Characterization of Rhodopsin Kinase Purified from Bovine Rod Outer Segments*

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Rhodopsin kinase was purified by sequential chromatography on DEAE-cellulose and blue-Sepharose. Kinase activity co-purified with a 62 kDa polypeptide, which bound light-dependently in the absence of ATP to purified vesicle-reconstituted rhodopsin. Purified rhodopsin kinase is free of any detectable arrestin or the retinal G-protein. Rhodopsin kinase is autophosphorylated on serine residues which is unaffected by the presence of bleached rhodopsin and results in a transition in molecular mass to 64 kDa. Autophosphorylation of the kinase did not appear to alter the overall rate of rhodopsin phosphorylation or the apparent $K_M$ (0.6 $\mu$M) for purified reconstituted rhodopsin. Peptides corresponding to sequences within opsin loops 3-4 and 5-6 and the COOH terminus inhibited kinase phosphorylation of bleached rhodopsin, suggesting at least three potential sites to account for the stable high affinity binding of rhodopsin kinase to the bleached photoreceptor molecule that are at least in part distinct from the substrate sites for phosphorylation. These sequences are similar to those proposed for receptor recognition of G-proteins and indicate that the domains involved in light-dependent binding of rhodopsin kinase and retinal G-protein are similar or overlapping.

Rhodopsin kinase catalyzes the phosphorylation of photolyzed rhodopsin (1-5), which in selected conditions may involve the modification of up to 9 serine and threonine residues (1-8). The phosphorylation of rhodopsin is thought to be a major regulatory mechanism for the control of the photorhodopsin transduction pathway (1-5). Recently, Bennett and Sitaramayya (14) described two effects of rhodopsin phosphorylation. First, multisite phosphorylation markedly blunted the ability of bleached rhodopsin to activate the retinal G-protein (G). Second, low phosphorylation stoichiometries enhanced the binding of arrestin, the retinal 48 kDa protein, to bleached rhodopsin which competitively inhibited G activation. Interestingly, multisite phosphorylation appeared to proceed despite the binding of arrestin to bleached rhodopsin (14), whereas G, binding to rhodopsin inhibited rhodopsin phosphorylation (16).

The kinetics of site-specific phosphorylation of rhodopsin by rhodopsin kinase in the presence or absence of arrestin or G, is unknown. In fact, no substrate-recognition sequence has been proposed for this kinase (1-5). Identification of a substrate-recognition sequence has been crucial to understanding the kinetics of site-specific multisite phosphorylation of other kinases (17). Our understanding of this kinase has also been hampered by commercially available preparations (13-15) have been extracts of rod outer segment membranes that have not been amenable to chromatographic purification to even allow certain identification of the protein (1-5, 13).

Here we describe a rapid two-step purification procedure for rhodopsin kinase that was previously shown to remove all immunologically detectable G, and arrestin (16) and to provide a rhodopsin kinase preparation that catalyzes multisite phosphorylation of purified bleached rhodopsin in phospholipid vesicles. Rhodopsin kinase was identified as a 62-kDa protein on SDS-PAGE which migrates as a 64-kDa phosphoprotein when autophosphorylated on serine residues.

**EXPERIMENTAL PROCEDURES**

**Purification of Rhodopsin Kinase—**Rhodopsin kinase was purified routinely at 0-4°C from 150 frozen dark-adapted bovine retinas. Leupeptin (40 $\mu$g/ml), aprotenin (0.3 $\mu$m/ml), and phenylmethanesulfonyl fluoride (100 $\mu$M) were included in all buffers used prior to chromatography on DEAE-cellulose and blue-Sepharose. Rod outer segment membranes were isolated in the light as previously described (18) except that the step gradients used were 25 and 30% (w/w) sucrose as suggested by Sitaramayya (13). The membranes were then extracted for 60 min on ice in the dark using 50 ml of 200 mM NaHepes, 20 mM EDTA, 2 mM DTT, pH 8.0, followed by centrifugation (100,000 $\times$ g, 15 min). The kinase extract (supernatant) was dialyzed overnight against 4 liters of 10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT (buffer A) and then loaded onto a 2-ml (1 cm diameter) DEAE-cellulose column equilibrated in buffer A. The column was washed with 10 column volumes of buffer A, followed by 10 column volumes of 35 mM NaCl in buffer A. Rhodopsin kinase was eluted using a 20-column volume linear gradient of 35-155 mM NaCl in buffer A. Ten 4-ml fractions were collected, and rhodopsin kinase activity which peaked at 80 mM NaCl began to elute during fraction 1 and was completely eluted by fraction 16. Fractions 2-8 were pooled, mixed, and immediately applied to a 2-ml (1 cm diameter) column of blue-Sepharose equilibrated with 75 mM NaCl in buffer A. The column was washed with 20 column volumes of 100

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§ The abbreviations used are: G, retinal G-protein, transducin; SDS, sodium dodecyl sulfate; ACN, acetonitrile; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.
Rhodopsin Kinase Activity—Rhodopsin kinase activity was determined as the rate of incorporation of [γ-32P]phosphate from [γ-32P]ATP into the rhodopsin monomer that migrated as a 34-kDa band in SDS-PAGE. Rhodopsin monomers were resolved by SDS-PAGE, and the Coomassie-stained and dried gels were used to prepare autoradiographs followed by excising the rhodopsin band from the gel and determining [γ-32P] incorporation by Cerenkov counting. After addition of SDS-sample buffer to terminate the phosphorylation reactions, caution was taken to neither heat nor freeze the samples to minimize artifactual formation of rhodopsin dimer which migrates as a broad band having a mobility similar to that of rhodopsin kinase. The standard assay used 30 µl of column fractions, and incubations were for 5 min. A 100-µl assay contained 10 mM Tris-HCl pH 7.4, 260 mM NaCl, 3.2 mM MgCl₂, 0.95 mM EDTA, 0.85 mM EGTA, 2.2 mM DTT. The reaction was initiated by simultaneous addition of [γ-32P]ATP and transfer of the samples from the dark to room temperature with room white light illumination. Kinase activity was linear with time and the amount of kinase preparation added to the reaction mixture. Rhodopsin used as substrate was prepared by extraction from rod outer segment membranes using 1.5% octyl glucoside from which rhodopsin kinase and transducin had been removed previously under dim red light. Rhodopsin was purified by chromatography on concanavalin A-Sepharose (21) and reconstituted into egg phosphatidylinositol/phosphatidylethanolamine vesicles (22) after addition of 5 mM EDTA to the rhodopsin/lipid mixture by dialysis against 10 mM Tris-HCl, 5 mM MgCl₂, 1 mM EDTA, 2 mM DTT. The rhodopsin-containing vesicles were isolated, washed by centrifugation (15,000 × g, 15 min), and stored in dialysis buffer at -70 °C until a concentration of 1 mg of rhodopsin as determined by the change in optical density at 498 nm induced by exposure to white light (18, 19).

Peptide Synthesis, Purification, and Sequence Analysis—Peptides L₁₋₁, L₁₋₂, and L₃₋₄ and L₄₋₆ were synthesized by Peninsula Laboratories. Peptides CT₁, CT₂, CT₃, and CT₄ were synthesized using an automated solid-phase peptide synthesizer (Applied Biosystems). All synthetic peptides were purified on a C₁₈ reverse-phase HPLC column. Peptide CT₁ was generated from purified CT₁, by incubation of 19 mg of the peptide with 3 mg of Staphylococcus aureus V8 protease for 4 h at 30 °C in 50 mM NaH₂PO₄, pH 7.8. The products were purified, as well as using a HPLC C₁₈ reverse phase column. The sequence of CT₁, as well as that of the other purified peptides, was confirmed by automated amino-terminal protein sequencing using an ABI model 470A gas phase sequenator equipped with an on-line model 120A phenylthiohydantoin analyzer.

Phosphorylation and Analysis of Synthetic Peptides—Reaction volumes were 400 µl containing 200 µl of purified kinase, routinely 1 mM peptide (395-425 µg) and high specific activity [γ-32P]ATP (55,000 cpm/µmol). Control samples lacking peptide were processed in parallel with the plus-peptide samples, and all samples were analyzed by flatbed electrophoresis and autoradiography. The phosphorylation reaction in each 400-µl assay which contained 10 mM Tris-HCl, pH 7.4, was terminated by the addition of 2 ml of 0.1% (v/v) trifluoroacetic acid. The sample was applied to a 1 ml C₈-Sep-Pak column prewashed with 20 ml of methanol, followed by 20 ml of 60% ACN in 0.1% trifluoroacetic acid and then 100 ml of 0.1% trifluoroacetic acid. Throughout, the flow rate was dropwise under positive pressure applied by an attached 20-ml syringe. After sample application, the column was washed with 8 × 5 ml of 0.1% trifluoroacetic acid, followed by 10 × 1 ml of 5% ACN in 0.1% trifluoroacetic acid, and then 100 ml of 60% ACN. This exhausts the trypsin activity, and last five 1 ml 5% ACN elutes together with the 60% ACN eluate contained all of the detectable peptide-specific radioactivity for each peptide analyzed. These fractions were pooled and used for analysis. Labeled phoshopeptides were dissolved in H₂O and 80-185 µg of total peptide was analyzed by flatbed electrophoresis and autoradiography in order to remove eluting phosphopeptide. In the case of L₁₋₁, the remainder of the sample was made 0.1% trifluoroacetic acid (0.8 ml) and applied to a 20-cm reverse-phase HPLC column equilibrated with 0.1% trifluoroacetic acid. After loading, the column was washed for 10 min with 0.1% trifluoroacetic acid, then eluted with a 60-min gradient of 0-60% ACN in 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min, and fractions of 0.25 ml were collected and counted as well as the absorbance at 214 nm determined. For each of the phosphorylated peptides (see Figs. 7-10), one to three peaks of peptide-specific radioactivity were eluted either together or just in front of the major peak of peptide-specific A₁₋₁, as absorbance. For each of the peptides the radioactive peaks together with the major peak were collected and dried, and analyzed by flatbed electrophoresis and phospho-amino acid analysis. The dried samples from the HPLC runs were dissolved in H₂O and loaded along the centerline of 20 × 20-cm 0.1-mm thickness cellulose sheets. After flatbed electrophoresis using 1000 V for 45-50 min at 4 °C in a pH 5.5 buffer (pyridine/acetic acid/ H₃O₊ 1:10:180) the sheets were dried, sprayed with ninhydrin reagent, redried, and autoradiographed using intensifying screens. Radiolabeled zones appearing in lanes containing plus-peptide samples, but not detected in controls, were excised, eluted with H₂O, and counted by Cerenkov radiation. The eluted peptides were analyzed for phospho-amino acid composition essentially as described (23).

Rhodopsin kinase, as assayed in the legend to Fig. 2, was purified from 150 bovine retinas by sequential chromatography on DEAE-cellulose and blue-Sepharose (Fig. 1). Rhodopsin kinase activity eluting from both the DEAE column (not shown) and the blue-Sepharose column correlated with 62-64-kDa protein bands on Coomassie-stained gels (Fig. 1C). Incubation of these fractions with [γ-32P]ATP in the absence (Fig. 1A) or presence (Fig. 1B) of rhodopsin resulted in detection of a 64-kDa phosphoprotein. As defined in Figs. 2 and 3, rhodopsin kinase becomes rapidly phosphorylated which alters its migration on SDS-PAGE.

### RESULTS

Rhodopsin kinase has been noted for its instability during purification (13, 26, 27). The purification procedure described under "Materials and Methods" allows the consistent isolation of 300 units of the kinase (1 unit = 1 pmol of phosphorylase activity min⁻¹) at a specific activity of 10 pmol min⁻¹ µg⁻¹ (Table I). Starting with a rhodopsin kinase extract from 150 bovine retinas, the enzyme can be purified within 4-5 h by sequential chromatography using DEAE-cellulose and blue-Sepharose (Fig. 1). Rhodopsin kinase activity eluting from both the DEAE column (not shown) and the blue-Sepharose column correlated with 62-64-kDa protein bands on Coomassie-stained gels (Fig. 1C). Incubation of these fractions with [γ-32P]ATP in the absence (Fig. 1A) or presence (Fig. 1B) of rhodopsin resulted in detection of a 64-kDa phosphoprotein. As defined in Figs. 2 and 3, rhodopsin kinase becomes rapidly phosphorylated which alters its migration on SDS-PAGE.

<table>
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<tr>
<th>Protein</th>
<th>Activity</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td>DEAE</td>
<td>39 (15)</td>
<td>136 (14) 881 (14)</td>
</tr>
<tr>
<td>Blue-Sepharose</td>
<td>31 (1) 926 (20) 10.50</td>
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Table I: Purification of rhodopsin kinase

Rhodopsin kinase, assayed as described in the legend to Fig. 2, was purified from 150 bovine retinas by sequential chromatography on DEAE and blue-Sepharose as described under "Experimental Procedures." Coomassie-stained SDS-PAGE gels of the preparations at each stage of the purification procedure are shown in Fig. 3. The removal of G and arrestin during the chromatography was previously characterized by immunoblot analysis (16). Since the final preparation of rhodopsin kinase (this table) contained about 18 µg of the 62-kDa kinase (Fig. 3), the preparation is estimated to be greater than 50% pure with respect to total protein.
SDS-acrylamide gels. The phosphorylation of rhodopsin kinase was independent of enzyme dilution and occurred at similar rates when the purified enzyme fractions were diluted more than 1,000-fold (not shown). This observation indicated that rhodopsin kinase phosphorylation was an intramolecular reaction and represents an autophosphorylation.

Fig. 2 shows the time course of rhodopsin kinase autophosphorylation. At early times (3–6 min) a 62- to 64-kDa phosphorylated doublet was readily detected, while longer incubations resulted in the conversion to a predominant 64-kDa phosphorylated protein. The autophosphorylation occurred in the absence (Fig. 2A) or presence (Fig. 2B) of rhodopsin, although the generation of phosphorylated rhodopsin dimer frequently made the time points longer than 9–12 min impossible to analyze (Fig. 2B). However, with different rhodopsin preparations the 64-kDa band was always present after short phosphorylation incubations (see Fig. 1, A and B). When the autophosphorylation reaction was performed on ice (0 °C) for 85 min the phosphorylated rhodopsin kinase migrated predominantly as a 62-kDa band (Fig. 2C) with both 63- and 64-kDa bands evident. Thus, the shift in mobility from 62 to 64 kDa resulted from the phosphorylation of rhodopsin kinase that involved an intermediate conformation of 63 kDa that could be trapped by performing the incubation at 0 °C. This may suggest more than one phosphorylation site on rhodopsin kinase altering the interaction of SDS with the polypeptide, but this has not yet been determined unequivocally. Nonetheless, no evidence for a change in rhodopsin phosphorylation activity was detected resulting from the autophosphorylation of rhodopsin kinase (Fig. 2D). Whereas the rhodopsin kinase phosphorylation appeared complete within 9–12 min, no change in the rate of rhodopsin phosphorylation was detected over a 40-min time course with a rhodopsin concentration 2.5-fold greater than its apparent K_m for rhodopsin kinase, indicating that the kinase phosphorylation did not significantly alter the activity of the enzyme.

Additional evidence that the 64-kDa band was derived from the 62-kDa band is shown in Fig. 3. Lane 4 of Fig. 3A shows the Coomassie-stained 62-kDa rhodopsin kinase band eluted from blue-Sepharose. When this preparation is incubated with 10 μM [γ-32P]ATP, its mobility shifts to a 64-kDa band (lane 5). B shows the autoradiograph of lanes 4 and 5 clearly demonstrating a predominant 64-kDa phosphoprotein with minor bands at 62 and 63 kDa. Phosphoamino acid analysis of the 64-kDa band showed that serine was phosphorylated in the rhodopsin kinase polypeptide under these conditions (C). Neither phosphothreonine or phosphotyrosine was detected in the analysis with different rhodopsin kinase preparations.

Two additional lines of experimental evidence indicated that the 62- to 64-kDa forms of rhodopsin kinase were functional and specifically bound to bleached rhodopsin. Fig. 4 shows that the phosphorylation of bleached rhodopsin is similar during a 3-min reaction whether or not the kinase is preincubated with ATP to convert the 62-kDa kinase to the 64-kDa form. During the 3-min kinase reaction approximately one-half of the kinase was converted to the 64-kDa form (Fig. 4A), but the phosphorylation of increasing concentrations of bleached rhodopsin was identical to that observed with the kinase preconverted to the slower migrating 64-kDa form identified on SDS-PAGE (Fig. 4B and C). This is consistent with the constant rate of rhodopsin phosphorylation over a 40-min time course (Fig. 3D), indicating neither the apparent rate nor K_m of the kinase was significantly altered by autophosphorylation. The apparent K_m of the fully phosphorylated enzyme was estimated to be 0.62 μM purified reconstituted rhodopsin compared to 0.64 μM for the minimally phosphorylated kinase. Consistent with these findings was the observation that both the 62- and 64-kDa forms of rhodopsin kinase bind to bleached but not unbleached rhodopsin (Fig. 5). Thus, rhodopsin kinase autophosphorylation alters the behavior of the kinase polypeptide on SDS-PAGE but does not have a dramatic effect on the kinetics of rhodopsin phosphorylation.

We had previously demonstrated that the purified rhodops-
Phosphorylation of vesicle-reconstituted rhodopsin by purified rhodopsin kinase. Rhodopsin kinase after chromatography on DEAE-cellulose and blue-Sepharose (see "Experimental Procedures," Fig. 3, and Table I) was incubated with 100 μM [γ-32P]ATP (5000 cpm/pmol) for 0–40 min at room temperature in the absence (A) or presence (B) of 1.45 μM rhodopsin. The 100-μl assay volume contained 0.1 μg of rhodopsin kinase (1.8 pmol). The reaction was initiated by simultaneous addition of [γ-32P]ATP and transfer of the samples from ice water and dim red light to room temperature and room white light illumination. Some samples were kept in the dark at room temperature, and other sets contained 10 mM NaF to inhibit potential phosphatase activity (see text and D). Reactions were terminated by the addition of SDS sample buffer followed by SDS-PAGE, staining, drying, and autoradiography. The autoradiographs shown were from a 16-h exposure (A) and 10-h exposure (B). Kinase autophosphorylation appears independent of the presence of rhodopsin and rhodopsin phosphorylation. C shows an autoradiograph of the autophosphorylated kinase incubated in ice water to allow the reaction. Note that the most intense phosphorylation is associated with the 62-kDa band and that an additional labeled band located between the 62- and 64-kDa bands is visible. Longer incubations of 3–4 h resulted in the 64-kDa band becoming predominant with a corresponding diminution of the 62- and 63-kDa bands (not shown). In D, phosphorylation of rhodopsin plotted as a function of time at room temperature was estimated in the absence (○) or presence (□) of the phosphatase inhibitor NaF (10 mM) from Cerenkov counts of the excised rhodopsin monomer bands (54 kDa). The data from duplicate assays containing 1.6 pmol of rhodopsin kinase and 145 pmol of rhodopsin/100 μl were averaged, and the mean values were expressed as picomoles of [γ-32P]phosphate incorporated/145 pmol of rhodopsin (RHO).

Purified Rhodopsin Kinase

Sin kinase was biochemically and immunologically free of arrestin and Gα (16). This allowed for the analysis of rhodopsin sequences that were directly involved in the binding of rhodopsin kinase to the purified, vesicle-reconstituted bleached photoreceptor. Table II lists nine peptides corresponding to specific cytoplasmic sequences of rhodopsin that were synthesized and tested as either substrates for rhodopsin kinase or for their ability to influence the phosphorylation of bleached rhodopsin. The CT peptides correspond to specific sequences within the COOH-terminal tail of rhodopsin, whereas the L1-2, L1-4, L6-8, and L6-48 peptides correspond to sequences within the first, second, and third cytoplasmic loops of rhodopsin. A 10th peptide, EGFR, was a small basic peptide having a single threonine residue that is an excellent substrate for protein kinase C and was used as a control. Four of these peptides (CT1, CT4, CT7, and L6-8) were substrates for the purified rhodopsin kinase in the presence or absence of bleached rhodopsin (not shown).
The major peptide recently native sequenced. Table II). The peptide was found to be a substrate for rhodopsin kinase. The CT1 peptide contains the sequence SXTXXXKXE which is conserved within both the COOH terminus and loop 5-6 of rhodopsin (Table II). With peptide CT1 in particular, at least three major phosphorylated peptide species were detected after a 30-min incubation (not shown). This finding suggested CT1 as well as possibly the other peptides were phosphorylated at multiple sites similar to that observed with rhodopsin. Phosphoamino acid analysis of rhodopsin kinase phosphorylated peptides CT1, CT3, and Ls-6 indicated phosphoserine and phosphothreonine in each peptide (not shown).

Similar to the findings of Palcewski et al. (15), four peptides (CT1, Ls-6, Ls-6, and Ls-6b) of the nine tested were found to inhibit rhodopsin phosphorylation specifically (Fig. 6). Of these, CT1 and Ls-6 are also phosphorylated by rhodopsin kinase, although Ls-6b is a poor substrate. Interestingly, peptide CT4 did not inhibit rhodopsin phosphorylation at concentrations where it served as a substrate for rhodopsin kinase. Table III summarizes the quantitation of the effect of each peptide on rhodopsin kinase-dependent phosphorylation of bleached rhodopsin. Loop 5-6 and CT1 peptides at 1 mM final concentration inhibit to the greatest extent, while Ls-6b and Ls-6 gave weaker inhibition of rhodopsin phosphorylation. Peptide CT4 appeared to actually give reproducible small stimulations of rhodopsin phosphorylation, but the effect was not statistically significant. The results indicate that peptides CT4, CT5, CT6, and CT1 (not shown) do not inhibit rhodopsin kinase-dependent phosphorylation of bleached rhodopsin even though they either overlap or are within the CT1 sequence. It should be noted that Palcewski et al. (15) found that a peptide corresponding to CT4 at a 3 mM concentration caused a 22% inhibition of rhodopsin phosphorylation by rhodopsin kinase. At 1 mM CT4 peptide we did not observe this inhibition. The unique amino acids in CT1 relative to the other CT peptides are KNP corresponding to residues 325-327 of the opsin polypeptide sequence, suggesting that either these 3 residues are major recognition determinants for rhodopsin kinase or they confer the proper conformation on additional residues in the peptide that are important for this interaction. At present, we have been unable to resolve this question with synthetic peptides. Peptide Ls-6b, which encodes the entire predicted second cytoplasmic loop of opsin, has no apparent homology in the primary sequence or predicted secondary structure with either peptides CT1 or Ls-6. Furthermore, the Ls-6 net charge at pH 7 is +1 compared to -2 for CT1, indicating that the inhibition observed with Ls-6 is specific for the Ls-6 sequence and is not related to any apparent conservation in sequence or structure with the COOH-terminal peptide CT1. Peptide Ls-6 does have some homology with CT1. Within both peptides is the sequence SXTXXKXE and just NH2-terminal to this sequence is DDEA in CT1 and QQA in Ls-6. However, peptide CT4 also contains the sequence DEBSXTXXKXE and has no inhibitory effect on rhodopsin kinase-dependent phosphorylation of bleached rhodopsin. Peptide Ls-6b is partially inhibitory in the phosphorylation assay and overlaps with Ls-6 at its COOH terminus, with the sequence KAEEK suggesting this sequence within the third cytoplasmic loop is important in rhodopsin kinase recognition of bleached rhodopsin. Peptide Ls-6b had no effect on rhodopsin kinase phosphorylation activity nor did the highly basic EGFR peptide. Thus, inhibition of rhodopsin kinase phosphorylation of bleached rhodopsin was specific for certain opsin peptides that were not related in either net charge or ability to serve as kinase substrates.

**DISCUSSION**

We have developed a simple, rapid purification procedure for rhodopsin kinase. The enzyme is isolated predominantly

**FIG. 3. Purification of rhodopsin kinase as a 62-kDa protein that alters its mobility on SDS-PAGE when autophosphorylated at serine residues.** A, Coomassie-stained 10% acrylamide SDS-PAGE gel of rhodopsin kinase purified by sequential chromatography on DEAE-cellulose and blue-Sepharose. Lanes represent 100 µl of 1) membrane extract, 2) pooled DEAE-cellulose eluate, 3) blue-Sepharose flow-through, 4) pooled eluate from blue-Sepharose. Fractions are from the preparation shown in Table I. Lane 5 represents a 100-µl aliquot of the blue-Sepharose eluate shown in lane 4 that was incubated with 10 µM [γ-32P]ATP for 12 min at 24 °C prior to the addition of SDS-sample buffer. B, Autoradiograph of lanes 4 and 5 from A showing incorporation of 32P into rhodopsin kinase and mobility shift to a 64-kDa band. C, Phosphoamino acid analysis of 64-kDa band labeled in B. Mobility of internal standards phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) after flat-bed electrophoresis is indicated by arrows.

5 mM or greater, which is at least 100-fold lower affinity than native bleached rhodopsin. Similar low affinities for rhodopsin synthetic peptides as substrates for rhodopsin kinase was recently described by Palcewski et al. (15). CT1 is the smallest peptide we have found to be a substrate for rhodopsin kinase. It causes bleaching.
as a 62-kDa polypeptide, but it is rapidly converted to a form that migrates as a 64-kDa band on SDS-PAGE as a result of what appears to be autophosphorylation at serine residues. Rhodopsin kinase phosphorylation was found to be independent of dilution indicating autophosphorylation and not a transphosphorylation of the kinase. No significant change in $K_m$ or rate of rhodopsin phosphorylation was detected resulting from autophosphorylation, suggesting the autophosphorylation was probably not a major regulatory mechanism for control of kinase activity. Phosphorylation of synthetic peptides corresponding to the COOH terminus and loop 5-6 of opsins is consistent with the known phosphorylation sites on the photoreceptor polypeptide (1-8). The peptides are poor substrates in terms of $K_m$ for rhodopsin kinase. This finding is similar to that of Palczewski et al. (15) who found that COOH-terminal and loop 5-6 peptides had high $K_m$ values as substrates for rhodopsin kinase. We found that the peptides were substrates even in the presence of rhodopsin and indicated that, when the kinase was bound to bleached rhodopsin, the peptides could still serve as substrates. This observation indicates that the kinase binds bleached rhodopsin at sites on the opsin polypeptide that are at least in part distinct from the sites of phosphorylation. Similar conclusions were recently proposed by Palczewski et al. (15).

Consistent with the hypothesis that multiple opsin sequences are required for proper binding of rhodopsin kinase and orientation of its active site relative to the COOH-terminal serine/threonine-rich phosphorylation region of the photoreceptor was the observation that peptides CT1, L5-6, L5-6, and L5-6 inhibited phosphorylation of bleached rhodopsin. Peptides CT, and L5-6 are substrates for rhodopsin kinase, whereas L5-6 and L5-6 are not substrates, but nonetheless inhibit rhodopsin phosphorylation. The regions in opsin (28) corresponding to these peptides (4) and sequences in the adrenergic (29-32) and muscarinic (33) receptors have been predicted to be major determinants for G-protein recognition and activation. This is particularly true for sequences within L5-6 and L5-6. In fact, site-directed mutagenesis of the $\beta_2$-adrenergic receptor defined amino acids in the second and third cytoplasmic loops (L5-6 and L5-6) and the NH2-terminal region of the cytoplasmic tail (CT) near the membrane that dramatically inhibited Gs activity (31). In rhodopsin, it was shown that mutation of Lys-245---Leu in the third cytoplasmic loop (L5-6) inhibited the ability of the bleached photoreceptor to activate Ga, the retinal G-protein (28). This residue is within both L5-6 and L5-6 peptides, both of which were effective in inhibiting rhodopsin kinase phosphorylation of bleached rhodopsin.

The overlap of G, and rhodopsin kinase recognition sites on bleached rhodopsin has been demonstrated by direct competition experiments using resolved and highly purified preparations of rhodopsin kinase and the $\alpha$- and $\beta_2$-subunits of Gi (16). The L5-6, L5-6, L5-6, and CT1 peptides that block rhodopsin kinase map the sites that are potentially similar to

![Diagram](image-url)
recognition sites for Gt to specific regions of the opsin polypeptide. In fact, Konig et al. (35) have also shown that loops 3-4, 5-6, and carboxyl terminus of opsin are involved in Gt interactions. As yet we have not succeeded in using peptides corresponding to opsin sequences to discriminate between α and βγ recognition sites on bleached rhodopsin; however, Ls-α and Ls-αγ-like peptides weakly activate Gt similarly to the action of mastoparan on other G-proteins (34).

In summary, these studies distinguish opsin sequences that serve as substrates versus recognition binding sites for rhodopsin kinase. The similarity in domains involved in rhodop-
kinase and G-protein recognition (35) suggests that the
two proteins probably have conserved tertiary structural do-
mains involved in the recognition of bleached rhodopsin.
Therefore, multiple sites on the receptor surface are involved
in recognition of receptor kinases as well as G-proteins. It is
necessary to understand the three-dimensional orientation of
these sequences in the receptor before the catalytic and reg-
ulatory properties of G-protein-coupled receptors is under-
stood.

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