Cooperativity during Multiple Phosphorylations Catalyzed by Rhodopsin Kinase: Supporting Evidence Using Synthetic Phosphopeptides†

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ABSTRACT: Rhodopsin kinase is a key component in the shutdown of visual transduction. The phosphorylation of rhodopsin's C-terminus was evaluated using synthetic peptides derived from the last 12 amino acids (337–348) as substrates and their phosphorylated counterparts as inhibitors. It was found that synthetic peptides were phosphorylated at the serine residue corresponding to Ser-343 in the primary sequence of bovine rhodopsin. The phosphopeptides were prepared by incorporating into the peptide chain a trityl-protected serine derivative at the site destined to contain the phosphoryl group. The trityl group was selectively released with 20% (v/v) dichloroacetic acid; the free hydroxyl group was then phosphitylated with di-*tert*-butyl *N,N*-diethylphosphoramidite, and the resulting phosphite derivative was oxidized with *m*-chloroperoxybenzoic acid. The phosphopeptides were found to have a greater affinity for the kinase compared with their nonphosphorylated counterparts; for the peptides corresponding to residues 337–348 of rhodopsin the affinity increased in the order VSKTETSQVAPA < VSKTETS[PO_3H_2]QVAPA < VS-[PO_3H_2]KTETS[PO_3H_2]QVAPA. The results are interpreted to support the cooperativity hypotheses proposed previously [Wilden, U., & Kühn, H. (1982) *Biochemistry 21*, 3014–3022; Aton, B. R., Litman, B. J., & Jackson, M. L. (1984) *Biochemistry 23*, 1737–1741].

The function of several receptor systems and the activities of a large number of enzymes are regulated by a phosphorylation–dephosphorylation mechanism involving specific kinases and phosphatases. The compounds which interfere with the activity of the latter class of enzymes, apart from producing profound biological effects, have proved invaluable tools in the elucidation of cellular mechanisms. For example, the oncogenic effects of phorbol esters are attributed to the stimulation of the activity of protein kinase C, while the diarrhetic effect of the causative agent in seafood poisoning (okadaic acid) is due to the inhibition of protein phosphatase-(s) which maintains the components of the contractile system in a phosphorylated state (Cohen et al., 1990). The inhibitors of the type cited above were discovered serendipitously and pose a challenge to the ingenuity of a biochemist to design their "user-friendly" counterpart by applying the knowledge of enzymology. We have addressed this problem in the vision field, which is prototypical of G-protein-coupled-receptor systems.

Rhodopsin, following absorption of light, is converted into an activated form, metarhodopsin II (symbolized as Rho*), that participates in the transmission of the visual signal. The signal is then terminated (McDowell & Kühn, 1977) by the action of rhodopsin kinase (Bownds et al., 1972; Kühn et al., 1973; Frank & Buzney, 1975), which phosphorylates several serine and threonine residues in the C-terminal domain of Rho* (Sale et al., 1978; Hargrave et al., 1980; Wilden & Kühn, 1982; Thompson & Findlay, 1984). We recently made the unexpected discovery that several peptides corresponding to the C-terminal sequence of rhodopsin are phosphorylated by rhodopsin kinase [(Fowles et al., 1988; Brown et al., 1992; also see Palczewski et al. (1988), Onorato et al. (1991), and Palczewski et al. (1991)], at rates which are about 10% that of the native receptor, but only in the presence of bleached rhodopsin. The mechanistic implications of these findings have been discussed elsewhere (Fowles et al., 1988; Brown et al., 1992), but in the present context these observations highlighted the fact that relatively small peptides can deputize for the large receptor molecule, though their affinities for the kinase are 3 orders of magnitude lower than that of the native receptor. We anticipated that the phosphorylated counterparts of these peptides may have useful properties, and in this paper we report a new method for the synthesis of such phosphopeptides, which were then used to study their interactions with rhodopsin kinase.

EXPERIMENTAL PROCEDURES

Materials. (Fmoc-L-alanyl)[4-(hydroxymethyl)phenoxy]-methyl copolymer of styrene–1% divinylbenzene was purchased from Bachem. Castro's reagent, (benzotriazol-1-yl)oxytris(dimethylamino)phosphonium hexafluorophosphate, and N^{α}-Fmoc amino acids, with appropriate side-chain protection to be removed with 95% trifluoroacetic acid (TFA)/H_2O, were purchased from Novabiochem; N^{α},N^{ϵ}-bis(*tert*-butoxycarbonyl)-L-lysine and N^{α}-(*tert*-butoxycarbonyl)-L-valine were purchased from Sigma; and all other chemicals were acquired from Aldrich or BDH. Di-*tert*-butyl-*N,N*-diethylphosphoramidite and dibenzyl *N,N*-diethylphosphoramidite were prepared by small modifications to published methods (Perich & Johns, 1988; Andrews et al., 1991).

Optiphase "Hisafe" 3 was obtained from LKB, and [γ-^{32}P]-ATP and NCS tissue solubilizer were obtained from Amersham. All other chemicals were obtained from Sigma, BDH, or Interchem.

Bovine retinae were collected from a local slaughterhouse (FMC Ltd., Salisbury, Wiltshire, U.K.).

Phosphopeptide Synthesis. (Fmoc-L-alanyl)[4-(hydroxymethyl)phenoxy]methyl copolymer of styrene–1% divinyl-

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benzene (200 mg containing 0.5 mmol/g Fmoc-alanine) was used for the assembly of the peptide chain, using the protocol described by Atherton and Sheppard (1989). The successive addition of Fmoc amino acids and finally an N-terminal butoxycarbonyl-protected amino acid was catalyzed by using Castro's reagent (Le-Nguyen et al., 1985) with hydroxybenzotriazole in dimethylacetamide. Couplings were generally completed in less than 40 min (monitored with ninhydrin), and the Fmoc group was removed by two treatments with 20% (v/v) piperidine in dimethylacetamide (3 and 7 min). After the synthesis of the parent peptide, the trityl group was selectively removed by shaking with 20% (v/v) dichloroacetic acid in methylene chloride. The release of the trityl cation group, followed by measuring incubation aliquots at 432 nm over the time course of the incubation, serves as a monitor for the availability of a free hydroxyl. Under these conditions the trityl protecting group was completely removed in less than 5 min. The excess acid was exhaustively washed from the peptidyl resin, and the free hydroxyl group was modified by three successive treatments with di-*tert*-butyl *N*,*N*-diethylphosphoramidite (249 mg, 1 mmol) and tetrazole (280 mg, 4 mmol) in dimethylacetamide (90 min) at room temperature. The phosphite-protected seryl residue was oxidized with *m*-chloroperoxybenzoic acid (345 mg, 2 mmol) in methylene chloride for 1 h, and the peptidyl resin was washed with *tert*-amyl alcohol (3 × 2 min), glacial acetic acid (2 × 2 min), *tert*-amyl alcohol (2 × 2 min), and ether (2 × 1 min). Cleavage was carried out with 95% TFA/H_2O for 2.5 h, and the liquors were reduced in vacuo. The oily residue was resuspended in AnalaR H_2O (20 mL) and lyophilized overnight to yield a white solid, which was desalted on Sephadex G-25 with 0.1% TFA/H_2O.

In the synthesis of phosphopeptide **1b** (Table I), the peptidyl resin (120 mg) generated a solid (58 mg) following gel filtration. An analytical HPLC profile of the solid is shown in the inset to Figure 1A. Five milligrams of the preceding solid, when subjected to C_8-analytical HPLC with a linear gradient of 0.1% TFA in acetonitrile, gave KTETS[PO_3H_2]-QVAPA (3.5 mg; overall yield 64%). The analytical HPLC of the purified phosphopeptide along with the ion-spray mass spectrum is presented in Figure 1A.

In the synthesis of phosphopeptide **2b**, the peptidyl resin (164 mg) after cleavage and gel filtration yielded a crude solid (72 mg; HPLC profile in inset to Figure 1B), a portion of which (13 mg) was purified to homogeneity by HPLC using a preparative C_{18} column (4.1 mg; 30% yield). The purity of the resulting phosphopeptide, VSKTETS[PO_3H_2]QVAPA, was corroborated with analytical HPLC, ion-spray mass spectrometry (Figure 1B), and amino acid sequencing.

For the synthesis of phosphopeptide **2c**, the peptidyl resin (141 mg) yielded a solid (54.3 mg), which gave the analytical HPLC profile represented by the inset to Figure 1C. An aliquot of this crude product (16.6 mg) was purified using preparative HPLC and yielded VS[PO_3H_2]KTETS[PO_3H_2]-QVAPA (4.0 mg; overall yield 24%), which was shown to be homogeneous by analytical HPLC profile and ion-spray mass spectrometry (see Figure 1C).

It is noteworthy that dibenzyl *N*,*N*-diethylphosphoramidite could be used to perform the phosphitylation reaction and yet, contrary to published work (Andrews et al., 1991), the yield of phosphopeptide was not any better than if the di-*tert*-butyl analogue were used. Other methods in the literature have utilized an unprotected hydroxyl group and subsequent postassembly phosphitylation and oxidation for the preparation of phosphopeptides (de Bont et al., 1990; Andrews et al., 1991; Staerkaer et al., 1991).

The analogous nonphosphorylated peptides were prepared using similar protocols to that above, but without subsequent postassembly phosphitylation and oxidation. In all cases the nonphosphorylated peptides were purified to homogeneity on HPLC, and their purity was substantiated with high-voltage thin-layer electrophoresis and FIB mass spectrometry.

Phosphorylation of Rhodopsin. Rod outer segments (ROS) from 100–140 bovine retinae were purified by sucrose density gradient as described previously (Fowles et al., 1988) and resuspended in 100 mM potassium phosphate buffer, pH 7.0, 2 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol. An aliquot of the ROS (5 μL) containing 2.4×10^{-4} M rhodopsin (calculated from $\epsilon_{500} = 4 \times 10^4$ M^{-1} cm^{-1}) was used throughout for the phosphorylation experiments. The final incubation (50 μL) contained 2.4×10^{-5} M rhodopsin, 2.4–3 mM [γ-^{32}P]ATP (50 000–150 000 cpm/nmol), and 0–3 mM peptide in phosphate buffer. After being mixed under red safelight conditions, the samples were sonicated for 1 min in a Decon FX100 sonication bath and then incubated at 30 °C for 5 min in a thermostat-controlled water bath to equilibrate. The phosphorylation reactions were initiated by illumination with continuous white light (150-W photoflood lamp at a distance of 30 cm). Dark controls were incubated while wrapped in foil. Under these conditions, 100% bleaching, as determined by the decrease in absorbency at 500 nm, was achieved within 10 min. ROS phosphorylation was determined using a modification of the method described by Kühn and Wilden (1982b). At the appropriate times, 5-μL aliquots were placed in 200 μL of 20% (w/v) trichloroacetic acid (TCA) at 0 °C. After 10 min the ROS were recovered by pelleting in an Eppendorf centrifuge at 10000*g* for 2 min, and the supernatant was discarded. The pellets were washed twice more in 500 μL of 10% (w/v) TCA containing 10 mM KH_2PO_4 at 0 °C for 5 min by the same method, and the final pellet was resuspended in 100 μL of NCS tissue solubilizer and incubated for 10 min at room temperature. Incorporated ^{32}P was determined by liquid scintillation counting in 900 μL of Optiphase "Hisafe" 3 using a Phillips PW470 liquid scintillation counter.

Phosphorylation of Peptides. Peptide incubations were performed as for the rhodopsin phosphorylation above, and some of the phosphate buffer was replaced with the appropriate peptide (1.25–15 μL of a 10 mM stock in phosphate buffer) to obtain a final concentration of 0.25–3 mM. At the appropriate times, aliquots (10–40 μL) were centrifuged in an Eppendorf centrifuge, and 5 μL of the supernatants was applied to a cellulose TLC plate and subjected to high-voltage electrophoresis using a pyridine/acetic acid/water (1:10:89) buffer, pH 3.5, at 7 °C at 1000 V for 2.5 h. Under these conditions, phosphopeptides as well as their unphosphorylated counterparts migrated from the positive terminal to the negative one, the unphosphorylated peptides migrating further. The plates were oven dried and autoradiographed at −70 °C for 15–20 h using Fuji NIF RX X-ray film. After the film was developed, enabling bands of phosphopeptide to be seen, these bands of cellulose were scraped off the plate and, after addition of 5 mL of Optiphase "Hisafe 3" and mixing, were analyzed by scintillation counting.

Pulse Liquid-Phase Sequencing of Peptides Phosphorylated by Rhodopsin Kinase. Aliquots (40 μL) of peptide incubations were subjected to high-voltage electrophoresis; the plates were air dried and then autoradiographed as described above. The phosphopeptide bands were scraped off, and the phosphopeptides were eluted with water and lyophilized. The phosphopeptides were sequenced on an Applied Biosystems 477A protein sequencer with an Applied Biosystems 120A

analyzer. Phosphopeptide (1 nmol) was covalently linked via the C-terminus to a Sequelin AA membrane (Milligen, U.K.), and anilinothiazolinone (AZT) amino acid derivatives were extracted with trifluoroacetic acid/methanol and analyzed by scintillation counting in Optiphase "HiSafe" 3 (5 mL).

RESULTS

Synthesis of Phosphopeptides. The key feature of our strategy for the synthesis of phosphopeptides was the use of the *O*-trityl derivative of serine, which was incorporated into the peptide chain using the Fmoc methodology. Following the completion of the assembly, the trityl group was removed with 20% (v/v) dichloroacetic acid in methylene chloride. Under these conditions the *tert*-butoxycarbonyl protecting group of the N-terminal amino acid was completely undisturbed. Although we were unable to monitor *on the resin* the effect of the dichloroacetic acid treatment on the stability of the *tert*-butyl groups used for the protection of both hydroxyl and carboxyl groups, it is well-known that the *tert*-butyl ethers and esters have greater acid stability than the *tert*-butoxycarbonyl group. It is therefore reasonable to assume that in the mild acid conditions under which the *tert*-butoxycarbonyl group is stable the other protecting groups used in the Fmoc methodology will also be stable. The support for this conclusion was provided by treatment of solutions of N^{α}-Fmoc-O^{β}-*tert*-butyl-L-serine and N^{α}-Fmoc-O^{δ}-*tert*-butyl-L-glutamic acid in 20% dichloroacetic acid in methylene chloride for 30 min when no hydrolysis was observed.

The serine hydroxyl group thus unmasked was phosphitylated with di-*tert*-butyl *N*,*N*-diethylphosphoramidite, and then the phosphite derivative was oxidized with *m*-chloroperoxybenzoic acid (Perich & Johns, 1988). The release of the peptide chain from the resin and the removal of all the protecting groups was achieved in a single step using 95% (v/v) TFA/H_2O. Three phosphopeptides, containing phosphoryl groups on positions which correspond to some sites of kinase action in the native receptor, were prepared with synthetic yields of 35–95%. The corroborative data is presented in Figure 1 and summarized in Table I.

The method described above allows the preparation of phosphopeptides using a protocol which retains all the attractive features of solid-phase peptide synthesis. The Fmoc-*O*-tritylserine could be incorporated into a wide range of peptide sequences without the need to alter the coupling conditions. Following the assembly of the peptide, the trityl group was also removed without posing any difficulty. The phosphitylation reaction, however, seemed to depend on the position of the seryl residue and the length of the peptide. In general we experienced no difficulty in the synthesis of phosphopeptides up to 12 residues long. Beyond this two problems became evident: the extent of phosphorylation of the peptide was low, but more unfortunately, longer phosphopeptides seemed to separate on HPLC poorly from their unphosphorylated counterparts, making the isolation of homogeneous compounds a laborious and uncertain task. Further improvements in the methodology are required to circumvent these problems.

Inhibitory Studies with Phosphopeptides. It is known that dark-adapted rhodopsin is resistant to the action of rhodopsin kinase but becomes an effective substrate following photoactivation (Bownds et al., 1972; Kühn et al., 1973; Frank & Buzney, 1975). Likewise, the phosphorylation of synthetic peptides corresponding to the C-terminal domain of rhodopsin by the kinase is 20–100-fold enhanced in the presence of photoactivated rhodopsin (Fowles et al., 1988; Palczewski et al., 1991; Brown et al., 1992). Rod outer segments (ROS), containing dark-adapted rhodopsin, as well as rhodopsin kinase, [γ-^{32}P]ATP, and 10-mer **1a** were incubated with appropriate controls by using the protocol described previously, and at various time intervals samples were removed to monitor peptide and protein phosphorylation. Figure 2A confirms the light-dependent phosphorylation of the two species (Rho* and the 10-mer) and also shows that, under the conditions of the experiment, peptide phosphorylation occurred at a rate that was 10% that of the native substrate. Furthermore, in the presence of 3 mM 10-mer **1a**, the rhodopsin phosphorylation was not significantly inhibited, which is not surprising since the affinity of the peptide for the kinase is about 6 mM compared to 3 μM for rhodopsin (Palczewski et al., 1988; Brown et al., 1992). In contrast with this result, Figure 2B shows that in the presence of 3 mM monophospho-10-mer **1b** the rhodopsin phosphorylation was inhibited to the extent of 37%.

Figure 2C shows a similar experiment in which the effects of 12-mer **2a** as well as its mono- and diphospho derivatives (**2b**, **2c**) on the phosphorylation of rhodopsin were studied, and it was found that the inhibitory potencies of these compounds increased with the extent of phosphorylation. Thus the parent peptide was without any effect, while its mono- and diphospho derivatives were significantly inhibitory.

Next the phosphorylation of the 12-mer in the presence of various concentrations of its mono- and diphospho derivatives was studied. Figure 3A shows that in the presence of 3 mM phosphopeptides (**2b** and **2c**) the phosphorylation of the 12-mer was inhibited by 70–85%. It should be noted that under these conditions the further phosphorylation of the phosphopeptides by the kinase was detected, but its level was too low for unambiguous characterization. Figure 4 shows that in synthetic peptides the residue corresponding to Ser-343 of rhodopsin is preferentially phosphorylated by rhodopsin kinase: in the phosphopeptides used in the present work, it is this residue that contains the phosphoryl group. In Figure 3B, another set of experiments in which the phosphorylation of the 10-mer **1a** was studied in the presence of monophospho-10-mer **1b** gave a profile similar to that obtained with 12-mer **2a**. The approximate K_i values for the phosphopeptides were calculated from the experimentally determined IC_{50} values according to the equation (Cheng & Prusoff, 1973)

$$K_i = \frac{IC_{50}}{1 + [s]/K_m}$$

It was found that compared to the K_m for the 12-mer **2a** of 7 mM the K_i values were 0.81 and 0.55 mM for the mono- and diphospho-12-mers (**2b** and **2c**), respectively. A similar improved affinity was evident when the comparison was made between 10-mer **1a** and monophospho-10-mer **1b**. See Table I.

It could be supposed that this improved affinity of rhodopsin kinase for the phosphorylated peptides would mean that these peptides were preferred as substrates to their unphosphorylated counterparts. However, we have discovered negligible phosphorylation of the phosphopeptides by rhodopsin kinase. In earlier work (Brown et al., 1992) we have shown that the ability of a peptide to bind to rhodopsin kinase does not necessarily coincide with its effectiveness as a substrate. A peptide that closely mimicked the C-terminal domain of rhodopsin, comprising the final 20 amino acids of the sequence, was found to effectively inhibit the phosphorylation of the best peptide substrate, the final 11 amino acids of the sequence, while itself being negligibly phosphorylated. Such observations provide support for the intuitively held view that whereas a myriad of closely related molecular interactions between a

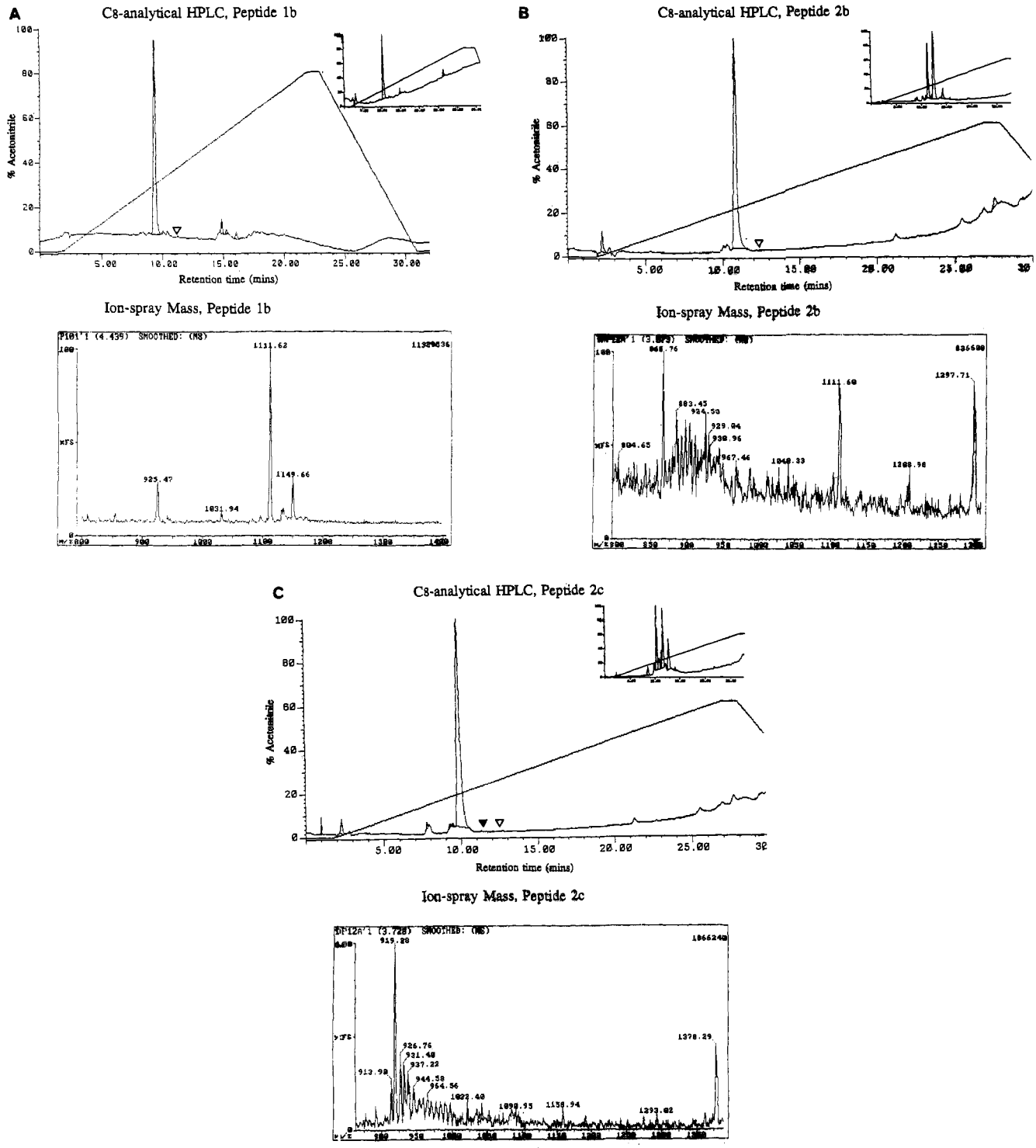

FIGURE 1: HPLC and ion-spray data for the synthetic phosphopeptides. C_8-analytical HPLC was performed on purified monophospho-10-mer **1b**, monophospho-12-mer **2b**, and diphospho-12-mer **2c** and is represented by the corresponding profiles A, B, and C (the insets are the HPLC profiles obtained from the crude products, prior to their purification). The symbols identify the positions to which the nonphosphorylated product (▽) and, in the case of phosphopeptide **2c**, the monophosphorylated product (▼) would have eluted in the impure mixture. Ion-spray mass analyses are shown for each HPLC profile. For phosphopeptide **1b**, the chelation of potassium led to the observation of a 1150 mass in the sample. For phosphopeptide **2b**, the peak at 1112 corresponding to the mass of phosphopeptide **1b** is entirely coincidental, since amino acid sequencing gave a single N terminal residue (Val) and failed to identify the presence of any phosphopeptide **1b** as an impurity.

flexible ligand and an enzyme may lead to the formation of inhibitory complexes, only one unique binding mode is needed to produce a catalytically competent complex.

That the phosphopeptides were not used as substrates by rhodopsin kinase means that their binding interactions with the enzyme lack the exquisite precision required for catalysis. The phosphopeptides were, however, the products of the kinase reaction, and this fact dictates that these bind to the active-site of the enzyme, though such a binding mode would not lead these to undergo a second round of phosphorylation. For this to occur, a rearrangement of the enzyme–phosphopeptide binary complex would be required so that the hydroxyl group of a new amino acid residue becomes available for phosphorylation. Such a state of affairs must operate when the catalysis involves the native substrate (that is, bleached rhodopsin), and it is in this respect that the synthetic peptides used in the present work differ from their physiological counterpart. The fact that despite this handicap the phosphopeptides showed

Table I: Affinity of Rhodopsin Kinase for Different Peptides and Phosphopeptides[a]

assignment	peptide	yield (%)	M_r	K_i (mM)	K_m (mM)
1a	^{339}KTETSQVAPA^{348} (OH)		1032		6.0 ± 0.23
1b	^{339}KTETSQVAPA^{348} (OPO_2H_2)	90–95	1112	0.57 ● 0.021	
2a	^{337}VSKTETSQVAPA^{348} (OH, OH)		1218		7.0 ± 0.2
2b	^{337}VSKTETSQVAPA^{348} (OH, OPO_2H_2)	45–50	1298	0.81 ± 0.015	
2c	^{337}VSKTETSQVAPA^{348} (OPO_2H_2, OPO_2H_2)	35	1378	0.55 ± 0.017	

[a] The residue numbers quoted on the peptides relate to the corresponding sequences of bovine rhodopsin. Molecular masses were found from the ion-spray mass (see Figure 1), and the yield represents the percentage of the phosphopeptide contained within the final cleaved product. This figure was obtained from analytical HPLC data presented in Figure 1. The yields of the purified products are described in the Experimental Procedures. The K_i's for the phosphopeptides were calculated as described in the text.

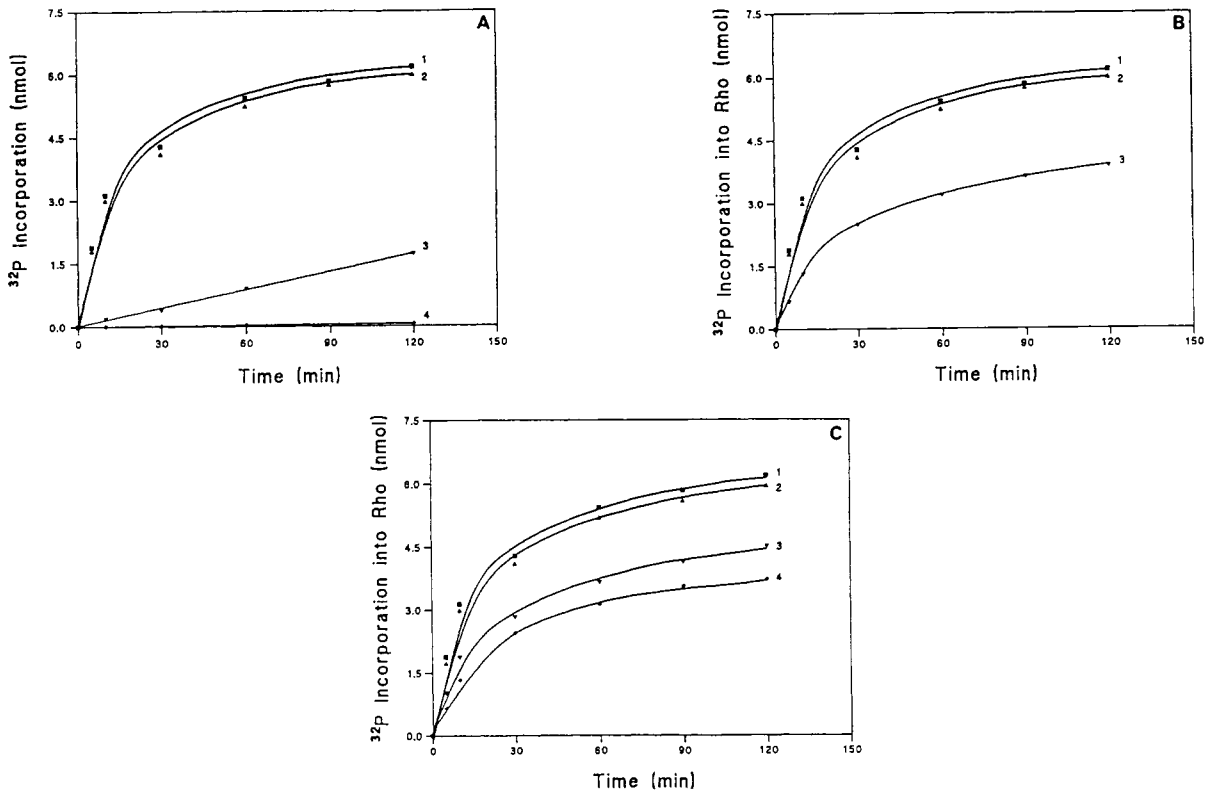

FIGURE 2: (A) Light-dependent phosphorylation of rhodopsin and the 10 mer. The incubations were performed as described in Experimental Procedures for 2 h at 30 °C under white light or in darkness in a total volume of 50 μL containing 3 mM 10-mer **1a**, 2.4×10^{-5} M rhodopsin in unextracted rod outer segments (which contain the endogenous kinase), and 2.6 mM [γ-^{32}P]ATP. At the indicated times, 5-μL samples were removed and processed for either rhodopsin or peptide phosphorylation as described in Experimental Procedures. Curve 1, rhodopsin phosphorylation in the absence of any peptide; curve 2, rhodopsin phosphorylation in the presence of 3 mM 10-mer **1a**; curve 3, phosphorylation of the peptide in the same incubation as for curve 1; and curve 4, phosphorylation of rhodopsin and peptide when both were present in the dark. The results shown are an average of several experiments where rhodopsin and peptide phosphorylation varied by less than 5%. (B) Phosphorylation of rhodopsin in the presence of 10-mer **1a** or monophospho-10-mer **1b**. The incubations were performed as described in Experimental Procedures in a total volume of 50 μL containing 3 mM peptide, 2.4×10^{-5} M rhodopsin in unextracted rod outer segments, and 2.6 mM [γ-^{32}P]ATP. The other details are the same as in (A). Curve 1, rhodopsin phosphorylation in the absence of any peptide; curve 2, rhodopsin phosphorylation in the presence of 10-mer **1a**; and curve 3, rhodopsin phosphorylation in the presence of monophospho-10-mer **1b**. The results shown are an average of several experiments where rhodopsin phosphorylation varied by less than 5%. (C) Phosphorylation of rhodopsin in the presence of 12-mer **2a**, monophospho-12-mer (**2b**), or diphospho-12-mer **2c**. The incubations were performed as described in Experimental Procedures in a total volume of 50 μL containing 3 mM peptide, 2.4×10^{-5} M rhodopsin in unextracted rod outer segments, and 2.6 mM [γ-^{32}P]ATP. The other details are the same as in (A). Curve 1, rhodopsin phosphorylation in the absence of any peptide; curve 2, rhodopsin phosphorylation in the presence of 12-mer **2a**; (curve 3) rhodopsin phosphorylation in the presence of monophospho-12-mer **2b**; and curve 4, rhodopsin phosphorylation in the presence of diphospho-12-mer **2c**. The results shown are an average of several experiments where rhodopsin phosphorylation varied by less than 5%.

higher affinity for the kinase than did the unphosphorylated peptide shows that the enzyme can favorably accommodate phosphorylated ligands, and we suggest that this property of the enzyme, when extended to the physiological substrate, may rationalize some interesting observations recorded in the literature and described below.

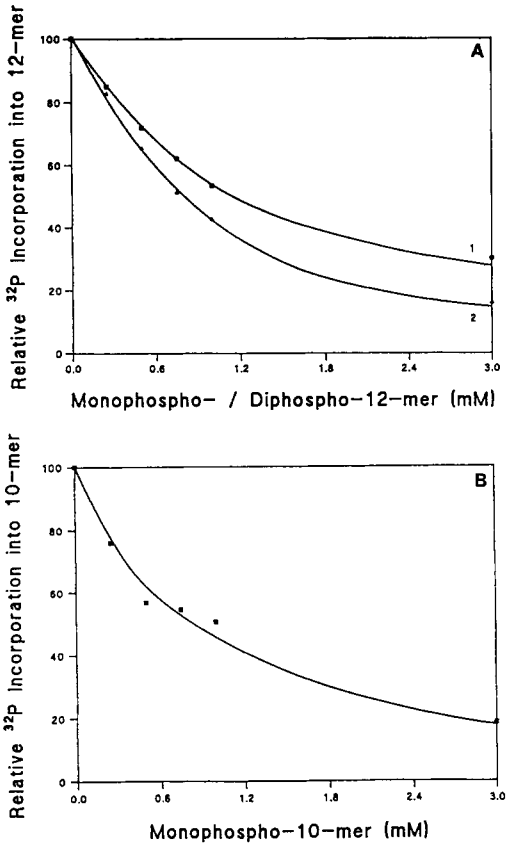

FIGURE 3: Phosphorylation of 12-mer **2a** in the presence of monophospho-12-mer **2b** or diphospho-12-mer **2c**. The incubations were performed as described in Experimental Procedures in a total volume of 50 μL containing 3 mM 12-mer **2a**, 0–3.0 mM monophospho-12-mer **2b** or diphospho-12-mer **2c**, 2.4×10^{-5} M rhodopsin in unextracted rod outer segments, and 2.6 mM [γ-^{32}P]ATP. The other details are the same as in Figure 2A. Curve 1, phosphorylation of 12-mer **2a** in the presence of monophospho-12-mer **2b**; curve 2, phosphorylation of 12-mer **2a** in the presence of diphospho-12-mer **2c**. The results shown are an average of several experiments where 12-mer **2a** phosphorylation varied by less than 7%. (B) Phosphorylation of 10-mer **1a** in the presence of monophospho-10-mer **1b**. The incubations were performed as described in Experimental Procedures in a total volume of 50 μL containing 3 mM 10-mer **1a**, 0–3.0 mM monophospho-10-mer **1b**, 2.4×10^{-5} M rhodopsin in unextracted rod outer segments, and 2.6 mM [γ-^{32}P]ATP. The other details are the same as in Figure 2A. The results shown are an average of several experiments where 10-mer **1a** phosphorylation varied by less than 7%.

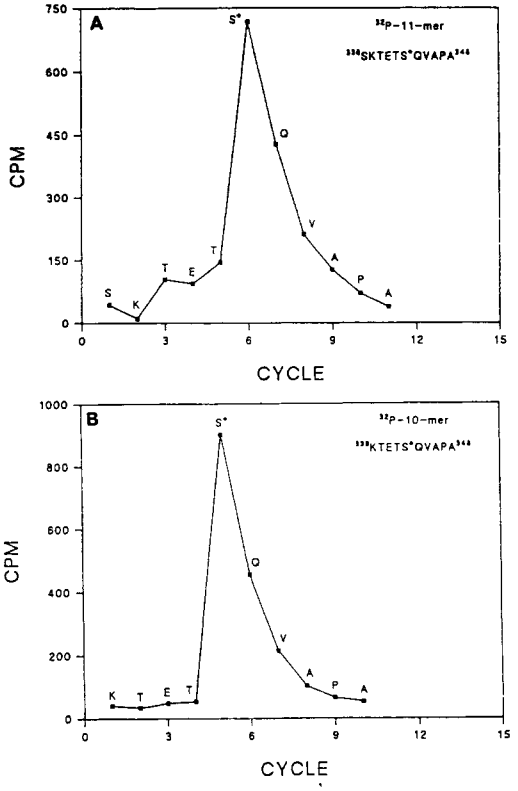

FIGURE 4: Pulse liquid-phase sequencing of two peptides phosphorylated by rhodopsin kinase: (A) 11-mer ^{338}SKTETS*QVAPA^{348} and (B) 10-mer ^{339}KTETS*QVAPA^{348}. In each case 1 nmol of the ^{32}P-labeled phosphopeptide obtained enzymically was sequenced as described in Experimental Procedures. S* is the residue associated with the release of the maximum amount of ^{32}P.

DISCUSSION

Recently the mechanism of action of rhodopsin kinase has been probed using synthetic peptides, and it was found that peptides corresponding to the C-terminal sequence of bovine rhodopsin were phosphorylated by the kinase (Palczewski et al., 1988; Onorato et al., 1991; Kelleher & Johnson, 1990), but the rate of this reaction was dramatically stimulated in the presence of photoexcited rhodopsin (Fowles et al., 1988, 1991; Palczewski et al., 1991; Brown et al., 1992). Our previous work has shown that in the Rho*-dependent phosphorylation system the best peptide substrates were those which contained the last 10–12 amino acids from the C-terminus of bovine rhodopsin (Brown et al., 1992). In the latter study indirect evidence was provided to show that the peptides were phosphorylated at the serine residue corresponding to Ser-343, and this feature has now been unambiguously established. In the light of this information the present study was performed, using two of the peptides which were found good substrates, and the phosphopeptides were designed to contain the phosphoryl group in the position (Ser-343) that is preferentially modified by the kinase.

The kinetic data now obtained are used to examine the multiphosphorylation pattern observed when rhodopsin kinase acts upon Rho*. It is not known whether uniquely phosphorylated intermediates are formed during the modification process, but if these are, they cannot be isolated in a form suitable for critical kinetic analysis. Notwithstanding this, there are some intriguing observations already recorded in the literature which pose interesting mechanistic questions. For example, Wilden and Kühn (1982a) carried out a careful study in which the time course of the formation of variously phosphorylated species of rhodopsin by the kinase was investigated. It was found that between 3 and 5 min after the initiation of phosphorylation unphosphorylated bleached rhodopsin and its tetraphosphorylated derivative were the predominant species present in the mixture. At this juncture the levels of the two species were nearly identical and 3–4 times greater than of any other phosphorylated derivative [deduced from Wilden and Kühn (1982a), Figure 6]. Thus the lack of accumulation of mono-, di-, and triphosphorylated species when a significant amount of unphosphorylated substrate remained was a notable observation in the aforementioned paper. A few years later the same phenomenon was reinvestigated by Aton et al. (1984) using an improved method for the analysis of variously phosphorylated forms of rhodopsin, and these workers confirmed the essential features of the finding of Wilden and Kühn (1982a) and further noted that throughout the time-course study the proportion of the monophosphorylated species in the mixture was extremely low. A possible explanation for these findings offered by Wilden and Kühn (1982a) was that there is cooperativity in the process; that is, once a phosphorylated species is formed, there is a higher probability that this is used for further phosphorylation than the original unphosphorylated substrate. As pointed out by Aton et al. (1984), however, there remained

the possibility that the phosphorylation profile noted in these experiments was due to a nonuniform distribution of the kinase in the photoreceptor membranes which lead to facile accessibility of the kinase only to some of the bleached rhodopsin molecules, which were rapidly phosphorylated at all their phosphorylation sites.

The observations made in the present study are particularly relevant to the above debate. We have now shown that the affinities of the monophosphorylated peptides for the kinase are about 10 times higher than those of the corresponding unphosphorylated peptides. There is a further 50% improvement in affinity when a second phosphoryl group is present in the peptide.

If the information on relative affinities of the variously phosphorylated peptides is extended to the native receptor, then it would be expected that the monophosphorylated bleached rhodopsin, once formed, owing to its superior affinity, is removed by the kinase for further phosphorylation in preference to the unphosphorylated original substrate. The results reported here thus greatly bolster the cooperativity hypothesis so perceptively envisaged by Wilden and Kühn (1982a) a decade ago and reemphasised by Aton et al. (1984).

There is another implication of the present work which merits attention. That peptides of 10–12 amino acids when deputizing for a protein of M_r 40 000 do by necessity lose a great deal of binding interactions, reflected by the poor affinity of peptides **1a** and **2a** for the kinase, is not surprising. It is, however, interesting that the affinity is considerably improved by the addition of phosphoryl groups, as is seen with the diphospho-12-mer (**2c**). The knowledge of the amino acid sequences in the peptides preferred by the kinase and the contributions which anionic sites in the peptides made to binding interactions are important findings which could act as a launch pad for designing more effective inhibitors, particularly those in which naturally occurring amino acids are replaced by metabolically inert templates. In view of the close similarity which exists between rhodopsin kinase and β-adrenergic receptor kinase (Benovic et al., 1988), the approach initiated in the present work should merit extensions to the development of inhibitors for the pharmacologically desirable members of the G-protein-coupled-receptor family.

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