid as previously described (Arendt et al., 1989). Peptides were deionized by passing over a column of Dowex 1 resin (formate). The purity of the peptides was checked by analytical HPLC on a Whatman Partisil 5 ODS-3 column (4.6 × 250 mm) and by amino acid analysis.

Clupeine and salmine (from Sigma) were additionally purified by RP-HPLC, and desalted, as described above. A synthetic peptide derived from the β-adrenergic receptor was the generous gift of Dr. J. Benovic of Duke University.

Preparation of the Native Phosphorylation Site Peptide. Peptide 318–348 (CB-3) is the carboxyl-terminal peptide from rhodopsin that contains most of the protein's phosphorylation sites. It can be conveniently prepared from rhodopsin that remains in the bleached rod outer segment membranes following extraction of soluble proteins for preparation of rhodopsin kinase (Palczewski et al., 1988b). Washed rod outer segment membranes (from 1200 retinas) were reduced, aminoethylated, and delipidated as previously described (Hargrave, 1977). Cyanogen bromide cleavage and peptide separation followed the procedure of Hargrave et al. (1982). Peptide CB-3 (1.56 μmol) was prepared in homogeneous form as determined by HPLC and amino acid analysis.

Isolation of Rod Outer Segments. Rod cell outer segments were prepared from frozen bovine retinas (Lawson, Inc., Omaha, NE) following the procedure of Wilden and Kühn (1982). The preparation was performed either under dim red light (to prepare a substrate for rhodopsin kinase) or under room light [for rhodopsin kinase extraction as described by Palczewski et al. (1988a)]. Substrate for the rhodopsin kinase assay, ROS[1] washed with 5 M urea, was prepared according to the method of Shichi and Somers (1978) during which contact of ROS with urea was limited to 15 min. Urea was removed by extensive washing with 67 mM sodium phosphate buffer, pH 7.5, containing 1 mM $Mg(OAc)_2$, 1 mM DTT, and 0.1 mM EDTA. Finally, the washed ROS were resuspended to a rhodopsin concentration of about 4 mg/mL in the above buffer and stored in small aliquots at −20 °C.

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).

Preparation of Rhodopsin Kinase. Rhodopsin kinase was extracted from ROS prepared under room light as described by Palczewski et al. (1988b). The kinase extract was loaded on a column of DEAE-cellulose (1.6 × 20 cm) that had been equilibrated with 75 mM Tris-HCl buffer, pH 7.8, containing 1 mM DTT and 1 mM $Mg(OAc)_2$. A gradient of KCl from 0 to 250 mM, in the same buffer, was used to elute rhodopsin kinase. Fractions containing the kinase were diluted with the Tris-HCl buffer to reduce the KCl concentration, and the enzyme was concentrated by rechromatography on DEAE-cellulose. The kinase was eluted with 110 mM KCl in the above buffer. About 35 μg of rhodopsin kinase in 2 mL was obtained from 400 retinas. The purity of the enzyme was greater than 80% as judged by SDS–PAGE.

Assay for Rhodopsin Kinase Using Urea-Washed ROS. Rhodopsin kinase activity was determined as described by Palczewski et al. (1988a). The standard reaction mixture contained 10 μM urea-washed ROS, 1 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP (2 × 10^4 cpm/nmol), 10–60 μL of rhodopsin kinase solution, and 2 mM DTT in 70 mM potassium phosphate buffer, pH 7.5. The reaction was terminated by addition of 10% TCA, and the excess radioactive ATP was removed (Palczewski et al., 1988a).

Assay for Rhodopsin Kinase Using Synthetic Peptides. Rhodopsin kinase (1–5 μg) was incubated with 400 μL of a solution containing peptide in potassium phosphate buffer, pH 7.5, containing 1 mM $Mg(OAc)_2$, 1 mM DTT, 0.1 mM EDTA, and 100 μM [γ-^{32}P]ATP (10^6 cpm/nmol). The mixture was incubated at 25 °C for 1–3 h. Excess radioactive ATP was removed by either ion-exchange (Kemp et al., 1976; Palczewski et al., 1988a) or reverse-phase HPLC (Kuenzel & Krebs, 1985; Palczewski et al., 1988a). Both methods gave equivalent results.

Protein determination was performed either by measuring absorbance at 280 nm or by using the Bio-Rad protein microassay with bovine serum albumin as a standard. Peptide concentrations were determined from amino acid analysis. Concentration of ATP was determined spectrophotometrically

[1] Abbreviations: DTT, dithiothreitol; EDTA, (ethylenedinitrilo)-tetraacetic acid; ROS, rod cell outer segments; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TCA, trichloroacetic acid.

by using the absorption coefficient 15.4 mM^{-1} cm^{-1} at 259 nm (Bock et al., 1956).

Chromatography of Rhodopsin Kinase on Poly(A)–Sepharose. Rhodopsin kinase extract from 40 retinas was loaded on a column containing poly(A)–Sepharose. The column (1 × 2 cm) had been equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM $Mg(OAc)_2$ and 1 mM DTT. Nearly all rhodopsin kinase activity was bound to the column under these conditions. The column was then washed at a flow rate of 10 mL/h until the absorbance at 280 nm dropped below 0.1. Rhodopsin kinase was eluted with 120 mM KCl in the above buffer.

RESULTS

Phosphorylation of Peptides by Rhodopsin Kinase. We have shown previously that synthetic peptides derived from the bovine rhodopsin sequence can serve as substrates for bovine rhodopsin kinase (Arendt et al., 1981; Palczewski et al., 1988a). In the current study we sought to test a variety of synthetic peptides as substrates for rhodopsin kinase to help delineate those features of primary structure that are important for kinase recognition. Peptides from the bovine rhodopsin sequences 231–252 and 337–348 both contain sites that are phosphorylated by the kinase in the intact protein, but these peptides are phosphorylated relatively poorly (Table I). The 14 amino acid carboxyl-terminal peptide 332–345 contains 7 serine and threonine phosphorylation sites in the carboxy-terminal region and is a better substrate. Further lengthening of the peptide to 21 amino acids (327–347), 25 amino acids (324–348), or 31 amino acids (318–348) makes it a better substrate by leading to a progressively lower K_m. The 25 amino acid length was chosen for further study.

When all of the threonines in the bovine rhodopsin 324–348 sequence are changed to serines, the peptide is a slightly better substrate as shown by a 3-fold increase in V_{max}/K_m (Table I). This effect is offset by removal of one of the phosphorylation sites, Ser^{342}. Interestingly, the carboxyl-terminal sequence of the human red or green cone visual pigment is a better substrate than the corresponding sequence from bovine rhodopsin itself, as shown by an 8-fold increase in V_{max}/K_m. The human cone pigment sequence is similar to that of bovine rhodopsin but richer in serines and contains 10 rather than 7 potential serine and threonine phosphorylation sites. The distantly related sequences from *Drosophila* rhodopsins also serve as substrates. The comparable 25 amino acid peptide from the principal *Drosophila* pigment is only one-third as effective a substrate compared to bovine rhodopsin due to its poorer K_m. Peptides from related receptors are also substrates for rhodopsin kinase. Peptides from both a β-adrenergic receptor and an M1 muscarinic receptor are better substrates than the rhodopsin carboxyl terminus due to an increased V_{max}.

Basic peptides and proteins that are substrates for other protein kinases (histones, clupeine, and salmine and a peptide from neurofilaments) are not substrates for rhodopsin kinase. In an effort to select a peptide substrate that was derived from a protein unrelated to rhodopsin, but that would more closely resemble characteristics of the rhodopsin sequence, we chose a sequence from α_{s1}B-casein. The 25 amino acid α_{s1}B-casein peptide contains five serines, two of which are flanked by acidic amino acids. This peptide is in fact as good a substrate for rhodopsin kinase as is the native carboxyl-terminal peptide of rhodopsin itself (Table I). This important and unanticipated result will be useful in shaping future studies to further delineate the substrate determinants for rhodopsin kinase.

Effects of Polyanions on Rhodopsin Phosphorylation. The influence of polyanions on rhodopsin kinase activity was determined by assaying for kinase activity in the presence of various concentrations of poly-L-Asp and poly-L-Glu homopolymers. Poly-L-Glu partially inhibits kinase activity (Figure 1A). We found poly-L-Asp (M_r = 11 500) is a competitive inhibitor with respect to rhodopsin (K_I = 300 μM), whereas it is a mixed-type inhibitor with respect to ATP. (It was interesting to note that a lower molecular weight poly-L-Asp showed a higher K_I, giving a similar extent of inhibition if expressed on a per-residue basis.) The random polymer Glu-Lys (6:4) has a small effect on rhodopsin kinase activity (Figure 1B). Below 1.3 mM the polymer does not influence the kinase activity. However, at 3 mM concentration, the

Table I: Kinetic Parameters for Rhodopsin Kinase Substrates[a]

Substrate	K_m (mM)	V_{max} (nmol Pi/min/mg)	V_{max}/K_m relative
peptides from bovine rhodopsin sequence			
231–252 KEAAAQQQESATTQKAEKEVTR	30	ND	
337–348 VSKTETSQVAPA	30	ND	
332–345 EASTTVSKTETSQV	18	ND	
327–347 PLGDDEASTTVSKTETSQVAP	8	ND	
324–348 GKNPLGDDEASTTVSKTETSQVAPA	7.8	5.3	12
318–348 (aminoethylated) VTTLC'C'GKNPLGDDEASTTVSKTETSQVAPA	1.4	7.7	100
324–348 (all T→S) GKNPLGDDEASSSVSKSESSQVAPA	6.6	14.7	40
324–348 (all T→S; S^{342}→A) GKNPLGDDEASSSVSKSEASQVAPA	11.0	12.4	20
peptides from other visual pigments			
human red/green cone 340–364 KVDDGSELSSASKTEVSSVSSVSPA	9.2	50.8	100
Drosophila 1-6, 349–373 FGKVDDGKSSDAQSQATASEAESKA	41	8.3	4
Drosophila ocelli, 369–381 SDTETTSEADSKA	62	6.4	2
peptides from related receptors			
β-adrenergic, 396–418 QGTVPSLSLDSQGRNCSTNDSPL	5.0	18.1	66
muscarinic M1, 280–304 EDEGSMESLTSSEGEEPGSEVVIKM	16.6	55.2	60
non related proteins and peptides			
Histones:			
II A		0	
II S		0	
protamines:			
cluepeine ARRRRSSSRPIRRRRPRRRTTRRRRAGRRRR		0	
salmine PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR		0	
human neurofilament, 614–638 KSPVKPSPVEEKGKSPVKPSPVEEKGK		0	
α_{s1}B-casein peptide, 61–85 EAESISSSEEIVPNSVQEKHIQKED	8.1	6	14

[a] The phosphorylation reactions were performed as described under Materials and Methods. Peptides were separated from unreacted radioactive ATP by either reverse-phase HPLC or ion-exchange chromatography. The sequences of these peptides are designated by the single letter code according to IUPAC–IUBCBN; C′ represents aminoethylcysteine. All serine (S) and threonine (T) residues are shown underlined to draw attention to potential sites of phosphorylation. Amino acid sequences are from the following references: bovine rhodopsin, Ovchinnikov et al. (1982), Hargrave et al. (1983); human red and green cone pigment, Nathans et al. (1986); *Drosophila* rhabdome 1–6 pigment, O'Tousa et al. (1985); *Drosophila* ocelli pigment, Cowman et al. (1986); β-adrenergic receptor, Dixon et al. (1986); muscarinic M1 acetylcholine receptor, Kubo et al. (1986); clupeine, Ando and Suzuki (1967); salmine, Dixon and Smith (1968); human neurofilament, Lee et al. (1988); and bovine α_{s1}B-casein, Mercier et al. (1971). ND, not determined.

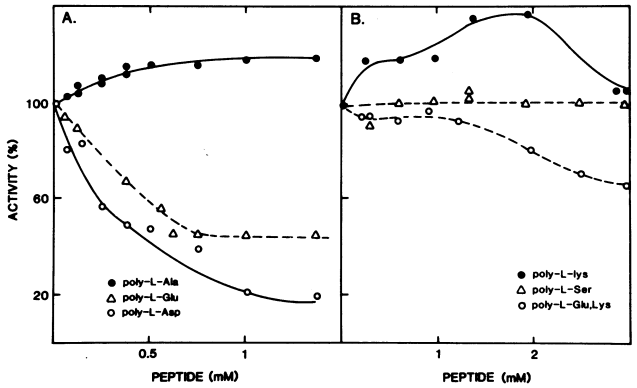

FIGURE 1: Influence of homopolymer peptides on the phosphorylation of rhodopsin by rhodopsin kinase. The activity of rhodopsin kinase is plotted as a function of the concentration of the effector, with 100% as the activity of rhodopsin kinase without added peptides. (A) Poly-L-Ala (●); poly-L-Glu (△); poly-L-Asp (○). (B) Poly-L-Ser (△); poly-L-Lys (●); poly-L-(Glu,Lys)6:4 (○).

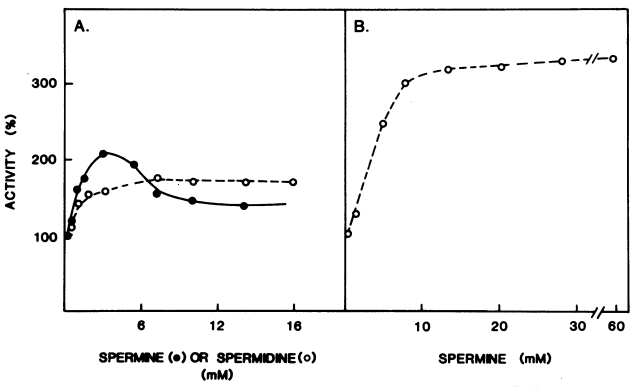

FIGURE 2: Effects of polyamines on phosphorylation of rhodopsin and rhodopsin peptide 324–348. The activity of rhodopsin kinase is plotted as a function of the concentration of the effector, where 100% is the activity of rhodopsin kinase without added polyamine. (A) Effects of spermine (●) and spermidine (○) on the phosphorylation of rhodopsin. (B) Effect of spermine on the phosphorylation of peptide 324–348 derived from the rhodopsin sequence.

extent of inhibition of rhodopsin phosphorylation is 30%. Even though rhodopsin kinase phosphorylates serine residues, poly-L-Ser has no effect on the phosphorylation of rhodopsin by its kinase. In contrast, the cationic poly-L-Lys activates the kinase by as much as 40% at 2 mM concentration (Figure 1B). The anionic polymers are weak inhibitors of rhodopsin kinase. I_{50} values for heparin and chondroitin sulfate C are high (200 μM and 6 mg/mL, respectively), whereas for poly(adenylic acid) and dextran sulfate the I_{50} values are relatively low (40 μM and 200 μg/mL, respectively) at 10 μM rhodopsin concentration (data not shown).

Binding to Poly(A)–Sepharose. Rhodopsin kinase binds to positively charged DEAE-cellulose at pH 7.5–7.8 (Palczewski et al., 1988b). However, at this pH, phosphorylation of rhodopsin by the kinase can be inhibited by polyanions. To determine if rhodopsin kinase has a region that can bind to polyanions, rhodopsin kinase was applied to a column containing poly(adenylic acid)–Sepharose. Under conditions described under Materials and Methods, the kinase bound to the poly(A)–Sepharose and could be eluted with 120 mM KCl (data not shown).

Effects of Polycations on Phosphorylation of Rhodopsin and Synthetic Peptide. Polycations such as spermine or spermidine activate phosphorylation of rhodopsin (Figure 2A). Activation of the reaction by spermidine is saturable at 5 mM concentration, reaching 210%. On the other hand, activation of the phosphorylation by spermine is more complex. Increasing the spermine concentration increases the kinase activity, reaching more than double the control activity at 3 mM spermine. At higher concentrations, the kinase activity decreases until at about 8–10 mM spermine the kinase activity is about 130% of the control activity (Figure 2A). At even higher concentrations (20–100 mM), spermine inhibits kinase activity (data not shown). Not all polyamines activate rhodopsin kinase; gentamycin, a polyamine aminoglycoside antibiotic, has no effect on rhodopsin phosphorylation even at 10 mM (data not shown). To explore the polycation effect on rhodopsin phosphorylation catalyzed by the kinase, the following questions were posed:

1. Does spermine interact with the substrate, the enzyme, or both?
2. Which kinetic parameters are affected by the activator?
3. If the kinase is affected by spermine, does spermine influence the ATP binding site or the rhodopsin binding site?

Spermine activated the phosphorylation of peptide 324–348 by rhodopsin kinase (Figure 2B). The kinase activity with this peptide as substrate showed up to a 3-fold stimulation in the presence of spermine, the effect saturating at about 8 mM spermine. Higher concentrations of spermine, up to 60 mM, caused no further increase in activity, nor did they cause the decrease in activity that was observed with rhodopsin as substrate (Figure 2A).

Table II: Influence of Spermine on K_m for Rhodopsin or Peptide 324–348

concn of spermine (mM)	K_m (μM)	V_{max} (nmol of P_i min^{-1} mg^{-1})	relative V_{max}/K_m
	Rhodopsin[a]		
0	4.04	650	100
0.33	1.1	600	340
0.66	4.6	1453	200
1.33	10.0	2993	180
	Peptide 324–348[b]		
0	7.8	5.3	100
5	1.3	5.4	610
10	0.78	5.0	940
30	0.76	5.6	1073

[a] The K_m's were determined as described under Materials and Methods. Different concentrations of spermine were added, and the reaction was carried out for 10 min. The reaction was stopped with 10% TCA, and rhodopsin was collected by centrifugation. [b] Effect of spermine on affinity of rhodopsin kinase for peptide 324–348 at 0, 5, 10, and 30 mM spermine. Assays were carried out as described under Materials and Methods.

The effect of spermine concentration on the kinetic parameters of the phosphorylation of rhodopsin kinase is shown in Table II. At low concentrations (0.33 mM), the K_m for rhodopsin is decreased by a factor of 4, while the V_{max} remains essentially unchanged. At a higher concentration of spermine (0.66 mM), both V_{max} and K_m are higher. Higher concentrations of spermine yield higher V_{max} and K_m values. This results in a maximum value of V_{max}/K_m at about 0.33 mM spermine and therefore a maximum in the activation of the phosphorylation. While increasing the spermine concentration above 0.33 mM causes a decrease in the activation, activation is still observed at high rhodopsin concentration with spermine concentrations in the millimolar range.

The K_m for ATP is hardly affected by the presence of spermine (data not shown). However, the K_m for peptide 324–348 is altered by spermine (Table II). The K_m for the peptide changes from 7.8 mM in the absence of spermine to 0.76 mM at 30 mM spermine. The V_{max} is slightly reduced with increasing spermine concentration, but the major effect is the change in K_m. K_m and V_{max} were not changed in going

Table III: Effect of Synthetic Peptides from the Cytosolic Surface of Rhodopsin on the Phosphorylation of Carboxyl-Terminal Peptide 324–348[a]

peptide	% of phosphorylation
control (3 mM peptide 324–348 without additional peptide)	100
loop I–II (65–75)	
1.5 mM	120
3 mM	200
loop III–IV (141–153)	
1.5 mM	100
3 mM	90
loop V–VI (231–252)	
1.5 mM	118
3 mM	120

[a] The phosphorylation reactions were performed as described under Materials and Methods. The concentration of C-terminal peptide (324–348) was 3 mM, whereas concentrations of additional peptides were as indicated.

from 10 to 30 mM spermine, consistent with the data of Figure 2.

Effects of Peptides from Cytosolic Surface of Rhodopsin on Phosphorylation of Carboxyl-Terminal Peptide 324–348. To see if other features of rhodopsin's surface might be involved in the phosphorylation reaction, synthetic peptides from the cytosolic surface regions of rhodopsin were tested for their ability to affect the phosphorylation of the carboxyl-terminal peptide 324–348, the primary phosphorylation site of rhodopsin (Table III). At 3 mM concentration, loop I–II increased the phosphorylation level by a factor of 2, whereas loop V–VI caused a 20% increase in phosphorylation of the mixture. The presence of loop III–IV in the phosphorylation reaction had no effect on the kinase activity (Table III).

Discussion

For many protein kinases, substrate specificities have been characterized by the use of synthetic peptides as substrates. The sequence of the synthetic peptide is generally derived from the native protein substrate. For protein kinase A, these studies have shown that a basic amino acid (particularly an arginine residue) on the amino-terminal side of the target serine acts as an important substrate specificity determinant (Kemp et al., 1975, 1976, 1977; Bramson et al., 1983). Although cGMP-dependent protein kinase shares some similarities with protein kinase A, the residues required for recognition by this kinase around the phosphorylation site in histone H2B are quite different (Glass & Krebs, 1979). Another protein kinase, phosphorylase kinase, requires a serine residue with an arginine residue on the amino-terminal side that is surrounded by hydrophobic groups. The conformation of the substrate peptide/protein is also important for recognition by phosphorylase kinase (Tessmer & Graves, 1973; Tessmer et al., 1977; Viriya & Graves, 1979). A single basic residue (preferably arginine) on the carboxyl- or amino-terminal side of the target serine residue is the primary requirement for recognition by protein kinase C, although additional basic residues as well as structural features strongly influence the kinetics of peptide phosphorylation (Turner et al., 1985; Su et al., 1986; Kondo et al., 1987; House et al., 1987; Sakanoue et al., 1987). A similar requirement for basic residues is exhibited by myosin light chain kinase (Kemp et al., 1982). However, it is clear that other (structural) features affect recognition by this enzyme. The best substrates for casein kinase II are those in which multiple acidic amino acids are present on both sides of the phosphorylatable serine or threonine. Acidic residues on the amino-terminal side of the serine greatly enhance the K_m's and V_{max}'s but are not absolutely required. An acidic amino acid located three residues on the carboxyl-terminal side of the residue to be phosphorylated is absolutely required. Aspartic acid residues serve to give better recognition than glutamic acid residues (Kuenzel et al., 1985, 1987; Fiol et al., 1987; Marchiori et al., 1988).

From previous studies, it appeared that rhodopsin kinase effectively utilized peptides as substrates (Arendt et al., 1981; Palczewski et al., 1988a). However, kinetic parameters were very different from those for rhodopsin. We had observed that the length of the peptide could play some role in affinity for the kinase, but the longest peptide tested then (327–347) had a K_m that was 3 orders of magnitude higher than that of freshly bleached rhodopsin, indicating that peptides were comparatively poor substrates (Palczewski et al., 1988a). In the work presented here we synthesized longer peptides containing 25 amino acids with a variety of different sequences and tested them as well as the native 31 amino acid carboxyl-terminal CNBr peptide. Although the aminoethylated native peptide had the lowest K_m compared to all other peptides, the K_m is still nearly 3 orders of magnitude higher than the K_m for freshly bleached rhodopsin. Converting all of the threonine residues in the carboxyl-terminal peptides to serines yielded a much better substrate for rhodopsin kinase, primarily by increasing V_{max}. Similar results have been observed for casein kinase II and protein kinase A, where substituting phosphorylatable threonine residues with serine residues increased V_{max} in all cases but with negligible changes in K_m for casein kinase II (Kuenzel et al., 1987) or with a 5-fold increase in K_m for protein kinase A (Kemp et al., 1977). Likewise, substituting a threonine for the phosphorylatable serine residue in a peptide phosphorylated by phosphorylase kinase yielded a peptide that was not a substrate for the enzyme (Tessmer et al., 1977). Another substitution in peptide 324–348 (all threonines substituted by serines) in which Ser^{342} was changed to alanine gave nearly a 2-fold increase in K_m, suggesting that the hydroxyl group at this position is important for recognition by the kinase.

Nearly all studies on phosphorylation of rhodopsin have been performed by using rod cell rhodopsin kinase and the rod cell visual pigment. Very little is known about phosphorylation of visual pigments in cone cells (Walter et al., 1986). Would the different visual pigments require different kinases? Could the rod cell kinase phosphorylate cone cell visual pigments? We began to approach these problems by asking whether bovine rhodopsin kinase can phosphorylate peptides with sequences derived from human cone cell visual pigments. We found that the human red/green cone sequence 340–364 has one of the best V_{max} of the peptides tested, although the K_m is similar to that for peptide 324–348 derived from bovine rhodopsin. Some specificity for vertebrate visual pigment sequences is suggested since the peptides from *Drosophila* visual pigments are comparatively poor substrates for rhodopsin kinase.

Benovic et al. (1986) have shown that, after agonist-induced conformational changes, rhodopsin kinase can phosphorylate the β-adrenergic receptor, although to a low extent when compared to rhodopsin. The carboxyl-terminal peptide derived from the β-adrenergic receptor sequence served as a good substrate for bovine rhodopsin kinase and has kinetic properties similar to those of the rhodopsin carboxyl-terminal peptide. Another peptide, derived from the sequence of an M1 muscarinic receptor, has the highest V_{max} among the peptides tested, but it also has a higher K_m, which reduces its effectiveness as a substrate.

One pattern that seems to emerge from the studies using peptide substrates is that rhodopsin kinase phosphorylates only

neutral or acidic peptides. Our most striking observation has been the finding that an acidic peptide from casein serves as a substrate for rhodopsin kinase. Positively charged serine-containing peptides such as histones, clupeine, or salmine are not phosphorylated by the kinase. In the peptides tested to date, rhodopsin kinase prefers to phosphorylate serines rather than threonines.

In general, free peptides are very poor substrates for rhodopsin kinase when compared to the protein rhodopsin. We propose that a multipoint binding of kinase to rhodopsin is required to obtain maximal activity. This is supported by the observation that serine 240, which is in the third cytoplasmic loop, also becomes phosphorylated. Conformation must also play a role since rhodopsin is a substrate for its kinase only after bleaching. The kinase could require a particular conformation of the phosphorylation region (secondary structure) or of its multipoint binding site (tertiary structure). However, our studies have shown that the 25 amino acid β-adrenergic carboxyl-terminal peptide is a better substrate for the kinase than the analogous peptide from rhodopsin. Since the β-adrenergic peptide forms about 20% α-helical structure and the rhodopsin peptide does not (unpublished data), this suggests that secondary structure is less important than higher order structure. Given the importance of binding of the kinase to rhodopsin and the wide variety of peptide sequences that the kinase can phosphorylate, rhodopsin kinase might be expected to phosphorylate these sequences (e.g., from α_{s1}B-casein) if they were present in the carboxyl-terminal sequence of a rhodopsin.

It is worth noting that the β-adrenergic receptor kinase, which is a receptor kinase with properties similar to those of rhodopsin kinase, can also phosphorylate α_2-adrenergic receptor in an agonist-dependent fashion (Benovic et al., 1987). Since the α_2-receptor has a very short C-terminal tail with no serine and threonine residues, phosphorylation of this receptor by β-adrenergic receptor kinase has to occur in the Ser/Thr-rich third cytoplasmic loop. Thus, it is likely that rhodopsin kinase would also phosphorylate an acidic peptide sequence rich in Ser/Thr residues if it were present in the third cytoplasmic loop.

Since acidic groups appear to be important in substrates for rhodopsin kinase, we investigated whether acidic compounds might prove to be inhibitors. We found that polyanions inhibit rhodopsin kinase. Poly(L-aspartic acid) is an effective inhibitor whose K_I is a function of the length of the polymer. The only other polyanions that are potent inhibitors [poly(adenylic acid), heparin, and dextran sulfate] have the common feature of areas of high density of negative charge.

Rhodopsin kinase has the property of being able to bind at the same pH to either a cation exchanger or an anion exchanger. This suggests that the protein has two surface regions, one basic and one acidic, that can bind to the cation or anion exchanger, respectively. Testing the effect of other homopolymers, we observed activation by a basic polypeptide, poly(L-lysine). Similarly, the polycation spermine decreased the apparent K_m (of casein) for casein kinase II and increased the maximum velocity of the reaction, yielding an effective 2.5-fold stimulation with spermine at physiological concentrations (Hathaway & Traugh, 1984; Feige et al., 1980). Casein kinase I is stimulated by spermine, by an unknown mechanism (Hegazy et al., 1988). Polyamines directly influence the phosphorylation of cytosolic proteins by protein kinase C (Monti et al., 1988). In contrast, the activity of protein kinase A is not altered by spermine (Mäenpää, 1977). Rhodopsin kinase is activated by spermidine (Figure 2A) as observed earlier by Shichi and Somers (1978), but the mechanism of the activation is unknown. The effect of another polycation, spermine, on rhodopsin kinase is complex and appears to affect both the substrate, rhodopsin, as well as the enzyme. With the peptide substrate, the activation saturated at about 8 mM spermine and the activation occurred primarily by decreasing the K_m for the peptide. The V_{max} of the reaction was essentially unchanged. Since the peptide is unchanged, we attribute these effects to interaction of the kinase and the polyamine. When rhodopsin was used as substrate, the effect was biphasic. At low concentrations of the polycation, a decrease in K_m was observed as well as a slight decrease in V_{max}. At higher concentrations both K_m and V_{max} increased. These effects must be due to interaction of kinase and polyamine as observed for the peptide substrate, but there must also be an effect of spermine on rhodopsin that causes additional changes at higher spermine concentration. For concentrations of spermine up to 0.5 mM, the K_m for ATP is not changed. This suggests that the effect of spermine is on the rhodopsin binding site(s) and not the ATP binding region.

The activation of rhodopsin kinase by polycations appears to require some structure of the polycation and is not caused by all molecules that have multiple positive charges. As shown, the aminoglycoside gentamycin which stimulates Ph-C2 kinase (equivalent of casein kinase II; Ahmed et al., 1988) did not enhance rhodopsin phosphorylation.

Two other observations may be related to the effect of polyamine stimulation of rhodopsin. Fowles et al. (1988) have reported that the phosphorylation of synthetic peptide from the rhodopsin sequence is stimulated by bleached rhodopsin. We find such peptide phosphorylation to be stimulated by a peptide from the rhodopsin surface—the basic cytoplasmic surface peptide, loop I–II (Table III). It is possible that when the kinase binds to light-exposed rhodopsin, the basic sequence of the loop I–II serves to stimulate the phosphorylation reaction and that the observed polyamine stimulation mimics this effect.

Recently, Benovic et al. (1989) showed that the β-adrenergic receptor kinase is inhibited by both polyanions and polycations. Since both β-adrenergic receptor kinase and rhodopsin kinase share many similarities, the effect of spermine (or other polycations) may prove useful as a way to distinguish them. Also, it is clear that the kinases show difference sensitivities to inhibition by polyanions (Benovic et al., 1989).

In conclusion, rhodopsin kinase is an enzyme that is very specific for its protein substrate. It binds to and phosphorylates a specific transient conformation of light-exposed rhodopsin. Its protein substrate specificity appears to be governed more by this recognition than by a sequence specificity, since it will phosphorylate serines and threonines in a variety of acidic peptides. The activation of rhodopsin kinase by certain basic compounds and inhibition by acidic ones may mimic its interactions with its protein substrate, light-activated rhodopsin.

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