Rhodopsin Kinase: Studies on the Sequence of and the Recognition Motif for Multiphosphorylations†

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ABSTRACT: Peptides of 10−12 amino acids in length, which overlapped with the sequence of the last 20 amino acids in the C-terminal tail of rhodopsin, were synthesised and used as substrates for rhodopsin kinase. In all cases the phosphorylation of the peptides was found to be greatly stimulated (>20-fold) by the presence of light-activated rhodopsin (Rho*). The incorporation of ^{32}P at seven Ser/Thr residues that are the potential sites of phosphorylation was quantified, and the results were analyzed in terms of two parameters. First, a global comparison of phosphorylation at each site was made when the propensity for the modification was found to be in the order: Ser 343 > Ser 338 > Thr 336 > Ser 334, Thr 342 > Thr 335, Thr 340. Second, the peptides were aligned on a hypothetical template with the residue to be phosphorylated occupying the P-position, and the manner in which the nature of the surrounding resides effected the phosphorylation was assessed. It was found that the optimal phosphorylation of the P-site Ser/Thr occurs if it has at least one residue on the amino side and five on the acyl side and also contains a neutral residue, preferably small (A, P, S, T) at the P+4 position. The salient features of the two analyses are combined into a model, and it is speculated that the multiphosphorylation of rhodopsin involves a sequence in which the first modification occurs at Ser 343, second at Ser 338, third at Thr 336, and fourth at Thr 342; the remaining three residues (Ser 334, Thr 335, and Thr 340) are poorly phosphorylated, and a choice from among these is discussed. These findings extend and in broad terms confirm the previous conclusions drawn from structural studies on the phosphorylated receptor.

The transmission and the termination of signals by most G-protein-coupled receptors occur by similar mechanisms [reviewed by Dohlman et al. (1991) and Stryer (1991)]. Rhodopsin kinase from bovine retina, because of its relative abundance, has served as a prototype for this class of receptors, and the knowledge gained from its study has been extensively used to probe the biochemical details of other members of the family [reviewed by Inglese et al. (1993)]. The absorption of light by rhodopsin leads to the sequential formation of several photo-intermediates and one of these, metarhodopsin II (hereafter referred to as Rho*), participates in signal transduction by a cascade mechanism. The signal is then terminated by the action of rhodopsin kinase, which also acts specifically on Rho*. Rhodopsin kinase catalyses multiphosphorylations on the C-terminal tail of the receptor (Kuhn & McDowell, 1977; Sale et al., 1978; Shichi & Somers, 1978; Hargrave et al., 1980; Thompson & Findlay, 1984) and in the process between seven and nine serine/threonine residues were thought to be modified (Wilden & Kuhn, 1982), though phosphorylated rhodopsin species containing that number of phosphates have not been detected (McDowell et al., 1993; Ohguro et al., 1993). The mechanism through which rhodopsin kinase displays its specificity and phosphorylates rhodopsin in a light-dependent reaction has recently been explored through the use of synthetic peptides corresponding to the C-terminal region of the visual receptor (Fowles et al., 1988; Palczewski et al., 1989; Onorato et al., 1991; Brown et al., 1992). It was found by us that the phosphorylation of synthetic peptides was enhanced (20−100 times) by the presence of Rho* (Fowles et al., 1988; Brown et al., 1992). This finding has received support from subsequent studies in the rhodopsin field (Palczewski et al., 1991) and has provided the stimulus for the same approach to be used on the β-adrenergic receptor kinase, the activity of which for the phosphorylation of synthetic peptides also was greatly increased by the presence of the stimulated form of the receptor (Kameyama et al., 1993; Kim et al., 1993; Haga et al., 1994). The latter requirement was rationalized in terms of a two-step mechanism for rhodopsin kinase (Figure 1), and, hence, by implication for βARK, which envisages that the enzyme is normally present in an inactive, resting state and is activated following interaction with rhodopsin (Fowles et al., 1988; Palczewski et al., 1991; Brown et al., 1992; Dean & Akhtar, 1993). Thereafter the activated kinase preferentially acts on Rho*, but can also phosphorylate, though poorly, the dark-adapted form of rhodopsin (Dean & Akhtar, 1993) as well as synthetic peptides (Brown et al., 1992). This study is concerned with a complementary aspect, seeking to examine the order in which the multiphosphorylations occur and define the structural features which can be recognized by the kinase. The specificities of several protein kinases are known, and in most cases the target hydroxyl group of serine/threonine/tyrosine is found to be the part of a specific amino acid sequence [reviewed by Edelman et al. (1987), Kemp and Pearson (1990), and Roach (1991)]. Rhodopsin kinase is an example of a unique type, since in it each potential

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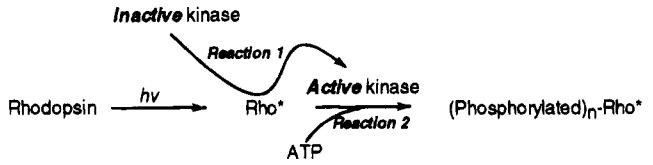

FIGURE 1: Two-step process for activation of rhodopsin kinase. Theoretical mechanisms for the activation are considered elsewhere (Brown et al., 1992; Dean & Akhtar, 1993). In the present context, a point to note is that it is the activated kinase formed in reaction 1 that promotes the phosphorylation of peptides.

phosphorylation site is surrounded by a different amino acid sequence; it appears that the specificity resides not in a particular primary sequence but in some form of higher-order structure. The purpose of the present study is to dissect the complex recognition process into its individual components, and for this purpose a strategy based on the use of synthetic peptides has been selected. We were attracted to this approach from the knowledge that judiciously selected peptides may deputize for the native protein with a high degree of fidelity. Ser 343 has been shown by us to be preferentially phosphorylated in some synthetic peptides (Pullen et al., 1993). Several independent studies, performed contemporaneously, have also shown that this residue is one of two amino acid sites most readily modified in the native receptor (McDowell et al., 1993; Ohguro et al., 1993; Papac et al., 1993).

EXPERIMENTAL PROCEDURES

Materials and Methods. Bovine eyes were obtained from freshly slaughtered bullocks (FMC Meat, Salisbury, Wilts, or more recently from H. M. Bennett, Funtley, Hants). Rod outer segments (ROS) were purifed as described previously (Brown et al., 1993), and all additions were made under red safelight conditions.

Synthetic peptides were prepared by manual solid-phase Fmoc-peptide synthesis as previously described (Pullen et al., 1993). Peptides were purified using HPLC after acidolytic liberation from the solid support and subjected to rigorous mass spectrometry and amino acid sequencing to ensure the purity.

[γ-^{32}P]ATP was obtained from the Radiochemical Centre (Amersham, Bucks). Optiphase 'HiSafe 3' scintillant was purchased from Pharmacia LKB. Radioactivity was visualized with RPN-6 Amersham Hyperfilm-MP. Plastic-backed cellulose plates were purchased from Eastman Kodak (Rochester, NY). Analytical grade AG1-X2 anion exchange material was supplied by Bio-Rad (Richmond, CA). The Sequelon AA attachment kit was supplied by Milligen/Biosearch (product no. GEN920033). All other reagents used were of sequencer grade purchased from Applied Biosystems Ltd. Densitometry was performed on a Joyce-Loebl UV scanner.

Purification of Rhodopsin Kinase from ROS Membranes. Rhodopsin kinase was extracted from ROS membranes and purified by heparin−agarose chromatography, using a modified method of Palczewski (Pulvermuller et al., 1993), and the final extract was assayed using urea-washed ROS membranes (30 μM) as described under 'peptide phosphorylation in a reconstituted system', but in the absence of peptide. The extract per microgram of protein, transferred 145 pmol of ^{32}P from [γ-^{32}P]ATP to bleached, urea-washed rhodopsin/min.

Peptide Phosphorylation Using Unextracted ROS Membranes. Peptide phosphorylation was performed for 60 min with unextracted ROS membranes (30 μM) in buffer containing 70 mM sodium phosphate (pH 7.25), 2 mM $MgCl_2$, and 0.1 mM EDTA at 30 °C ± light as described previously (Brown et al., 1992). Reactions were performed on a 50-μL scale, but scaled up when necessary.

The ^{32}P-labeled phosphopeptide products were separated from the ROS membranes by centrifugation and diluted within 7% (v/v) acetic acid (500 μL). The supernatants were applied to individual, preequilibrated AG1-X2 columns (2 mL), and the phosphopeptides eluted from the contaminant [γ-^{32}P]-ATP with 7% (v/v) acetic acid (5 mL). Fractions were collected, the Cerenkov radioactivity was measured, and the appropriate fractions were pooled and lyophilized. The ^{32}P-labeled phosphopeptides were further purified by electrophoresis (pH 3.5, 1 KV, 2.5 h) on plastic-backed cellulose plates. The ^{32}P-labeled phosphopeptides were either subjected to scintillation counting directly or excised and eluted from the cellulose matrix with 0.1% (v/v) trifluoroacetic acid. Aliquots of the excised ^{32}P-labeled phosphopeptide, after lyophilization, were subjected to phosphoamino acid analysis and sequencing.

Peptide Phosphorylation in Reconstituted System. Rhodopsin kinase (2.2 μg) was incubated with 2 mM peptide I (^{338}SKTETSQVAPA^{348}) and 3 mM [γ-^{32}P]-ATP in a buffer containing 50 mM BTP/HCl (pH 7.5), 2 mM $MgCl_2$, and 0.1 mM EDTA (50 μL). Phosphorylation was initiated in the presence or absence of urea-washed ROS membranes (30 μM) ± light at 30 °C (15 min). Incubations were centrifuged (10000g), to remove the rhodopsin, and the supernatants were harvested under red safelight and processed as above.

Phosphoamino Acid Analysis. Purified ^{32}P-labeled phosphopeptides were resuspended with 6 M HCl (110 μL) in sealable Pyrex tubes (10 mL). Peptide hydrolysis (Cooper et al., 1983) was initiated by heating to 110 °C (1.25 h), the tubes were cooled, and the excess acid and water were removed over KOH and P_2O_5. The residues were resuspended in analar H_2O (1 mL) and lyophilized overnight. The hydrolysate was resuspended with phosphoamino acid standards and spotted on cellulose plates for electrophoresis at (pH 3.5, 1 KV constant voltage, 55 min). The spots of radioactivity were visualized by autoradiography. The relative proportions of phosphoserine to phosphothreonine were determined by both densitometric measurements and direct scintillation counting.

Attachment of ^{32}P-Labeled Phosphopeptides to Sequelon AA Membrane and Subsequent Amino Acid Sequencing to Identify Positions of Phosphorylation. Aliquots of each ^{32}P-labeled phosphopeptide (12 000−20 000 cpm) were deposited on Sequelon AA membrane and treated overnight with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide according to manufacturer's instructions. The efficiency of the attachment as measured by the elution of nonbound counts after washes with H_2O (1 mL × 5), MeOH (1 mL × 5), and finally 12.5% trifluoroacetic acid/37.5% H_2O/50% AcCN (1 mL × 5) was usually greater than 65%. Using standard automated Edman degradation chemistry, the N-terminal anilinothiazolinone amino acid derivatives were eluted from the membrane once with 100% trifluoroacetic acid and then with methanol, and the combined eluates were analyzed by scintillation counting. Peptide sequencing was performed on an Applied Biosystems

Table 1: Relative Distribution of Monophosphorylated Species When Peptides Derived from C-Terminus of Rhodopsin Are Used as Substrates[a]

sequence	rel. peptide phosphorylation	% PhosSer:PhosThr	% distribution of isomer monophosphorylated at						
			Ser 334	Thr 335	Thr 336	Ser 338	Thr 340	Thr 342	Ser 343
^{338}SKTETSQVAPA^{348} (I)	100	97:3	NA	NA	NA	2	3	6	89
^{339}KTETSQVAPA^{348} (II)	74.5 ± 3.4	93:7	NA	NA	NA	NA	3	7	90
^{337}VSKTETSQVA^{346} (III)	34.9	86:14	NA	NA	NA	29	7	6	56
^{337}VSKTETSQVAPA^{348} (IV)	56.3 ± 4.8	94:6	NA	NA	NA	25	7	23	45
^{337}VSKTETS[PO_3H_2]QVAPA^{348} (IVa)	0								
^{333}ASTTVSKTET^{342} (V)	94.8 ± 3.6	80:20	20	13	26	36	4	1	NA
^{333}ASTTVS[PO_3H_2]KTET^{342} (Va)	9.3 ± 1.8	52:48							
^{333}AS[PO_3H_2]TTVSKTET^{342} (Vb)	28.6 ± 2.7	69:31							
^{329}GDDEASTTVSK^{339} (VI)*	8.9 ± 1.3	89:11	60	8	28	4	NA	NA	NA
^{334}STTVSKTETS^{343} (VII)	40.5	71:29	6	3	44	38	5	2	2
^{339}KTET[Cys]QVAPA^{348}	12.4 ± 2.0	0:100	NA	NA	NA	NA	39	61	0
^{337}VSKTET[Cys]QVAPA^{348}	5.54 ± 0.4	62:38	NA	NA	NA	72	19	9	0
^{337}V[Cys]KTETSQVAPA^{348}	39.5 ± 1.6	91:9	NA	NA	NA	0	13	11	76

[a] Peptides were phosphorylated by rhodopsin kinase in the presence of light-activated rhodopsin as described in Experimental Procedures. The % distribution of phosphorylation is calculated by determining the release of ^{32}P in those cycles containing a phosphorylatable residue; as an illustration, the ^{32}P at sites in peptide VII was calculated for cycles 1, 2, 3, 5, 7, 8, and 9, whose combined values totaled 100% (see Figure 3). This method does not to include carryover ^{32}P released in those cycles not containing a phosphorylatable group. Where information on a particular amino acid phosphorylation is not applicable, NA is recorded. The peptides Va and Vb could not be sequenced while IVa was not phosphorylated. The analysis of the phosphopeptide obtained from VI* was performed following proteolytic digestion as described in Experimental Procedures.

477A protein sequencer coupled with ^{32}P-counting on a Raytest 4700 scintillation counter.

Endopeptidase Glu-C Cleavage of Peptide VI, ^{32}P-[^{329}GDDEASTTVSK^{339}]. The large contributions of N-terminal carboxyl side chains had made the sequencing of peptide VI difficult. This problem was circumvented by cleavage with the endopeptidase Glu-C from *Staphylococcus aureus* V8 before sequencing. Peptide VI (ca. 200 000 cpm; 150 pmol) was resuspended in 25 mM NH_4HCO_3 (pH 7.8; 60 μL) and added to endopeptidase Glu-C (25 μg) in a sealed vial. The reaction was allowed to proceed overnight at 30 °C, and the cleaved fragments were purified by electrophoresis (pH 3.5, 1 KV, 2 h). Autoradiography revealed two bands of radioactivity (rf 0.58 and rf 0.52) with differing intensity. The lower band was approximately 2.4 times the intensity of the upper band. Both bands were excised, eluted with 0.1% trifluoroacetic acid, lyophilized, and subjected to sequencing and phosphoamino acid analysis. The distribution of ^{32}P for each band, at each putative phorphorylation site, was determined from the respective sequencing profiles. For the lower band the major identified phosphopeptide corresponded to AS[PO_3H_2]TTVSK; the less intense upper band sequencing profile identified a second phosphopeptide, ASTT[PO_3H_2]VSK. Data from both sequencing profiles were combined, and the relative proportions of ^{32}P at each possible phosphorylation site (Ser 334, Thr 335, Thr 336 and Ser 338) in the original uncleaved peptide were determined. When this was done, the ratio of Ser 334 to Thr 336 phosphorylation was approximately 2.1, similar to the observed band intensities following the original proteolysis and electrophoresis. These data are expressed in Table 1.

Synthesis of Phosphopeptides Va and Vb. The synthesis of phosphopeptide IVa has been documented previously (Pullen et al., 1993). The synthesis of phosphopeptides Va and Vb (Table 1) was achieved using a similar protocol to that described. Both of these peptides were successfully made starting with Wang Thr-(*O-tert*-butyl)-Fmoc resin (0.47 mmol/g; 0.1 mmol) and finishing with the incorporation of Boc-Ala. The syntheses differed only in the position of the Ser-*O*-trityl derivative. Phosphorylation of the two peptides was achieved by a 'phosphitylation and oxidation approach' (Andrews et al., 1991). Phosphorylation for phosphopeptide Va was performed with *bis-tert*-butyl *N,N*-diethylphosphoramidite, but phosphorylation for phosphopeptide Vb could only be achieved with *bis*-benzyl-*N,N*-diethylphosphoramidite. Both peptides were cleaved from their supports with 95% (v/v) trifluoracetic acid to yield crude solids (phosphopeptide Va, 79 mg from 155 mg of peptidyl resin; phosphopeptide Vb, 67 mg from 146 mg of peptidyl resin) that were then submitted to gel filtration on G25. Purification with C18-analytical HPLC yielded pure phosphopeptide Va (5.3 mg from 15 mg of desalted peptide, 35%) and phosphopeptide Vb (1.4 mg from 15 mg of desalted material, 9.8%).

RESULTS

Phosphorylation of Synthetic Peptides by Rhodopsin Kinase and Analysis of Phosphopeptides. The peptides chosen for this study were selected in the light of two types of previous observations. First, in our original study, it was found that peptides of 9−12 amino acids, from the C-terminal region of rhodopsin, were the optimal substrates for the kinase. Longer peptides, even when these closely resemble the sequence of the phosphorylation domain of the receptor, were poorly phosphorylated, presumably due to the formation of dead-end enzyme−ligand complexes (Brown et al., 1992). Second, all the potential phosphorylation sites in rhodopsin which had initially been located in the C-terminal CNBr peptide fragment (comprising residues 318−348) were subsequently narrowed down to the most terminal 19 amino acids (McDowell et al., 1993; Ohguro et al., 1993; Papac et al., 1993). Using this information the peptides detailed in this study were 10−12 amino acids in length and overlapped the sequence of the last 20 amino acids in the rhodopsin C-terminal tail. Three phosphopeptides and three mutant peptides, in which the important Ser residue(s) were replaced by Cys, were also included in the study.

The peptides were phosphorylated using the protocol described previously (Fowles et al., 1988; Brown et al., 1992). In preliminary studies, the peptides were incubated with rhodopsin kinase and [γ-^{32}P]-ATP, with or without urea-washed rod outer segments (ROS) in the presence or absence of light (Figure 2). These experiments confirmed that in all

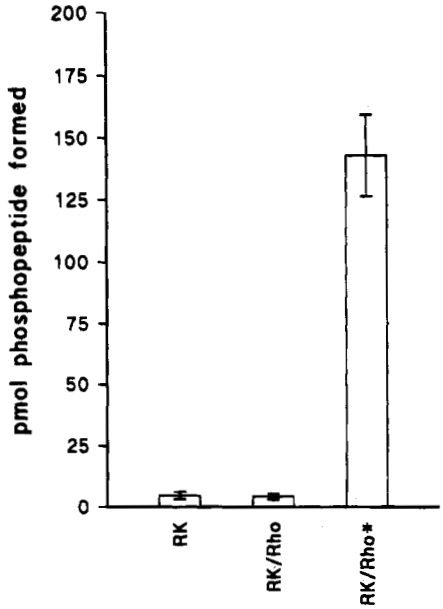

FIGURE 2: Rho* dependence of peptide phosphorylation catalyzed by rhodopsin kinase. The phosphorylation of peptide I (^{339}SKTETSQVAPA^{348}) by purified rhodopsin kinase is detailed in the methods section. These data are plotted as an average of two experiments (± SEM): RK, incubation with rhodopsin kinase; RK/Rho, incubation with rhodopsin kinase and urea-washed rod outer segments in the dark; RK/Rho*, same as the previous experiment but in the light.

cases light and urea-washed ROS were essential for the convincing phosphorylation of the peptides. In the absence of ROS or light the level of phosphorylation was less than 5% of that in the complete system. In preparative experiments in which the biosynthesized phosphopeptides were required in larger amounts for structural studies intact ROS membranes, which contain endogenous rhodopsin kinase, were employed, and the products were separated by electrophoresis on preparative cellulose plates. When the peptides (I, II, III, IV, V, VI and VII; Table 1), overlapping with the last 20 amino acids of rhodopsin, were the substrates, only monophosphorylated products were detected; since in all of the cases the ^{32}P-containing band migrated in the position of an authentic sample of monophosphorylated peptide. The formation of singly phosphorylated species under the conditions of our experiment is not surprising since the initial substrate was used in large excess and during the course of the incubation less than 1% of the material was consumed. We shall see, however, that in most cases the phosphorylated peptides were a mixture of singly modified isomers, which were not clearly separated with electrophoresis. The distribution of each monophosphorylated isomer in the mixture was determined by Edman degradation in which the peptides were covalently linked to a NH_2-containing membrane using carbodiimide coupling and sequenced using a solvent regime that allowed a good recovery of ^{32}P-containing products at each round of the degradation. Figure 3 presents a typical profile of the release of ^{32}P in the analysis of one of the peptides (VII), and Table 1 shows the distribution of each mono-phosphorylated isomer in the mixture. In the calculation of the values in Table 1, the total radioactivity released is obtained from that measured in those cycles containing the known hydroxy amino acids. This method of calculation errs on the side of caution, underestimating the intrinsic phosphorylation at each site, by ignoring carryover of radioactivity from one sequencing cycle to the next. Column 2 in Table 1 highlights that the

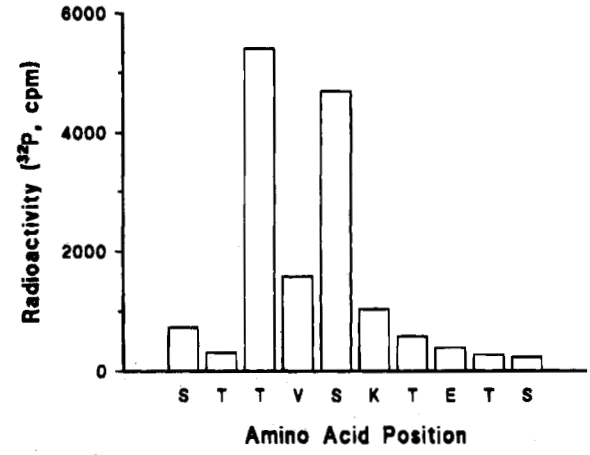

FIGURE 3: Elution of ^{32}P from sequence analysis of peptide VII, ^{334}STTVSKTETS^{343}. Peptide VII was phosphorylated by rhodopsin kinase, in Rho*-dependent fashion, as described in the text. The purified ^{32}P-labeled phosphopeptide was immobilized via its C-terminus, using a carbodiimide-catalyzed functionalization to a NH_2-membrane support. ^{32}P-elution was measured at each stage of the Edman degradation by scintillation counting and plotted here as a function of the sequence. For the calculation of % distribution in Table 1, the ^{32}P released in only those cycles which contain the potential phosphorylation sites is taken as 100%. This is exemplified for peptide VII, when the 100% value is obtained from the summation of cpm in parentheses: ^{334}Ser (728); ^{335}Thr (306); ^{336}Thr (5401); ^{338}Ser (4689); ^{340}Thr (578); ^{342}Thr (270); ^{343}Ser (235).

enzymic phosphorylation of peptides occurred to different extents. Therefore, to allow a global comparison of the propensity of phosphorylation of each singly modified peptide the data were normalized as

$$\text{propensity of phosphorylation} = \text{\% distribution of } ^{32}\text{P in Site (by Edman)} \times \text{relative peptide phosphorylation by kinase}/100 \quad (1)$$

Before we attempt to analyze the data, it is important to draw attention to the problems which are encountered in the quantitative analysis of the phosphorylation sites in phosphoproteins and phosphopeptides. There are several possible sources of error. The incorporation of phosphate at different positions in a polypeptide effects the physiochemical and enzymic properties of the resulting species in unpredictable manners. During the proteolytic degradation of phosphoproteins (and peptides), the phosphorylated fragments maybe isolated in varying yields, due to differing cleavage efficiencies during proteolytic digestion and/or selective losses in the often multi-step purification. These problems are minimized in the present work since in all experiments the phosphopeptides were isolated by a standard method which allows a quantitative recovery of the products formed from the incubation mixtures, and in all, except one case, the phosphopeptides were analyzed without recourse to enzymic digestion.

The other problems are, however, common to the analysis of all phosphopeptides, whether obtained directly from peptides by enzymic phosphorylations or through the degradation of a larger protein. This first involves the efficiency through which each species is analyzed: in the Edman degradation used here uncertainty exists at the stage of the covalent attachment of peptides to the solid support when one isomer may link in preference to an other. Then there are problems in the final analysis: in the present study the sequencing efficiency is susceptible to variation due to a variety of reasons known to the protein chemist. These

Table 2: Alignment of Phosphorylation Sites in Peptides[a]

Entry No.	P-1 ↓	P ↓	P+4 ↓	Propensity Index
1	S-K-T-E-T-	S^{343}	-Q-V-A-P-A	89
2	K-T-E-T-	S^{343}	-Q-V-A-P-A	67
3	V-S-K-T-E-T-	S^{343}	-Q-V-A-P-A	25
4	V-c-K-T-E-T-	S^{343}	-Q-V-A-P-A	30
5	V-S-K-T-E-T-	S^{343}	-Q-V-A	20
6	V-	S^{338}	-K-T-E-T-S-Q-V-A-P-A	25
7	V-	S^{338}	-K-T-E-T-c-Q-V-A-P-A	4
8	A-S-T-T-V-	S^{338}	-K-T-E-T	35
9	G-D-D-E-A-S-T-T-V-	S^{338}	-K	0.4
10		S^{338}	-K-T-E-T-S-Q-V-A-P-A	2
11	V-	S^{338}	-K-T-E-T-S-Q-V-A	10
12	S-T-T-V-	S^{338}	-K-T-E-T-S	15.4
13	A-S-T-	T^{336}	-V-S-K-T-E-T	25
14	S-T-	T^{336}	-V-S-K-T-E-T-S	17.8
15	G-D-D-E-A-S-T-	T^{336}	-V-S-K	2.5
16	G-D-D-E-A-	S^{334}	-T-T-V-S-K	5
17	A-	S^{334}	-T-T-V-S-K-T-E-T	19
18	L-G-D-D-E-A-	S^{334}	-T-T-V	0
19		S^{334}	-T-T-V-S-K-T-E-T-S	2.5
20	V-S-K-T-E-	T^{342}	-S-Q-V-A-P-A	13
21	K-T-E-	T^{342}	-S-Q-V-A-P-A	5
22	K-T-E-	T^{342}	-c-Q-V-A-P-A	8
23	S-K-T-E-	T^{342}	-S-Q-V-A-P-A	6
24	V-S-K-T-E-	T^{342}	-S-Q-V-A	2
25	A-S-	T^{335}	-T-V-S-K-T-E-T	13[b]
26	S-	T^{335}	-T-V-S-K-T-E-T-S	1.2
27	G-D-D-E-A-S-	T^{335}	-T-V-S-K	0.7
28	K-	T^{340}	-E-T-S-Q-V-A-P-A	3
29	V-S-K-	T^{340}	-E-T-S-Q-V-A-P-A	4
30	S-T-T-V-S-K-	T^{340}	-E-T-S	2

[a] The residue to be phosphorylated is designated as P, and those around it are denoted by P−1, P+1, P+2, P+3, P+4, etc. The values in Table 1 were used to calculate the propensity index according to eq 1. [b] See text for discussion.

problems are, to some extent, highlighted when one compares the relative proportions of phosphoserine to phosphothreonine obtained by acid hydrolysis with those inferred from the Edman degradation profiles. It should, however, be noted that the deficiencies of this type also equally apply to the rival and immensely powerful mass spectrometric method when used for the purpose of quantification. In this technique, the intensity of a particular ion retaining the phosphate group depends on the ease of fragmentation, which is governed by subtle stereoelectronic factors.

Propensity of Phosphorylation at Various Serine and Threonine Residues. Keeping those reservations in mind, we analyzed the data obtained in two stages, initially focusing on the relative propensity of monophosphorylation at each position and then the alignment of sequences to visualize a common specificity determinant. From Table 2 it may be seen that Ser 343 was present in five sequences, and in every case the residue was phosphorylated with high propensity. Ser 338 was the next most favorable site for phosphorylation. The low phosphorylation of Ser 338 in entry 7 may be attributed to the presence of a Cys mutation introduced at residue 343, and its (Ser 338) negligible phosphorylation in entries 9 and 10, as we shall see later, is due to the absence of the required number of C-terminal residues and an essential N-terminal amino acid, respectively. Thr 336 was available for phosphorylation in three sequences and showed good reactivity in two cases; its poor performance in entry 15 provided an important test for the hypothesis developed in the next stage of the analysis. With this information Thr 336 qualifies, but only just, to be placed third in the ranking order. From the data in Table 2, Ser 334 shows an adequate to respectable phosphorylation in entries 17 and 19, and Thr 342, though showing lower propensity, was consistently phosphorylated in all the five sequences (entries 20−24); therefore, these two residues are not separated from each other and are jointly placed in the fourth place. The situation with respect to Thr 335 is equivocal; it was satisfactorily phosphorylated (entry 13) in a peptide which was an excellent substrate for the kinase. However, the extent of its phosphorylation was lower than that of Ser 338, Thr 336, and Ser 334 but higher than Thr 340. Thr 340 was poorly phosphorylated in all sequences (Table 1), and in the cases when significant results were obtained, the data were processed and carried forward in Table 2. These two residues, Thr 335 and Thr 340, thus appear to be the least preferred sites and are nominally placed in the fifth position. It could be argued that the ranking order concluded from the preceding analysis may have been initially deduced by merely glancing at the spread of data in Table 1.

Search for Motifs Recognized by Rhodopsin Kinase. For the next stage in the analysis, the peptides were aligned on a hypothetical template with the hydroxy amino acid to be phosphorylated, designated as the P residue. The surrounding sequences were then examined to see whether a diagnostic pattern exists (Table 2). Ser 343 is the most preferred site of phosphorylation and in the native receptor contains five C-terminal residues which may be required for interactions with the complementary sites on rhodopsin kinase; these by adapting a widely used convention are designated as P+1, P+2, P+3, P+4, and P+5. The occupancy of how many of these sites is required for an optimal interaction with the enzyme is not revealed by our study, though attention is drawn to the fact that with the exception of the data in entry 5, the presence of fewer than four C-terminal residues following the target hydroxy amino acid adversely affected the phosphorylation reaction. When the peptides are aligned as in Table 2, the most significant pattern observed is at the P+4 site. In almost all the peptides undergoing medium to good phosphorylation, this site is populated by Pro, Ala, Ser, or Thr. Entry 25 (Table 2) is the only exception in which Thr 335 was phosphorylated to a satisfactory level, yet it had a positive residue, Lys 339, at the P+4 site. The propensity index of 13 calculated for the phosphorylation of Thr 335 is derived from the analysis of a peptide (V) in which high levels of ^{32}P were also released in the two rounds of Edman degradation corresponding to Ser 334 and Thr 336. Thr 335 is sandwiched between these two residues, hence there is a possibility that due to the carryover and previewing of radioactivity, the level of phosphorylation for this residue may have been overestimated. This possibility was scrutinized by using peptide VII (^{334}STTVSKTETS^{343}) as a substrate for the kinase. It was hoped that the absence of a residue toward the amino side of Ser 334 may impair its phosphorylation (see below for explanation) and hence reduce the carryover of ^{32}P into the cycle of the Edman degradation releasing Thr 335. The results in Tables 1 and (entry 25) clearly show the poor phosphorylation of Thr 335 in this peptide, which may be attributed to the inhibitory role of the charged amino acid at the P+4 site. As a further test of this hypothesis, two synthetic phosphopeptides (Va and Vb) were prepared and found to be substrates for rhodopsin kinase in a Rho*-mediated fashion. The distribution of ^{32}P in the diphosphorylated species was not determined because the peptides could not be coupled to the solid support for Edman sequencing; however, from the phosphoamino acid analysis

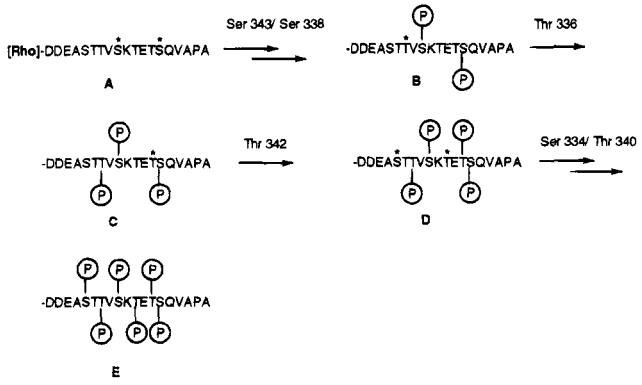

FIGURE 4: Sequential phosphorylation of rhodopsin by rhodopsin kinase. Those residues are to undergo phosphorylation in the next round are identified by an asterisk (*). A, symbolizes Rho* showing its 19 most C-terminal amino acids. B−E are the postulated products of sequential phosphorylation.

and the knowledge that in each case a single serine is available for modification, it is possible to estimate the propensity of phosphorylation for Ser 334 and Ser 338 which was 4.8 and 20 respectively. The data showed that the phosphorylation of Ser 334 in the peptide (Va) which would have a phosphoamino acid in the P+4 site was more significantly affected than of Ser 338 in the peptide (VIb) that contains the phosphoserine toward the N-terminal of the P site. The results then support the view that the occupancy of the P+4 site with a charged amino acid (i.e., phosphoamino acid) is less satisfactory than when this position is filled with a neutral residue. There is, however, a need for caution here since a third phosphopeptide (IVa), which should phosphorylate well according to our model, was not a substrate for rhodopsin kinase. The synthetic phosphopeptide IVa had a good affinity for the enzyme, but it failed to recruit all the interactions necessary for catalysis.

The four amino acids (A, P, S, and T) that are most frequently found at the P+4 positions of peptides phosphorylated well fall into the same group according to the classification of Dayhoff and Maclachlen [reviewed by Taylor (1986)]. These amino acids are related to one another by virtue of frequency of mutation, chemical properties, and size.

Good phosphorylation of Ser 338 in peptides ^{337}VSKTETSQVAPA^{348} (entry 6) and ^{333}ASTTVSKTET^{342} (entry 8) but not in ^{338}SKTETSQVAPA^{348} (entry 10) may be rationalized by assuming that *at least* one residue toward the N-terminal of the phosphorylation site is also required for the optimal interaction of the substrate with rhodopsin kinase; this site is designated as P-1.

In summing up this section, we conclude that the phosphorylation of a serine/threonine residue in a peptide occurs optimally if it is surrounded by at least one residue on the amino side and five on the acyl side and also has a neutral, preferably small, residue at the P+4 position.

The implication of the latter statement being that there will be a bias against the phosphorylation of the residue that contains a charged amino acid at the P+4 site. The tendency for the presence of neutral residues (A, S, T, or Q) at the P-1 and P+2 sites is also worthy of note though these features have not yet been incorporated in our prediction model.

Concerning Order of Phosphorylations. In the light of the ranking order deduced in Table 2 and the assumptions made above regarding the specificity-conferring residues, an attempt is made to analyze the order in which the phosphoryl groups may be introduced in the 19 amino acid tail constituting the phosphorylation domain of rhodopsin (Figure 4). We start with the phosphorylation of the two most preferred sites, Ser 343 and Ser 338, to give the diphosphorylated species (Figure 4B) and then in accordance with the ranking order select Thr 336 for the third phosphorylation. Ser 334 and Thr 342 were jointly accorded the fourth position in the analysis, but in the triphosphorylated species (Figure 4C) when Ser 334 occupies the P site for phosphorylation, the P+4 position designed for a neutral residue will be occupied by [Phos]-Ser 338. Using the examples of the difference in propensity for phosphorylation of Ser 338 and Ser 334 in the phosphopeptides (Va and Vb), as described above, Ser 334 is given a lower priority and Thr 342 selected to produce the tetraphosphorylated derivative (Figure 4D). A choice now must be made between Ser 334, Thr 335, and Thr 340 for the fifth phosphorylation. We do not favor the selection of Thr 335, because a large basic moiety, Lys 339, would occupy the crucial P+4 site. Though it is not possible to reliably separate the remining two sites (Thr 340 and Ser 334), Thr 340 may be preferred for the fifth phosphorylation since the modification of this residue when resident at the P position may be less extensively impaired by the presence of uncharged Gln 344 at the P+4 than will be, as mentioned above, of Ser 334 which will have phosphoserine 338 at the P+4 position.

It needs to be stressed that these inferences are made on a purely speculative basis, but nevertheless form a working model for further analysis. Furthermore, we make no attempt to predict the order of further phosphorylations since the results obtained in our laboratory confirm the recent findings of others that species containing more than five phosphate groups, if formed, are present as minor components. In our study, when the C-terminal CNBr fragment obtained from the exhaustive phosphorylation of rhodopsin was analyzed, mono-, di-, tri-, tetra-, penta-, and hexaphosphorylated species were found to be present in a ratio of 1:0.56:?:0.4:0.25:0.13 respectively (Fowles, 1988). The triphosphorylated peptide could not be quantified because it was not separated from the bulk impurities. Nor is it possible from the present study to deduce the sequence of further phosphorylations of those species which may be initially monophosphorylated at residues other than Ser 343 and Ser 338.

CONCLUSIONS

The statement in the introduction that in the multiphosphorylations catalyzed by rhodopsin kinase the molecular recognition is governed, not by readily discernible features contained in the primary sequence, but by a higher-order organization has been re-emphasized by the results described in the paper. Thus it is not possible, *a priori*, to tell whether a peptide with a given sequence will be a substrate for rhodopsin kinase; however, when it does phosphorylate, the process shows a discernible pattern of hierarchy which is reminiscent of the behavior of the native receptor. For example Ser 343 and Ser 338, found in this study to be the top ranking sites of phosphorylation in peptides, were also shown to be the two most readily phosphorylated amino acids in the native receptor as demonstrated almost simultaneously by three groups (McDowell et al., 1993; Ohguro et al., 1993; Papac et al., 1993). In these studies the phosphorylation domain of rhodopsin was isolated following an overnight digestion with endopeptidase Asp-N and the C-terminal 19

amino acid peptide analyzed either by Edman degradation or mass spectrometry. In two of the studies the monophosphorylated form of the peptide was found to be a mixture of two isomers modified at either Ser 343 or Ser 338, while the diphosphorylated species was phosphorylated at Ser 343 as well as Ser 338. These groups, without indicating any specific preferences, concluded that Ser 343 and Ser 338 are the two initial residues modified by rhodopsin kinase (McDowell et al., 1993; Papac et al., 1993). Ohguro et al. (1993) on the other hand also used the aforementioned degradation protocol in conjunction with mass spectrometry but found that the monophosphorylated form of the C-terminal peptide was predominantly modified at Ser 338 and the diphosphorylated material was modified at Ser 343 and Ser 338. It was therefore concluded that Ser 338 was modified first and then Ser 343. Attention may be drawn to the fact that when a mixture of similar peptides, obtained using ATPγS instead of ATP, was analyzed the singly modified species were about equally thiophosphorylated ar Ser 338 and Ser 343 (Ohguro et al., 1994). This pattern was attributed to the altered nucleotide binding site of rhodopsin kinase with ATPγS compared with ATP. This might well be the case, but it should be remembered that conventionally thiophosphorylation has found use in enzymology because the thiophosphoryl group is resistant to the action of a wide range of phosphatases. Notwithstanding the minor differences of emphasis in the studies cited, the common message is that Ser 343 and Ser 338 are the preferred sites for the action of rhodopsin kinase. Two of the groups also isolated a triphosphorylated C-terminal peptide from the above experiments and found it to be phosphorylated at Ser 343, at Ser 338, and at one of the four threonine residues (McDowell et al., 1993; Ohguro et al., 1993). That Thr 336 was the third site was established by Oghuro et al. (1993). The latter conclusion is fully in agreement with the ranking order deduced from the present work and used to predict the order of phosphorylation in the native receptor. We believe that the ideas developed here and embodied into a working hypothesis shall provide a focus for future structural studies.

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