Sequential Phosphorylation of Rhodopsin at Multiple Sites†

Hiroshi Ohguro,‡ Krzysztof Palczewski,*,‡,§ Lowell H. Ericsson,∥ Kenneth A. Walsh,∥ and Richard S. Johnson∥

Departments of Ophthalmology, Pharmacology, and Biochemistry, University of Washington, Seattle, Washington 98195

Received January 8, 1993; Revised Manuscript Received March 23, 1993

ABSTRACT: Photolyzed rhodopsin is phosphorylated at multiple serine and threonine residues during the quenching of phototransduction. Sites of phosphorylation by rhodopsin kinase have been localized to the C-terminal region of rhodopsin, but no information was available on the kinetics and identity of phosphorylated residues. To determine the kinetics of phosphorylation at specific residues, the phosphorylated C-terminal peptide of rhodopsin (^{330}DDEASTTVSKTETSQVAPA) obtained by proteolysis of rhodopsin with endoproteinase Asp-N was subjected to further subdigestion followed by electrospray mass spectrometry. Analysis of monophosphorylated peptide revealed that the major initial phosphorylation site is ^{338}Ser. The analysis of di- and triphosphorylated peptides indicated that ^{343}Ser or ^{336}Thr residues are subsequent phosphorylation sites. These three residues, located in the C-terminal region of rhodopsin, are likely to be key phosphorylation sites of rhodopsin during the quenching of phototransduction. Identification of the kinetics of phosphorylation will facilitate understanding the functional significance of rhodopsin phosphorylation at multiple sites and the mechanism of rhodopsin kinase action.

Protein phosphorylation is one of the most important controlling mechanisms in signal transduction pathways. In phototransduction, phosphorylation of rhodopsin (Rho) is a key step in quenching of the amplification cascade of reactions and initiates regeneration of the photolyzed receptor [reviewed by Palczewski and Benovic (1991) and Hofmann et al. (1992)]. Specifically, phosphorylation of photolyzed rhodopsin (Rho*) leads to uncoupling of a photoreceptor-specific G-protein (transducin) and subsequent binding of arrestin (Wilden et al., 1986; Miller et al., 1986; Bennett & Sitaramayya, 1988). Rhodopsin kinase (RK) catalyzes phosphorylation of Rho* at multiple Ser and Thr residues, located exclusively in the C-terminal region of Rho (Palczewski et al., 1991). The position of phosphates in the primary sequence of Rho was not completely characterized, primarily because of problems in sequencing phosphorylated Ser/Thr-rich regions by Edman degradation and the lack of a suitable proteolytic method for isolation of the C-terminal peptides. In preliminary studies, Thompson and Findlay (1984) have isolated CNBr C-terminal peptide from sheep Rho and reported that ^{334}Ser, ^{338}Ser, ^{343}Ser, ^{335}Thr, and ^{336}Thr are the major phosphorylation sites; however, no information was provided regarding the kinetics and functional significance of the site-specific phosphorylation. Recently, we found that endoproteinase Asp-N selectively cleaves the C-terminal region of Rho and eliminates sites phosphorylated by RK (Palczewski et al., 1991). This digestion method permits rapid isolation of a 19-amino acid peptide containing the phosphorylation sites and has allowed us to address these questions.

In this paper, we describe the kinetics of Rho phosphorylation. The C-terminal peptide obtained by proteolysis of Rho by endoproteinase Asp-N was subjected to further subdigestion followed by electrospray mass spectrometry (ES/MS) (Covey et al., 1988; Fenn et al., 1989; Smith et al., 1991). Although the molecular weights of the phosphopeptides permitted their identification, this was insufficient to locate their phosphoryl groups within peptides containing multiple Ser or Thr residues. Thus, tandem mass spectrometry (MS/MS) was employed for both verification of sequence (Biemann, 1990a,b; Hunt et al., 1986) and identification of phosphorylation sites (Biemann, 1992). The observation that residues ^{338}Ser, ^{343}Ser, and ^{336}Thr were found to be sequentially phosphorylated in the early stage of the kinase reaction may provide insight into the mechanism whereby RK plays a role in quenching phototransduction.

MATERIALS AND METHODS

Unless otherwise stated, all procedures with native rhodopsin were performed on ice using ice-cold solutions.

Isolation of Bovine Rod Outer Segments (ROS). Fresh bovine retinas were obtained from a local slaughterhouse, and dissected under dim red light to avoid rhodopsin bleaching. ROS were isolated using the discontinuous sucrose gradient method (Papermaster, 1982).

Time Course of Rhodopsin (Rho) Phosphorylation. Time course of Rho phosphorylation was studied as previously described (Wilden & Kühn, 1982). Briefly, ROS were suspended at a final concentration of 0.7 mg/mL in 100 mM potassium phosphate buffer, pH 7.2, containing 2.8 mM ATP and 10 mM $MgCl_2$. Rho phosphorylation was triggered by illuminating the sample from a distance of approximately 10 cm with 150-W lamp. The temperature of the reaction was held at 30 °C. At time 0, 4, 7.5, 15, 30, and 45 min, an aliquot of the mixture was withdrawn and added to an equal amount of 250 mM potassium phosphate buffer, pH 7.2, containing 200 mM EDTA, 5 mM adenosine, 100 mM KF, and 200 mM KCl to quench rhodopsin kinase and phosphatase activities. ROS membranes were collected by centrifugation at 16000*g* for 10 min, and the pellet was subsequently washed with (1) water, (2) 200 mM KCl, and (3) 10 mM Hepes, pH 7.5, containing 100 mM KCl (approximately 2 mL of each these solutions per ROS preparation obtained from 10 retinas).

† This research was supported by USPHS Grants EY09339 and EY01730 from the National Institutes of Health, by the Human Frontiers in Science Program, and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research. K.P. is the recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship.

* To whom correspondence should be addressed at the Department of Ophthalmology, RJ-10, University of Washington, Seattle, WA 98195-0001. FAX: (206) 543-4414.

‡ Department of Ophthalmology.

§ Department of Pharmacology.

∥ Department of Biochemistry.

0006-2960/93/0432-5718$04.00/0

Digestion of Rho by Endoproteinase Asp-N. Endoproteinase Asp-N (Boehringer Mannheim, obtained from a *Pseudomonas fragi* mutant, was dissolved in H_2O (20 μg/mL), mixed with washed ROS membranes (3.5 mg/mL) in a 1:7000 (w/w) ratio in 10 mM Hepes, pH 7.5, containing 100 mM KCl (Palczewski et al., 1991), and incubated in the dark at room temperature for 24 h. The peptides were separated from membranes by centrifugation at 200000*g* for 20 min, and then purified on a reverse-phase HPLC column (2.1 × 250 mm, C18 column, Vydac 218TP52) employing a linear gradient from 0 to 47% acetonitrile and from 0.08 to 0.1% trifluoroacetic acid for 27 min. Peptides were detected at 220 nm. The C-terminal peptide (^{330}DDEASTTVSKTETSQVAPA) was eluted at approximately 37% acetonitrile.

Subdigestion of ^{330}DDEASTTVSKTETSQVAPA. The C-terminal peptide (^{330}DDEASTTVSKTETSQVAPA; approximately 5–20 nmol) was treated with 0.2 nmol of trypsin-TPCK (Worthington), 15 pmol of thermolysin (Boehringer Mannheim), or 2 nmol of *Staphylococcus aureus* protease V8 (Boehringer Mannheim) in 80 mM Tris-HCl (pH 8.0) at 30 °C for 1, 16, or 2 h, respectively. Peptides were purified by HPLC as above, but with a linear gradient from 0 to 24% acetonitrile and from 0.08 to 0.1% trifluoroacetic acid for 60 min.

Digestion of ^{333}ASTTVSKTE Peptide by Carboxypeptidase P. The C-terminal amino acids of ^{333}ASTTVSKTE (approximately 2 nmol) were removed by 0.2 nmol of carboxypeptidase P (Boehringer Mannheim) in 50 mM sodium acetate buffer, pH 4.6 at 30 °C, for 0.5, 2, or 16 h. Residual peptides were purified by HPLC as above.

Digestion of ^{342}TSQVAPA Peptide by Leucine Aminopeptidase. The N-terminal amino acids of ^{342}TSQVAPA (approximately 2 nmol) were removed by 120 pmol of microsomal leucine aminopeptidase (Sigma) in 80 mM Tris-HCl buffer, pH 8.0, containing 2 mM $MgCl_2$ at 30 °C for 2 h. Residual peptides were purified by HPLC as above.

Electrospray and Tandem Mass Spectrometry. Electrospray mass spectra were acquired on a Sciex API III triple-quadrupole mass spectrometer fitted with a nebulization-assisted electrospray ionization source (PE/Sciex, Thornhill, Ontario). Peptide fractions were concentrated to dryness and reconstituted in 1:1 methanol/H_2O (v/v), 0.1% in formic acid. Samples were infused into the electrospray source via a 50-μm inner diameter fused silica transfer line using a Harvard Apparatus pump. The mass spectrometer was scanned repetitively with a mass step of 0.1 Da and 1 ms/step. Resolution was adjusted to a 20% valley between adjacent isotope peaks in a singly charged cluster.

For tandem mass spectrometry, precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation (at approximately 30 eV) with argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.25 Da and 1 ms/step. Parent-ion transmission was maximized by reducing the resolution of Q1 to transmit a 2–3 m/z window about the selected parent ion, and Q3 resolution was adjusted to approximately 50% valley between peaks 3 Da apart.

RESULTS

Time Course of Rhodopsin Phosphorylation. Rhodopsin phosphorylation by endogenous rhodopsin kinase was studied in homogenate ROS in which all of the components of the phototransduction cascade are present under saturable light conditions. The phosphorylation reaction was started by exposing ROS to light and terminated at 0, 4, 7.5, 15, 30, or 45 min by addition of a buffer containing inhibitors of rhodopsin kinase and phosphatase (EDTA, adenosine, KF, and high concentrations of phosphate). After ROS membranes were washed, initial proteolysis of Rho was performed with endoproteinase Asp-N, which removes the C-terminal 19 amino acids, ^{330}DDEASTTVSKTETSQVAPA, containing all potential phosphorylation sites (Palczewski et al., 1991). Then the C-terminal peptide of Rho was purified as a single peak by HPLC (C18) using a relatively steep gradient of acetonitrile (Figure 1A) so that phosphorylated and non-phosphorylated peptides would coelute. Mass spectra of this peptide at the various time points showed $(M+2H)^{2+}$ ions at *m/z* 969.1, 1009.3, 1049.3, and 1089.0 indicating non-, mono-, di-, and triphosphorylated peptide, respectively (Figure 1B). No ions were observed for more highly phosphorylated species.

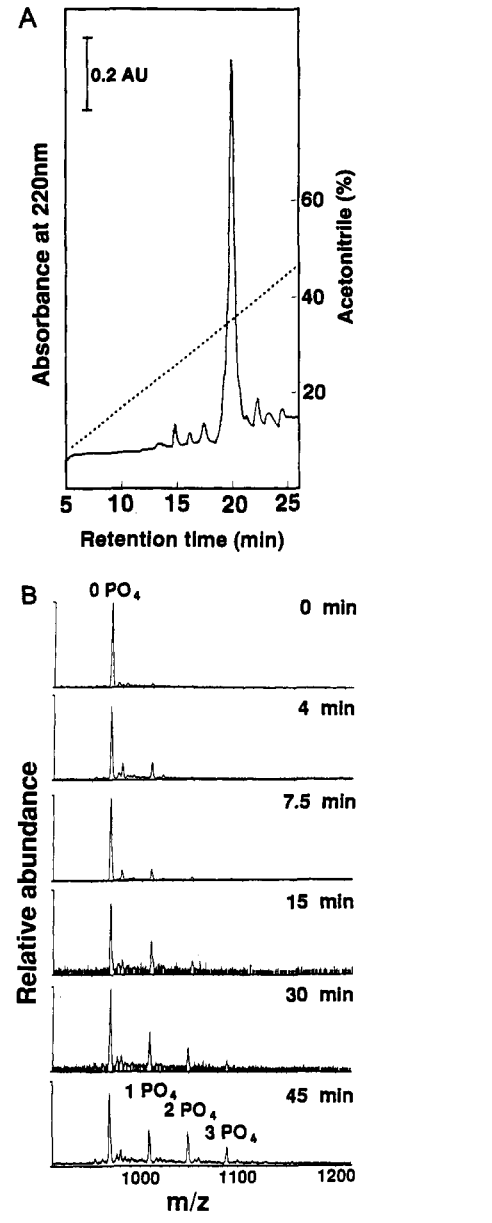

FIGURE 1: Isolation of Rho C-terminal peptide (^{330}DDEASTTVSKTETSQVAPA) (A) and ES/MS spectra of the peptides at 0-, 4-, 7.5-, 15-, 30-, and 45-min points (B). (A) After digestion of unphosphorylated or phosphorylated Rho at several time points with endoproteinase Asp-N, C-terminal peptide, ^{330}DDEASTTVSKTETSQVAPA, was purified by reverse-phase (RP) HPLC with a linear gradient from 0 to 47% acetonitrile and from 0.08 to 0.1% trifluoroacetic acid. Peptides were detected at 220 nm. (B) The purified peptides were directly analyzed by ES/MS. The ions marked with 0 PO_4, 1 PO_4, 2 PO_4, and 3 PO_4 represent the corresponding peptide with zero, one, two, and three phosphates, respectively.

Table I: Tryptic Peptides

peak designation[a]	observed MW[b]	calculated MW[c]	sequence	phosphate no.
1	1051.4	1051.5	DDEASTTVSK	0
2	902.5	902.4	TETSQVAPA	0
3	2176.6	2177.0	DDEASTTVSKTETSQVAPA	3
4	2096.4	2097.0	DDEASTTVSKTETSQVAPA	2
5	2017.0	2017.0	DDEASTTVSKTETSQVAPA	1
w	1211.8	1211.4	DDEASTTVSK[d]	2
	1291.8	1291.4	DDEASTTVSK[d]	3
x	1131.6	1131.5	DDEASTTVSK[d]	1
y	982.5	982.4	TETSQVAPA	1
z	902.6	902.4	TETSQVAPA	0

[a] Peak designations correspond to those of Figure 2. [b] Molecular weights determined by ES/MS. [c] Monoisotopic masses for all peptides except for the higher molecular weight uncleaved peptides, which are reported as average masses. [d] Minor components of the fraction.

It seems likely that the relative abundance of ions within each spectrum (Figure 1B) reflects the percentage of each phosphorylated form present at each time point. It could be argued that the addition of a phosphate might reduce the ionization efficiency at the pH of the electrospray solution (pH 3); thus, the trend toward increased phosphorylation with time may be even more exaggerated than what is depicted in Figure 1B.

The identification of phosphorylation sites could, in principle, be carried out by MS/MS of the phosphorylated forms of the peptide. In practice, this was not feasible using the low-energy collisions of the triple-quadrupole instrument. The MS/MS spectra of the $(M+2H)^{2+}$ ion of either the nonphosphorylated or the phosphorylated peptides yielded very abundant ions for the cleavage of the ^{346}Ala–Pro bond. This preferential cleavage seemed to have a detrimental effect with respect to obtaining a more complete series of sequence-specific fragment ions, and identification of phosphorylation sites was not therefore accomplished by MS/MS alone. Instead, the C-terminal peptide was subjected to additional proteolyses with trypsin (at ^{339}Lys; Molday & MacKenzie, 1985), thermolysin (at ^{337}Val and ^{345}Val; Hargrave & Fong, 1977), and *S. aureus* protease V8 (at ^{332}Glu and ^{341}Glu; Findlay et al., 1981). These fragments were then analyzed by ES/MS and MS/MS to determine the number of phosphate groups and their sequence location.

Tryptic Digestion of ^{330}DDEASTTVSKTETSQVAPA. Previous studies have shown that the C-terminal peptide of nonphosphorylated rhodopsin can be cleaved by trypsin whereas the corresponding phosphorylated peptides cannot (Molday & MacKenzie, 1985). This observation was confirmed and found to be a useful means of separating the phosphorylated C-terminal peptides from the nonphosphorylated one. The nonphosphorylated peptide is cleaved into two peptides (peaks 1 and 2 in Figure 2A), which were shown by ES/MS to be ^{330}DDEASTTVSK and ^{340}TETSQVAPA (Table I). Tryptic digestion of the partially phosphorylated C-terminal peptides at the 4- and 45-min time points resulted in three additional HPLC peaks that by ES/MS corresponded to the uncleaved C-terminal peptide plus one phosphate (peak 5), two phosphates (peak 4), and three phosphates (peak 3) (Figure 2B,C, Table I).

In addition to peaks 1–5, four minor peaks designated w–z were observed (Figure 2B,C). Peaks w and x were found to contain trypsin autolysis products and traces of peptides corresponding to ^{330}DDEASTTVSK with one to three phosphates (Table I). The MS/MS spectra indicated that these phosphates appeared to be located within the ^{334}Ser-Thr-Thr sequence. Peak y was shown to be ^{340}TET (phospho-S)QVAPA. The ES/MS spectrum of peak z was identical to peak 2. Either different conformations of the peptide ^{340}TETSQVAPA are separated by HPLC (e.g., *cis*- versus *trans*-proline), or the fraction cut for peak z included an overlap of peak 2.

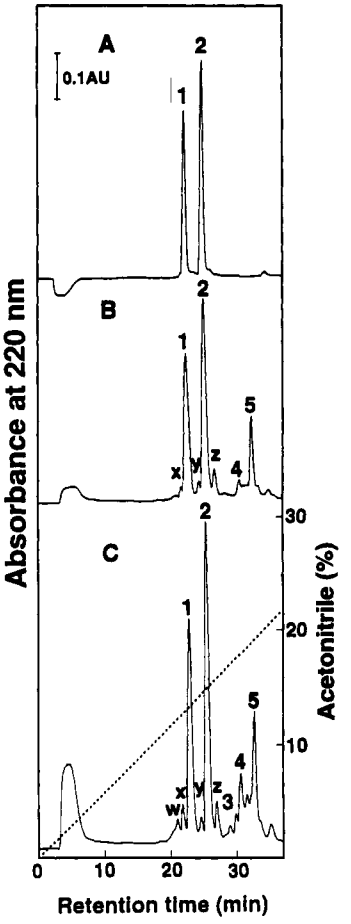

FIGURE 2: Tryptic digestion of unphosphorylated and phosphorylated C-terminal peptides of Rho. The C-terminal peptide (approximately 5–20 nmol each), ^{330}DDEASTTVSKTETSQVAPA, isolated 0 (A), 4 (B), or 45 min after the initial light flash (C) time point was incubated with 0.2 nmol of trypsin-TPCK in 50 μL of 80 mM Tris-HCl, pH 8.0, at 30 °C for 1 h. Digested fragments were separated from each other by RP-HPLC as described in the legend to Figure 1A, but with a linear gradient from 0 to 24% acetonitrile. The peak designations correspond to those in Table I.

Thermolytic Digestion of ^{330}DDEASTTVSKTETSQVAPA. The nonphosphorylated and the mono-, di-, and triphosphorylated peptides obtained by tryptic digestion were subjected to further proteolysis using thermolysin. The elution profiles of their proteolytic peptides are shown in Figure 3, and the identification of each peak by ES/MS is summarized in Table II. In Figure 3, peaks 4, 6, 8, and 9 represent uncleaved non-, mono-, di-, and triphosphorylated peptides, respectively. Thermolysin cleaved the nonphosphorylated

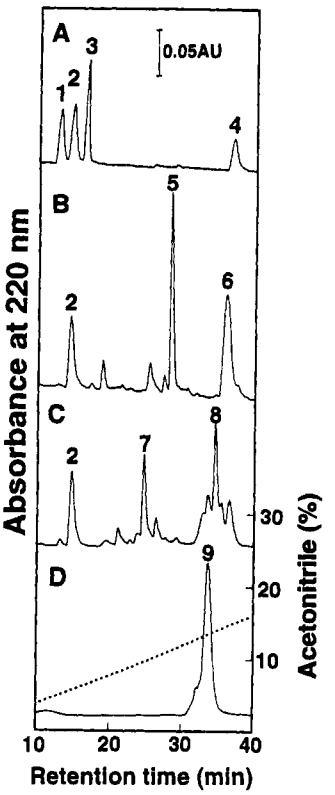

FIGURE 3: Comparison of elution profiles of thermolytic peptides of non-, mono-, di-, and triphosphorylated C-terminal peptides of Rho. Non- (A), mono- (B), di- (C), or triphosphorylated ^{330}DDEASTTVSKTETSQVAPA (D) (approximately 5–20 nmol each) was digested with 15 pmol of thermolysin in 50 μL of 80 mM Tris-HCl, pH 8.0, at 30 °C for 16 h. Fragments were separated from each other by RP-HPLC as described in the legend to Figure 2. The peak designations correspond to those in Table II.

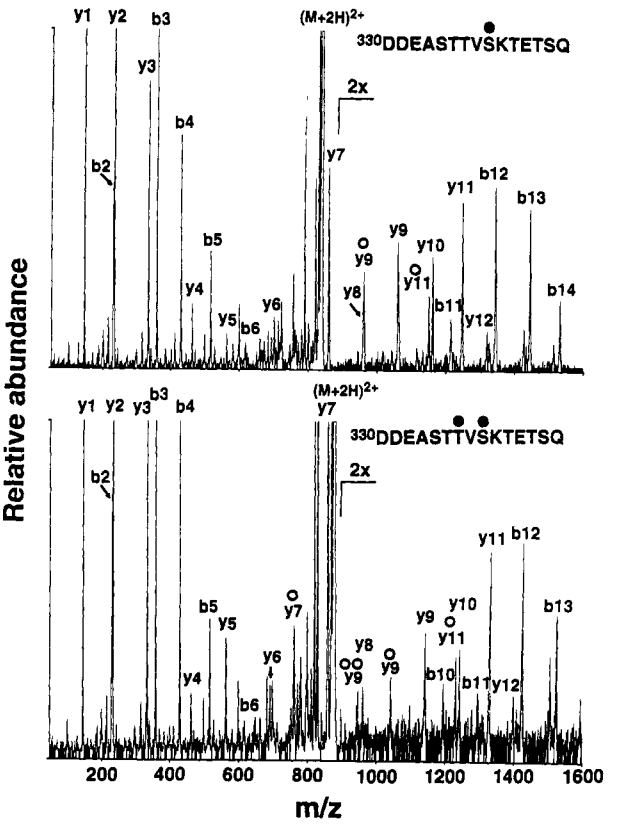

FIGURE 4: MS/MS spectra of mono- and diphosphorylated ^{330}DDEASTTVSKTETSQ. *Upper panel*: MS/MS spectrum of the singly phosphorylated $(M+2H)^{2+}$ ion of m/z 840.0. The ion nomenclature is that proposed by Biemann (1990b), where the y-type ions are numbered from the C-terminus and the b-type ions are numbered from the N-terminus. The abundant ion at m/z 790.9 corresponds to the β-elimination of phosphate from the precursor ion. *Lower panel*: MS/MS spectrum of the doubly phosphorylated $(M+2H)^{2+}$ ion of m/z 879.8. Those ion designations with one or two open circles above them indicate additional β-elimination of one or two phosphates, respectively. Closed circles above the sequence indicate phosphorylation sites.

peptide into three fragments that were identified as ^{330}DDEASTT (peak 1), ^{345}VAPA (peak 2), and ^{337}VSKTETSQ (peak 3) (Figure 3A, Table II). The mono- or diphosphorylated C-terminal peptides were proteolyzed to give ^{345}VAPA (peak 2) and ^{330}DDEASTTVSKTETSQ plus one or two phosphates (Figure 3B,C; peaks 5 and 7, respectively). The triphosphorylated C-terminal peptide was not cleaved at all by thermolysin (Figure 3D).

The differences in the cleavage profiles among the four peptides suggested that a phosphate group was inhibiting cleavage at ^{337}Val. MS/MS analysis of the singly phosphorylated peak 5 (Table II) $(M+2H)^{2+}$ precursor ion (m/z 840.0) was consistent with the sequence ^{330}DDEASTTV(phospho-S)KTETSQ (Figure 4, upper panel). The masses of y1–y12, and related ions resulting from the elimination of H_3PO_4 (98 Da, indicated by open circles), confirm that the phosphate group is located at ^{338}Ser. The y5 and y6 ions that differentiate between phosphorylation of ^{338}Ser and ^{340}Thr are of low abundance and are located in a crowded region of the MS/MS spectrum. This ambiguity was resolved by carboxypeptidase P digestion and is described below. The MS/MS spectrum of the $(M+2H)^{2+}$ precursor ion (m/z 879.8) of the same peptide doubly-phosphorylated (Figure 4, lower panel) yielded product ions, y1–y8 and b2–b6, that were identical to those of the monophosphorylated peptide. However, the ions y9–y12, b12, and b13 are 80 Da higher than the corresponding ions in the monophosphorylated peptide. The absence of an ion at m/z 1060.5 (the calculated value of y9 if ^{336}Thr is unphosphorylated) and the presence of an ion of m/z 1140.9 demonstrate that the second phosphate is at position ^{336}Thr. Furthermore, there are additional ions related to y9 by the elimination of one or two molecules of H_3PO_4 (loss of 98 or

Table II: Thermolytic Peptides

peak designation[a]	observed MW[b]	calculated MW[c]	sequence	phosphate no.
1	737.3	737.3	DDEASTT	0
2	356.2	356.2	VAPA	0
3	878.4	878.4	VSKTETSQ	0
4[d]	1936.2	1937.0	DDEASTTVSKTETSQVAPA	0
5	1678.2	1678.6	DDEASTTVSKTETSQ	1
6[d]	2016.8	2017.0	DDEASTTVSKTETSQVAPA	1
7	1758.6	1758.6	DDEASTTVSKTETSQ	2
8[d]	2096.4	2097.0	DDEASTTVSKTETSQVAPA	2
9[d]	2176.6	2176.9	DDEASTTVSKTETSQVAPA	3

[a] Peak designations correspond to those of Figure 3. [b] Molecular weights determined by ES/MS. [c] Average masses reported for peptides 4–9 and monoisotopic masses for peptides 1–3. [d] Uncleaved starting material.

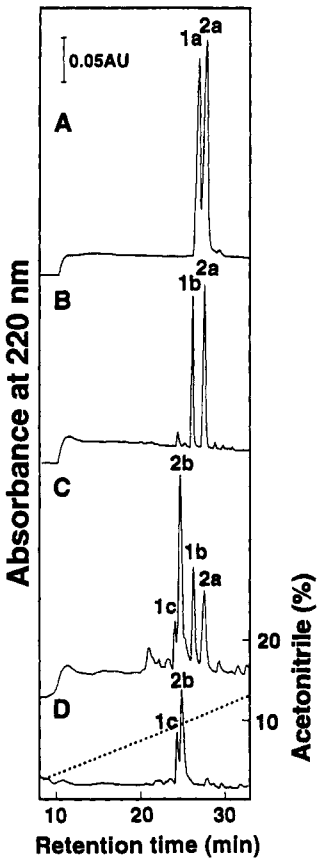

FIGURE 5: Comparison of elution profiles of *S. aureus* proteolytic peptides of non-, mono-, di-, and triphosphorylated C-terminal peptides of Rho. Non- (A), mono- (B), di- (C), or triphosphorylated ^{330}DDEASTTVSKTETSQVAPA (D) (approximately 5–20 nmol) was digested with 2 nmol of *S. aureus* protease V8 in 50 μL of 80 mM Tris-HCl, pH 8.0, at 30 °C for 2 h. Fragments were separated from each other by RP-HPLC as described in the legend to Figure 2A. The peak designations correspond to those in Table III. Peptides 1a–1c are identified as ^{333}ASTTVSKTE in various phosphorylation states (Table III); similarly, peptides 2a and 2b refer to ^{342}TSQVAPA.

196 Da, indicated by single or double open circles in Figure 4, lower panel).

Identical data were obtained for the monophosphorylated peptide from the 4- and 45-min time points, for the diphosphorylated peptide from the 7.5- and 45-min time points, and for the triphosphorylated peptide from the 30- and 45-min time points. Thus, these results suggest that ^{338}Ser is the major phosphorylation site of the monophosphorylated peptide and ^{338}Ser and ^{336}Thr are both phosphorylated in the diphosphorylated peptides. Since the triphosphorylated peptide could not be cleaved by thermolysin (Figure 3D), we could not determine their phosphorylation sites. In addition, there was a significant amount of the undigested components in both the mono- and diphosphorylated peptide (Figure 3B,C), which was not cleaved even when digested for 48 h. Thus, it seemed likely that the phosphorylation sites of the undigested peptides would be different. Additional subdigestions were employed to examine this possibility.

S. aureus Protease V8 Digestion of ^{330}DDEASTTVSKTETSQVAPA. Figure 5 shows the elution profiles after *S. aureus* protease V8 digestion of non- (A), mono- (B), di- (C), and triphosphorylated (D) peptides of the C-terminus of rhodopsin. In all cases, *S. aureus* protease V8 was capable of complete digestion, and each elution profile of digested peptides from the mono-, di-, or triphosphorylated peptide was identical in any time course point examined. The identification of each peak by ES/MS is summarized in Table III. The nonphosphorylated C-terminal peptide is cleaved as

Table III: *S. aureus* Proteolytic Peptides

peak designation[a]	observed MW[b]	calculated MW[c]	sequence	phosphate no.
1a	922.7	922.5	ASTTVSKTE	0
1b	1002.6	1002.5	ASTTVSKTE	1
1c	1082.4	1082.4	ASTTVSKTE	2
2a	672.5	672.4	TSQVAPA	0
2b	752.4	752.4	TSQVAPA	1

[a] Peak designations correspond to those of Figure 5. [b] Molecular weights determined by ES/MS. [c] Calculations used monoisotopic masses.

expected to give ^{333}ASTTVSKTE and ^{342}TSQVAPA (peaks 1a and 2a in Figure 5A), whereas the small peptide ^{330}DDE presumably eluted with the solvent front. *S. aureus* protease V8 digestion of the monophosphorylated C-terminal peptide also resulted in only two major HPLC peaks (Figure 5B)—peak 2a plus a new peak (1b), which by ES/MS corresponded to monophosphorylated ^{333}ASTTVSKTE.

The results for the diphosphorylated peptide are more complicated. Both mono- and diphosphorylated ^{333}ASTTVSKTE (peaks 1b and 1c) and non- and monophosphorylated ^{342}TSQVAPA (peaks 2a and 2b) are observed, thereby verifying that ^{342}TSQVAPA includes a secondary phosphorylation site (Figure 5C, Table III). This heterogeneity disappears in the triphosphorylated C-terminal peptide, which upon digestion with *S. aureus* protease V8 yielded two main HPLC peaks containing diphosphorylated ^{333}ASTTVSKTE and monophosphorylated ^{342}TSQVAPA (peaks 1c and 2b in Figure 5D, Table III). These data suggest that the diphosphorylated C-terminal peptide of rhodopsin is a mixture of two peptides, both phosphorylated on ^{338}Ser, and differing in secondary phosphorylation on ^{336}Thr, or in ^{342}TSQVAPA. It is apparent from the ratio of the monophosphorylated peptide 2b to the nonphosphorylated peptide 2a in Figure 5C that the ^{342}TSQVAPA sequence is the favored secondary phosphorylation site.

The MS/MS spectrum of the $(M+2H)^{2+}$ ion of monophosphorylated ^{333}ASTTVSKTE is shown in the upper panel of Figure 6. This spectrum confirms the deduction from the spectra of Figure 4. Neither spectrum, however, was unambiguous with respect to differentiating between phosphorylation at ^{338}Ser or ^{340}Thr. As shown in Figure 7, carboxypeptidase P digestion of this monophosphorylated peptide yielded three truncated *phospho*peptides which, by ES/MS, corresponded to ^{333}ASTTVSKT (peak 1), ^{333}ASTTVSK (peak 2), and ^{333}ASTTVS (peak 3) (digestion is greatly reduced at phosphorylated residues). Thus, ^{338}Ser was confirmed as the only phosphorylation site in this singly phosphorylated peptide.

The MS/MS spectrum of diphosphorylated ^{333}ASTTVSKTE is shown in the lower panel of Figure 6, which demonstrates that ^{336}Thr is phosphorylated in addition to ^{338}Ser. Identical MS/MS spectra were acquired from different sources; i.e., the spectra of 1b derived from the mono- and diphosphorylated C-terminal peptides of Rho were identical, as were the spectra of 1c derived from the di- and triphosphorylated peptides. On the other hand, MS/MS spectra of monophosphorylated ^{342}TSQVAPA yielded no collision-induced dissociations between ^{342}Thr and ^{343}Ser, and this assignment remained undifferentiable. To make this determination, monophosphorylated ^{342}TSQVAPA from di- and triphosphorylated peptide obtained by *S. aureus* protease V8 digestion were treated separately with leucine aminopeptidase, and residual peptides were purified by HPLC (Figure 8). ES/MS and MS/MS spectra from either peptide demonstrated the presence of monophosphorylated ^{343}SQVAPA (peak 2) and monophosphorylated ^{343}SQVAP (peak 3), but no sig-

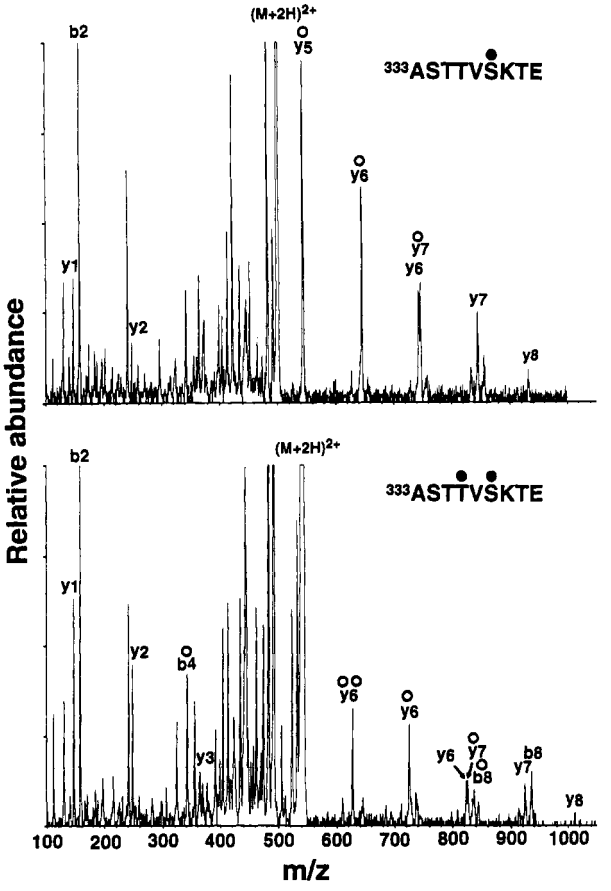

FIGURE 6: MS/MS spectra of mono- and diphosphorylated ^{333}AST-TVSKTE. *Upper panel*: MS/MS spectrum of the singly phosphorylated $(M+2H)^{2+}$ ion of m/z 502.0. *Lower panel*: MS/MS spectrum of the doubly phosphorylated $(M+2H)^{2+}$ ion of m/z 542.0. The abundant ion at m/z 241.9 in both panels corresponds to elimination of H_2O from b3. The ion at m/z 423.2 in the upper panel is doubly charged y7; its counterpart in the lower panel (m/z 462.8) is obscured by several abundant ions corresponding to multiple eliminations of H_3PO_4 and H_2O. Those ion designations with one or two open circles above them indicate additional β-elimination of one or two phosphates, respectively. Closed circles above the sequence indicate phosphorylation sites.

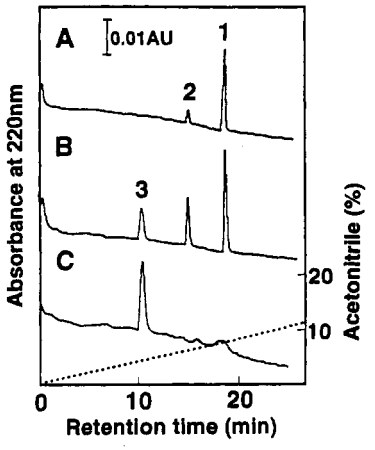

FIGURE 7: Elution profiles of carboxypeptidase P digestion of monophosphorylated ^{333}ASTTVSKTE. *Upper panel*: Monophosphorylated ^{333}ASTTVSKTE (approximately 2 nmol) was digested with 0.2 nmol of carboxypeptidase P in 50 μL of 50 mM sodium acetate buffer, pH 4.6, at 30 °C for 0.5 h (A), 2 h (B), and 16 h (C). The residual peptides were purified by RP-HPLC as described in the legend to Figure 2.

nificant signals were obtained from peak 1. Evidently, the aminopeptidase was contaminated with a carboxypeptidase that was unable to proteolyze beyond the C-terminal proline.

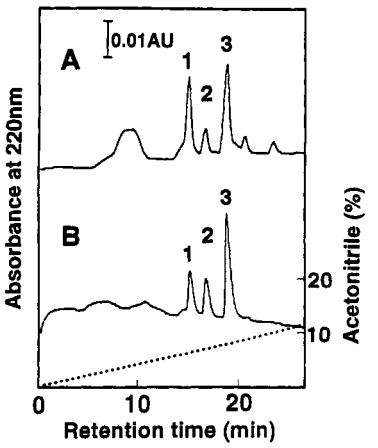

FIGURE 8: Elution profiles of leucine aminopeptidase digestion of monophosphorylated ^{342}TSQVAPA. Monophosphorylated ^{342}TSQVA-PA (approximately 2 nmol) from di- (A) or triphosphorylated peptide (B) was digested with 120 pmol of leucine aminopeptidase in 50 μL of 80 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$ at 30 °C for 2 h. The residual peptides were purified by RP-HPLC as described in the legend to Figure 2.

Taken together, these studies demonstrate that ^{338}Ser is the initial phosphorylation site and that ^{343}Ser and ^{336}Thr are subsequently phosphorylated. Additional phosphorylation may occur at other locations, but at very low levels (e.g., peak w in Figure 2 and Table I).

DISCUSSION

In this study, we employ techniques of proteolysis and mass spectrometry to identify the consecutive phosphorylation sites on Rho. A homogenate of ROS was used to maintain all of the components of phototransduction present during the phosphorylation reaction, and saturable light condition was employed. We found that phosphorylation is sequential with the first phosphorylation site at ^{338}Ser followed by phosphorylation at either ^{343}Ser or ^{336}Thr. These findings are consistent with earlier studies of the phosphorylation of Rho, which suggested that Ser was phosphorylated to a greater extent than Thr (Kühn & Dreyer, 1972). Similar positions of phosphorylation were identified by Thompson and Findlay (1984) using direct Edman degradation, a method less reliable for identification of the phosphorylation sites.

Recent studies suggest that binding of RK to the V–VI loop of Rho* (Figure 9) is essential for RK activation and that the interaction with the site of phosphorylation, the C-terminal region, is weak (Fowles et al., 1988; Palczewski et al., 1991; Brown et al., 1992), perhaps to permit multiple phosphorylations. RK is known to prefer an acidic residue near phosphorylatable residues (Palczewski et al., 1989; Onorato et al., 1991). During RK activation, the phosphorylation of ^{338}Ser may neutralize the neighboring positively charged ^{339}Lys and facilitate subsequent phosphorylation. Second phosphorylation sites are located close to Val residues (^{337}Val or ^{345}Val), suggesting that hydrophobic interaction may be required for the second site of phosphorylation (Figure 9).

In photoreceptor cells, quenching of the light signal starts with phosphorylation of Rho* by RK [reviewed by Palczewski and Benovic (1991) and Hofmann et al. (1992)]. The extent and half-life of phosphorylation *in vivo* are unknown, but biochemical data suggest that one to nine phosphates could be incorporated into Rho* during the phosphorylation reaction (Wilden & Kühn, 1982; Miller et al., 1986). Functionally, on the other hand, it was suggested that one to two phosphate moieties per Rho are required for rapid inactivation of Rho* (Sitaramayya, 1986). It is conceivable that more extensive

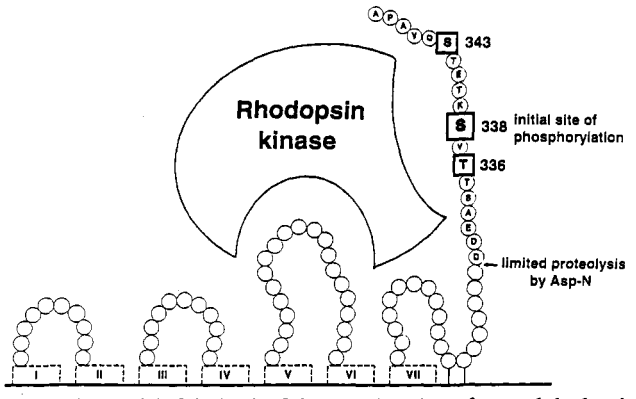

FIGURE 9: Model of the bovine Rho cytoplasmic surface and rhodopsin kinase. This model is a modification of the secondary structure of rhodopsin by Dratz and Hargrave (1983) and Karnik and Khorana (1990). I–VII are putative transmembrane α-helical segments. RK binds the V–VI loop (Palczewski et al., 1991; Brown et al., 1992) and interacts with the C-terminal segment of Rho (Palczewski et al., 1988, 1991; Fowles et al., 1988). The C-terminal 19 amino acids, ^{330}DDEASTTVSKTETSQVAPA, are cleaved from rhodopsin by endoproteinase Asp-N (Palczewski et al., 1991). ^{338}Ser is the major initial phosphorylation site, and ^{343}Ser and ^{336}Thr are subsequent phosphorylation sites.

Rho phosphorylation (4–7 P_i/Rho) is an artifact of biochemical procedures and that only these three ^{338}Ser, ^{343}Ser, or ^{336}Thr residues are critical for binding of arrestin and quenching of the phototransduction cascade. Identification of these sites allows us to now address this problem. The newly developed methodology allows further evaluation of the phosphorylation sites in Rho using intact retina and mutants of Rho lacking these phosphorylatable residues. Furthermore, these procedures may facilitate a similar approach for the evaluation of phosphorylation of other G-protein-coupled receptor systems, such as β-adrenergic receptor.

REFERENCES

Bennett, N., & Sitaramayya, A. (1988) *Biochemistry 27,* 1710–1715.

Biemann, K. (1990a) *Methods Enzymol. 193,* 455–479.

Biemann, K. (1990b) *Methods Enzymol. 193,* 886–887.

Biemann, K. (1992) *Annu. Rev. Biochem. 61,* 977–1010.

Brown, N. G., Fowles, C., Sharma, R., & Akhtar, M. (1992) *Eur. J. Biochem. 208,* 659–667.

Covey, T. R., Bonner, R. F., Shushan, B. I., & Henion, J. (1988) *Rapid Commun. Mass Spectrom. 2,* 249–256.

Dratz, E. A., & Hargrave, P. A. (1983) *Trends Biol. Sci. 8,* 128–131.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989) *Science 246,* 64–71.

Findlay, J. B. C., Brett, M., & Pappin, D. J. C. (1981) *Nature 293,* 314–316.

Fowles, C., Sharma, R., & Akhtar, M. (1988) *FEBS Lett. 238,* 56–60.

Hargrave, P. A., & Fong, S.-L. (1977) *J. Supramol. Struct. 6,* 559–570.

Hofmann, K. P., Pulvermüller, A., Buczyłko, J., Van Hooser, P., & Palczewski, K. (1992) *J. Biol. Chem. 267,* 15701–15706.

Hunt, D. F., Yates, J. R., Shabanowitz, J., Winston, S., & Hauer C. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83,* 6233–6237.

Karnik, S. S., & Khorana, H. G. (1990) *J. Biol. Chem. 265,* 17520–17524.

Kühn, H., & Dreyer, W. J. (1972) *FEBS Lett. 20,* 1–6.

Miller, J. L., Fox, D. A., & Litman, B. J. (1986) *Biochemistry 25,* 4983–4988.

Molday, R. S., & MacKenzie, D. (1985) *Biochemistry 24,* 776–781.

Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., & Benovic, J. L. (1991) *Biochemistry 30,* 5118–5125.

Palczewski, K., & Benovic, J. L. (1991) *Trends Biol. Sci. 16,* 387–391.

Palczewski, K., McDowell, J. H., & Hargrave, P. A. (1988) *Biochemistry 27,* 2306–2313.

Palczewski, K., Arendt, A., McDowell, J. H., & Hargrave, P. A. (1989) *Biochemistry 28,* 8764–8770.

Palczewski, K., Buczyłko, J., Kaplan, M. W., Polans, A. S., & Crabb, J. W. (1991) *J. Biol. Chem. 266,* 12949–12955.

Papermaster, D. S. (1982) *Methods Enzymol. 81,* 48–52.

Sitaramayya, A. (1986) *Invest. Ophthalmol. Visual Sci., Suppl. 27,* 217.

Smith, R. D., Loo, J. A., Ogorzalek, R. R., Busman, M., & Udseth, H. R. (1991) *Mass Spectrom. Rev. 10,* 359–451.

Thompson, P., & Findlay, J. B. C. (1984) *Biochem. J. 220,* 773–780.

Wilden, U., & Kühn, H. (1982) *Biochemistry 21,* 3014–3022.

Wilden, U., Hall, S. W., & Kühn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83,* 1174–1178.