Mass Spectrometric Identification of Phosphorylation Sites in Bleached Bovine Rhodopsin†

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ABSTRACT: Deactivation of the visual cascade is initiated by the phosphorylation of rhodopsin. We report here identification of the two major sites of phosphorylation in bleached bovine rhodopsin using tandem mass spectrometry in conjunction with synthetic phosphopeptide standards. Both bleached and unbleached rod outer segments were cleaved with endoproteinase Asp-N to release the C-terminal fragment, residues 330–348, containing seven potential sites of phosphorylation. High-performance liquid chromatographic separation of soluble cleavage products from both unbleached and bleached rod outer segments gave a peak which was identified by tandem mass spectrometry and comparison to synthetic standards as monophosphorylated (serine 338) DDEASTTVSKTETSQVAPA. Present only in the chromatogram of bleached ROS were two peaks identified as monophosphorylated (serine 343) and diphosphorylated (serines 338 and 343) derivatives of DDEASTTVSKTETSQVAPA. These results identify serines 338 and 343 as the major sites of phosphorylation within the C-terminal region of bleached bovine rhodopsin and constitute the first example of mass spectrometric characterization of phosphorylation sites in a G-protein coupled receptor.

Rhodopsin, the light receptor protein of retina rod cells, is the prototype of a large family of G-protein[1] coupled receptors. Upon irradiation with light, 11-*cis*-retinal bound via a Schiff base linkage to rhodopsin isomerizes to *all-trans*-retinal and causes a conformational change in the protein. This activated form of rhodopsin (R^*) is capable of binding and subsequently activating the G-protein, transducin (Kühn, 1984). After binding to rhodopsin, transducin exchanges GDP for GTP and releases the transducin α-subunit–GTP complex (Kühn, 1984). The α-subunit–GTP complex stimulates a cGMP phosphodiesterase which decreases the level of cGMP, ultimately leading to a hyperpolarization of the rod cell (Hurley, 1987; Stryer, 1991).

Deactivation of the visual cascade is achieved at multiple sites in the biochemical pathway. Transducin itself has intrinsic GTPase activity which inactivates itself over time (Chabre & Deterre, 1989). However, inactivation of the α-subunit–GTP complex is insufficient to quench the photocycle. R^* must also be deactivated, and this is accomplished by the phosphorylation of the C-terminal portion of rhodopsin by rhodopsin kinase (Shichi, 1989). This phosphorylation of rhodopsin promotes the binding of arrestin which prevents further activation of transducin (Wilden et al., 1986).

Bleached rhodopsin has been reported to incorporate up to 9 mol of phosphate per mole of rhodopsin (Wilden & Kühn, 1982). On the basis of proteolysis studies, the sites of phosphorylation by both rhodopsin kinase and protein kinase C have been shown to occur in the C-terminal portion of the molecule (Palczewski et al., 1991; Newton & Williams, 1991). Concentrated between residues 333 and 343 in the C-terminus of rhodopsin are seven hydroxyamino acids, the region thought to contain most of the phosphorylation sites (Palczewski et al., 1991; Thompson & Findlay, 1984). Using mass spectrometry and synthetic reference standards we have identified the two major sites of phosphorylation, serines 338 and 343, within the 19 amino acid C-terminal segment of bleached bovine rhodopsin.

MATERIALS AND METHODS

Materials. Frozen bovine retinae were obtained from Hormel (Austin, MN). Endoproteinase Asp-N, endoproteinase Glu-C, alkaline phosphatase, okadaic acid, adenosine triphosphate (ATP), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). Acetonitrile and trifluoroacetic acid (TFA) were obtained from Burdick and Jackson (Muskegon, MI).

Generation of C-Terminal Peptides. Rod outer segments (ROS) were isolated from 50 bovine retinae by the method of McDowell and Kühn (1977). After the first centrifugation step, the float was incubated with 17 OD (measured at 379 nm in ethanol) of 11-*cis*-retinal in an ice bath for 45 min. Following the isolation, half of the ROS were bleached with white light and half were treated as the bleached ROS, except they were not illuminated. Bleaching was performed on the ROS in 0.1 M sodium phosphate (pH 7.4) containing 1 mM $MgCl_2$ and 4 mM ATP under continuous illumination with white light for 30 min at 37 °C as described by Wilden and Kühn (1982). After 30 min, 1 M NaF was added to a final concentration of 10 mM to inhibit phosphatase activity (Cohen et al., 1991). The ROS were then washed with 10 mM Tris-HCl (pH 7.8) containing 50 mM NaF and 10 mM $NaPP_i$ and cleaved with endoproteinase Asp-N overnight at 37 °C employing an enzyme to substrate ratio of 1:6000 by weight. The reaction was terminated by the addition of 0.5 M EDTA

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[1] Abbreviations: G-protein, guanyl nucleotide-binding regulatory protein; R^*, activated rhodopsin; GDP, guanosine diphosphate; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; ROS, rod outer segments; HPLC, high-performance liquid chromatography; Boc, *tert*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; LSIMS, liquid secondary ion mass spectrometry; MS/MS, tandem mass spectrometry.

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(pH 8.0) to bring the final concentration to 50 mM. The samples were then centrifuged at 100000*g*, and the supernatant was evaporated on a Speed Vac dryer (Savant Instruments Inc., Farmingdale, NY). Samples were then dissolved in 0.1% aqueous TFA and separated by reversed-phase high-performance liquid chromatography (HPLC).

Reversed-Phase HPLC. Separation of the endoproteinase Asp-N and Glu-C digests was performed on a Vydac 218TP54 C-18 column 4.6 × 250 mm (Hesperia, CA). The mobile phase consisted of 0.1% aqueous TFA (A) and acetonitrile containing 0.084 % TFA (B). The gradient was 98% A held for 10 min and then ramped linearly over 80 min to 40% B. The effluent was monitored at 214 nm, and fractions were collected in 2.0-mL polypropylene tubes and then lyophilized.

Endoproteinase Glu-C Cleavage of C-Terminal Peptides. The peptide DDEASTTVSKTETSQVAPA and its phosphorylated derivatives were digested overnight at 37 °C with 3 μg of endoproteinase Glu-C in 0.1 M sodium phosphate (pH 7.8). Samples were then injected directly into the HPLC system, and fractions were collected and lyophilized.

Alkaline Phosphatase Treatment of C-Terminal Peptides. The phosphorylated derivatives of DDEASTTVSKTETSQVAPA were dephosphorylated overnight at 37 °C in 10 mM Tris (pH 9.8) with 1.5 μg of bovine intestinal mucosa alkaline phosphatase. Dephosphorylated samples were injected directly into the HPLC system, and fractions were collected and then lyophilized.

Synthesis of Reference Peptides. The peptide DDEASTTVSKTETSQVAPA was prepared on an ABI 430A synthesizer using standard *tert*-butyloxycarbonyl (Boc) chemistry. All monophosphorylated derivatives of the peptide ASTTVSKTE were manually prepared as described (Knapp et al., 1993). Syntheses of the monophosphorylated derivatives of TSQVAPA and DDEASTTVSKTETSQVAPA were performed on "Wang" resin using standard solid-phase 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry using the same synthesis system (Knapp et al., 1993). Serine or threonine residues to be phosphorylated were coupled with the hydroxyl group unprotected. Phosphitylation and oxidation were performed as previously described (Andrews et al., 1991) except the phosphitylation reaction was increased to 24 h, and the oxidation was performed with *m*-chloroperbenzoic acid for 2 h. Deprotection and cleavage was achieved with TFA. Following evaporation of the cleavage solution under argon, the crude phosphopeptides were washed with ether and purified by reversed-phase HPLC. Sequences of the peptides were verified by tandem mass spectrometry.

Mass Spectrometry. Mass spectra were obtained on a JEOL HX110/HX110 four sector tandem mass spectrometer (JEOL, Tokyo, Japan). Samples were dissolved in 10 μL of 1 N HCl and applied to the probe in a matrix of 1 N HCl/thioglycerol. Eighteen-kilovolt cesium ions were used for liquid secondary ion mass spectrometry (LSIMS). Single stage mass analysis was performed with an accelerating voltage of 10 kV and a resolution of 1500. The tandem mass spectrometry (MS/MS) experiments were performed with the collision cell operated at 3 kV and filled with enough helium to attenuate the ion current of the C-12 monoisotopic peak by 70%.

RESULTS AND DISCUSSION

Our goal was to identify the sites of phosphorylation in bleached bovine rhodopsin. We focused on identifying the phosphorylation sites within the hydroxyamino acid rich C-terminus of rhodopsin, since most of the phosphorylation occurs between residues 334 and 348 (Palczewski et al., 1991).

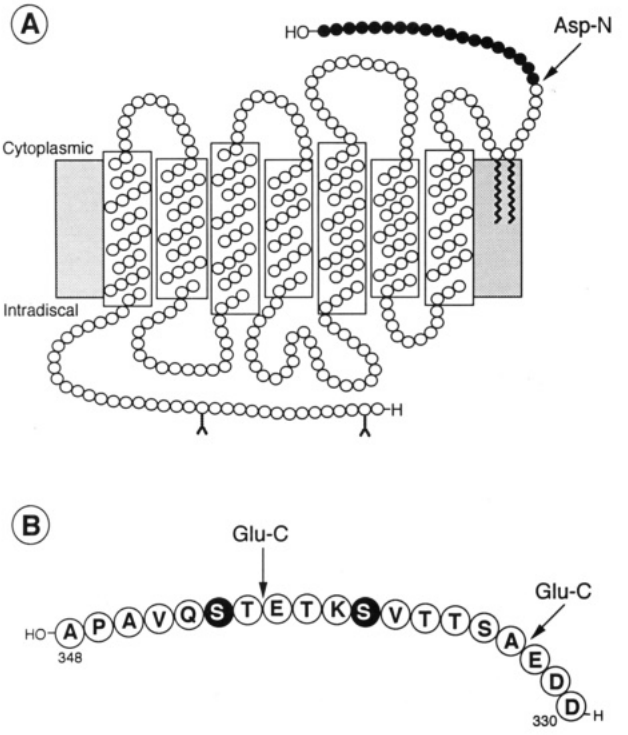

FIGURE 1: Sites of enzymatic cleavage within bovine rhodopsin. (A) The 348 amino acid residues of bovine rhodopsin are depicted crossing the lipid membrane seven times. Also depicted in the drawing are the two palmityl groups attached to cysteines 322 and 323 embedded in the lipid bilayer and the two oligosaccharides on the N-terminus of the protein. Asp-N indicates the site of cleavage by endoproteinase Asp-N, and the shaded spheres indicate the C-terminal peptide released into the supernatant upon cleavage. (B) An enlarged view of the C-terminal peptide which contains seven hydroxyamino acids. Glu-C indicates the sites of cleavage within the C-terminal peptide by endoproteinase Glu-C. The shaded spheres indicate the two sites of phosphorylation on serine 343 and serine 338.

Endoproteinase Asp-N selectively cleaves ROS and releases a peptide containing the last 19 amino acids of rhodopsin, residues 330–348 (Palczewski et al., 1991) (Figure 1A). Use of this enzyme combined with tandem mass spectrometry has enabled us to identify two sites of phosphorylation on serines 338 and 343 of bleached bovine rhodopsin (Figure 1B).

Following isolation and bleaching of ROS, both bleached and unbleached ROS were cleaved with endoproteinase Asp-N and subsequently centrifuged. In the supernatant are found the C-terminal peptide (residues 330–348), its phosphorylated derivatives, and other soluble peptides and proteins. The lyophilized supernatants were separated by reversed-phase HPLC (Figure 2). The major peak present in both Figure 2A and 2B, eluting at 50.2 min, is the unmodified C-terminal peptide, DDEASTTVSKTETSQVAPA. The peptide had the same MH^+ ion mass of 1936.8 Da by LSIMS and the same retention time as the synthetic standard.

The chromatogram of bleached ROS contains three large peaks not obviously apparent in the chromatogram of the unbleached ROS (Figure 2A). Peaks 2 and 3 have retention times of 47.8 and 48.7 min, respectively (inset, Figure 2B). When analyzed by LSIMS, both peaks contained peptides with MH^+ ions of 2016.8 Da. This corresponded to an increase in molecular mass of 80 Da over the MH^+ ion for the C-terminal peptide DDEASTTVSKTETSQVAPA, suggesting the incorporation of either one phosphate or sulfate group. The presence of phosphate in both peptides from peaks 2 and 3 was verified by treating the peptides with alkaline phosphatase. HPLC separation of the peptides generated from

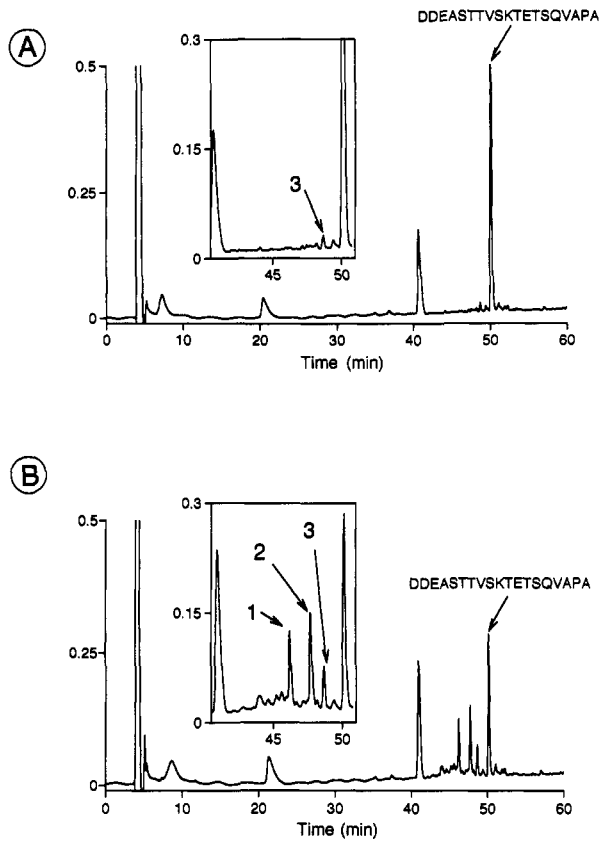

FIGURE 2: Reversed-phase HPLC separation of the supernatant from the endoproteinase Asp-N cleavage of ROS. (A) Chromatogram from the supernatant of unbleached ROS cleaved with endoproteinase Asp-N. The large peak at 50.2 min corresponds to the indicated peptide sequence for the C-terminal 19 amino acids of bovine rhodopsin. The inset shows an expanded region of the chromatogram between 41.5 and 51.5 min. The "3" indicates the peak which contains the phosphorylated peptide DDEASTTVS(p)KTETSQVAPA. (B) Chromatogram from the supernatant of bleached ROS cleaved with endoproteinase Asp-N. The large peak at 50.2 min corresponds to the indicated peptide sequence for the C-terminal 19 amino acids of bovine rhodopsin. The inset shows an expanded region of the chromatogram between 41.5 and 51.5 min. The numbered peaks correspond to the diphosphorylated species DDEASTTVS(p)KTETS(p)QVAPA (1) and the two monophosphorylated derivatives, DDEASTTVSKTETS(p)QVAPA (2) and DDEASTTVS(p)KTETSQVAPA (3).

alkaline phosphatase treatment of the peptides in both peaks 2 and 3 showed a shift in retention time to 50.2 min, suggesting that a phosphate group was removed from the peptide DDEASTTVSKTETSQVAPA. This suspicion was confirmed by LSIMS analysis which showed a shift of the MH^+ ion from 2016.8 to 1936.8 Da, a decrease of 80 Da corresponding to a loss of phosphate (data not shown). To help identify which of the monophosphorylated derivatives of DDEASTTVSKTETSQVAPA peaks 2 and 3 corresponded to, all seven of the monophosphorylated derivatives of the peptide DDEASTTVSKTETSQVAPA were synthesized. The retention times of the synthetic monophosphorylated derivatives were compared to the retention times for peaks 2 and 3. The synthetic peptides DDEASTTVSKTETS(p)QVAPA and DDEASTTVS(p)KTETSQVAPA were found to have the same retention times as peaks 2 and 3, respectively.

The LSIMS data, alkaline phosphatase experiments, and comparison of retention times to synthetic standards indicated we had isolated two monophosphorylated derivatives of DDEASTTVSKTETSQVAPA. To identify which amino acid was phosphorylated in the two monophosphorylated peptides, MS/MS of the peptides was required. However, due to the size of the peptides and their very polar nature, insufficient ion signal was obtained to generate a readily interpretable MS/MS spectrum to allow localization of the phosphate groups within the sequence. We therefore cleaved the monophosphorylated peptides with endoproteinase Glu-C to obtain smaller peptides containing the phosphorylated residues, which would be easier to sequence (Figure 1B). Endoproteinase Glu-C was chosen because it has not demonstrated resistance to cleavage by neighboring phosphoamino acid residues as has been shown for trypsin (Petrilli et al., 1986; Cohen et al., 1991). Cleavage of DDEASTTVSKTETSQVAPA with endoproteinase Glu-C generated two major fragments eluting at 27.8 and 29.1 min (data not shown). When analyzed by LSIMS, the fragment at 27.8 min had a MH^+ ion of 923.4 Da which corresponded to the MH^+ ion predicted for the peptide ASTTVSKTE. The fragment at 29.1 min had a MH^+ ion of 673.3 Da corresponding to the MH^+ ion predicted for the peptide TSQVAPA. When both peptides were analyzed by MS/MS, interpretable spectra were obtained and the sequences verified. Using this enzyme cleavage strategy, we could reduce the number of potential phosphorylation sites on a single peptide from 7 to 5 and 2, plus generate smaller peptides which could be more easily fragmented to obtain sequence information. The expected peptide DDE was not usually observed, especially when long cleavage times were employed.

Cleavage of the peptide in peak 2 with endoproteinase Glu-C yielded two major fragments with retention times of 25.3 and 27.8 min. LSIMS analysis of the two peptides revealed MH^+ ions of 753.3 Da for the peak eluting at 25.3 min and 923.4 Da for the peak eluting at 27.8 min. On the basis of the molecular weights observed and the retention time of the two peaks, the peak eluting at 27.8 min corresponded to the peptide ASTTVSKTE. The retention time of the peptide eluting at 25.3 min was almost 4 min less than the peptide TSQVAPA, implying that the polar phosphate group was contained on TSQVAPA. The appropriate increase in molecular mass of 80 Da was observed for this peptide. MS/MS verified that the phosphate group was on serine 343 (Figure 3). The most important ions for verifying the location of the phosphate group were the immonium ion at m/z 140.0, which indicates the presence of phosphoserine, and the a_1 ion at m/z 74.0, which indicates the threonine is not modified.[2] The b_2 ion at m/z 269.1 is observed and is shifted to higher molecular mass by 80 Da over the b_2 ion for the corresponding unmodified peptide, indicating that the phosphate is associated with the serine residue. Also observed in the spectra is a large loss of 98 Da due to a loss of H_3PO_4 corroborating the earlier results with the alkaline phosphatase, namely, that the peptide is modified by phosphate. Because so few ions are predicted to differ between the MS/MS spectrum for the peptides T(p)SQVAPA and TS(p)QVAPA, the synthetic standards were analyzed by MS/MS and compared (data not shown). Comparison of the MS/MS spectra from the synthetic standards to the isolated peptide confirmed our assignment of the phosphate to serine 343.

Cleavage of the peptide in peak 3 with endoproteinase Glu-C yielded two major fragments with retention times of 26.9 and 29.1 min. On the basis of the retention time, the fragment at 29.1 min was the peptide TSQVAPA. This peptide assignment was confirmed by observance of the predicted MH^+ ion at m/z 673.3 by LSIMS. The peptide at 26.9 min eluted earlier than the peptide ASTTVSKTE (retention time 27.8

[2] Peptide fragment ion nomeclature from Biemann (1990).

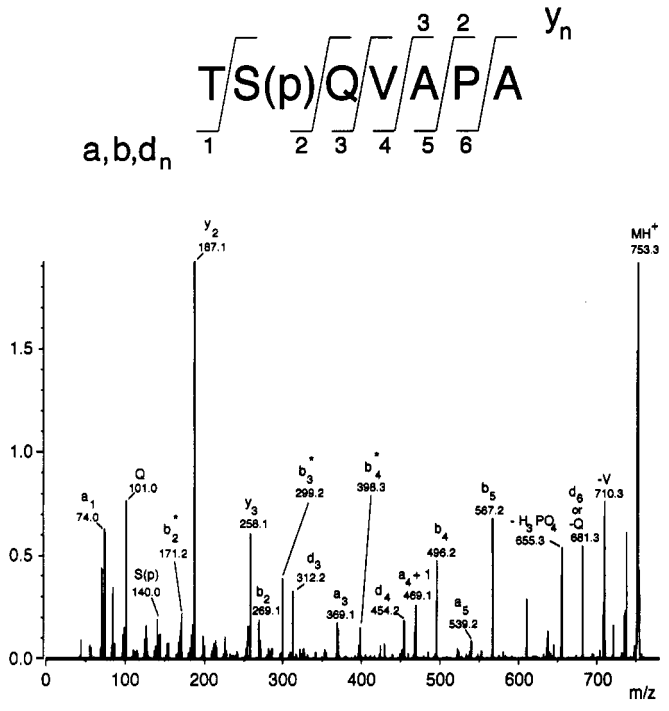

FIGURE 3: Tandem mass spectrum of the monophosphorylated peptide TS(p)QVAPA. Product ion spectrum of a fragment of m/z 753.3 generated by endoproteinase Glu-C digestion of the monophosphorylated peak 2 (Figure 2B). The asterisks indicate ions with an additional loss of H_3PO_4. The numbering above the single-letter code refers to the y_n-ions formed, and the numbering below refers to the a_n-, b_n-, and d_n-ions formed by cleavage of the peptide. Peptide fragment nomenclature is from K. Biemann.[2]

min) indicating that the phosphate group was on the peptide ASTTVSKTE. When analyzed by LSIMS, a MH^+ ion of 1003.4 Da was observed, 80 Da higher than the peptide ASTTVSKTE (MH^+ = 923.3 Da), confirming the presence of a phosphate group. MS/MS analysis of the MH^+ ion at 1003.4 Da yielded a fragment ion spectrum; however, due to losses of H_2O and H_3PO_4, location of the phosphorylation site was ambiguous. Although no clear ion sequence was observed to readily determine the exact location of the phosphate group, MS/MS analysis of all five synthetic monophosphorylated derivatives of ASTTVSKTE yielded clearly discernible spectra which could be compared to the isolated peptide. Comparison of the five spectra indicated that serine 338 is the site of attachment of the phosphate in the isolated peptide. Analysis of the fragmentation patterns of the five different phosphopeptides indicated that the peptides containing phosphothreonine demonstrate a characteristic side chain cleavage loss of 125 Da. This loss of 125 Da was not observed for the phosphoserine-containing derivatives of ASTTVSKTE or the isolated phosphopeptide, thereby reducing the possible sites of phosphorylation to one of two serine residues. The presence of the b_2 ion at m/z 159.1 indicated that the phosphate group was not on serine 334. The presence of a w_8 ion at m/z 899.4 and an x_4 ion at m/z 570.3 indicated that the phosphate was indeed attached to serine 338.

A small peak observed in the chromatogram for unbleached ROS (Figure 2A inset) had the same retention time (48.7 min) and MH^+ ion (2016.8 Da) as peak 3 from bleached ROS. The location of the phosphate on the peptide in peak 3 from unbleached rhodopsin was determined by MS/MS and shown to also occur at serine 338. The presence of a phosphorylated peptide from unbleached ROS suggests the possibility of constitutive phosphorylation of serine 338; alternatively, the phosphorylation of serine 338 may have occurred during harvesting of the retinae. To ensure that any ROS bleached during harvesting were in a dark-adapted state, 11-*cis*-retinal was added to the float during the first step of isolation. Addition of 11-*cis*-retinal alone, however, may be insufficient to bring bleached ROS back to a fully dark-adapted state in frozen retinae. The question therefore remains as to whether rhodopsin is constitutively phosphorylated or whether we observed a fraction of bleached rhodopsin in our "dark-adapted" preparation.

Peak 1 observed in the chromatogram of bleached ROS had a retention time of 46.3 min, 1.5 min earlier than the earliest eluting monophosphorylated derivative (Figure 2B, inset). Analysis of this peptide by LSIMS produced an MH^+ ion of 2096.8 Da suggesting a diphosphorylated derivative of DDEASTTVSKTETSQVAPA. Dephosphorylation with alkaline phosphatase shifted the retention time to 50.2 min and the MH^+ ion to 1936.8 Da indicating that the peptide was a diphosphorylated derivative of DDEASTTVSKTETSQVAPA. Endoproteinase Glu-C digestion of this peptide and subsequent chromatography produced two major fragments with retention times of 25.3 and 26.9 min. The retention time of both peaks was decreased relative to TSQVAPA and ASTTVSKTE, suggesting that one phosphate was located on each peptide fragment. LSIMS analysis confirmed that both peptides were monophosphorylated since MH^+ ions of m/z 753.3 and 1003.4 Da were observed. MS/MS analysis confirmed the location of the two phosphates on serines 338 and 343.

LSIMS analysis of the smaller peaks present in the chromatogram of bleached ROS revealed several di-, tri-, and tetraphosphorylated derivatives of DDEASTTVSKTETSQVAPA based upon MH^+ ions of 2096.8, 2176.8, and 2256.8 Da, respectively. These minor components were too small to obtain sequence information. The average number of phosphates incorporated into rhodopsin after bleaching varies from 1 to 7 mol of phosphate per mole of rhodopsin, depending upon the isolation and bleaching conditions used (Aton et al., 1984; McDowell & Kühn, 1977; Sale et al., 1978; Thompson & Findlay, 1984; Wilden & Kühn, 1982). To ensure maximal phosphorylation of rhodopsin, we used the bleaching method of Wilden and Kühn (1982) since they have reported the highest degree of phosphorylation. Our average level of phosphorylation was closer to one than to seven. Our low average level of phosphorylation may have been a result of our isolation conditions (McDowell & Kühn, 1977) or our use of frozen instead of fresh retinae; both of which could have resulted in loss of the kinases responsible for phosphorylation.

On the basis of the areas for peaks 1, 2, and 3, serines 338 and 343 are the major sites of phosphorylation in the C-terminal portion of bleached bovine rhodopsin (Figure 2B). Ovine rhodopsin has the same sequence from residue 319 to the C-terminal as bovine rhodopsin (Thompson & Findlay, 1984). Thompson and Findlay have sequenced phosphorylated ovine rhodopsin by Edman degradation and have found that both serines 338 and 343 are phosphorylated, as well as serine 334 and threonines 335 and 336 (Thompson & Findlay, 1984). Potential phosphorylation of neighboring sites combined with inherent carry-over of ^{32}P during Edman degradation leaves some ambiguity in the assignments of phosphorylation of the threonine residues. The amount of ^{32}P released at the specific cycles of Edman degradation in this work suggests that serines 338 and 343 are the major sites of phosphorylation in bleached ovine rhodopsin, analogous to our findings in bovine rhodopsin (Thompson & Findlay, 1984). Brown et al. (1992) using

synthetic peptide fragments from bovine rhodopsin have shown that serine residues are predominantly phosphorylated, supporting our findings in the intact protein. Additionally, rhodopsin kinase has been suggested to have a preference for serine, and our results support this hypothesis (Palczewski et al., 1989a). Brown et al. (1992) concluded that serine 343 is the preferred site of phosphorylation. The peak areas of peaks 2 and 3 (Figure 2B) support the notion that serine 343 is the preferred site of phosphorylation; however, no conclusions can be drawn from our work regarding the order of phosphorylation of serines 338 and 343.

Interestingly, when phosphatase inhibitors were omitted during endoproteinase Asp-N cleavage, only peaks 2 and 3 were observed, with peak 3 being twice the area of peak 2. This chromatographic profile suggests an order to the dephosphorylation of bovine rhodopsin with serine 343 being preferentially dephosphorylated. The slower dephosphorylation of serine 338 may be the result of the serine being preceded by a lysine. Phosphorylation sites preceded by a lysine have been shown to be resistant to dephosphorylation by alkaline phosphatase (Xu et al., 1992). A phosphatase has been purified from ROS that has specificity toward phosphorylated rhodopsin. (Palczewski et al., 1989b; Fowles et al., 1989) The apparent dephosphorylation in our experiments also suggests that this phosphatase may be membrane associated, since it would have been lost during the washing steps prior to endoproteinase Asp-N cleavage. No observable dephosphorylation of peptides occurred during endoproteinase Glu-C digestion. This was verified by performing the digestion in the presence of 5 μM okadaic acid. The presence of a phosphatase in endoproteinase Asp-N or Glu-C is unlikely since the both enzymes are HPLC purified.

Mass spectrometry offers advantages over conventional methods used to study protein phosphorylation. Mass spectrometry can be used to determine constitutively phosphorylated sites that cannot be measured by techniques requiring radiolabeled phosphate. With particular regards to the visual system, use of ^{32}P causes generation of Cerenkov radiation which produces light and bleaches the system being measured (Binder et al., 1990). Furthermore, the mass spectrometric approach allows more physiologically relevant experiments to be performed since disruption of the ROS is not required to allow [^{32}P]phosphate to enter the cell. We have identified the major sites of phosphorylation in the C-terminal region of bovine rhodopsin (serines 338 and 343), and the data indicate that this region may be constitutively phosphorylated. Our observations also suggest the possibility of a site specific order in the dephosphorylation of the receptor. This work demonstrates the utility of mass spectrometry in investigating the functional significance of phosphorylation in rhodopsin and the other G-protein-coupled receptors.

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