Phosphorylation Sites in Bovine Rhodopsin†

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ABSTRACT: Bovine rhodopsin has been phosphorylated in rod outer segments by ATP and endogenous rhodopsin kinase. Mono-, di-, and triphosphorylated rhodopsins have been prepared by chromatofocusing. Nearly all of the phosphate is found in peptide 330–348, formed by digestion of phosphorhodopsins with endoproteinase Asp-N. Sequence analysis of the phosphopeptides shows that monophosphorylated rhodopsin consists of a mixture containing rhodopsins phosphorylated at ^{338}Ser and ^{343}Ser. Diphosphorylated rhodopsin is phosphorylated at both ^{338}Ser and ^{343}Ser. When rhodopsin becomes triphosphorylated it does not become phosphorylated on ^{334}Ser but appears to become phosphorylated on one or more of the four threonine residues: ^{335}Thr, ^{336}Thr, ^{340}Thr, and ^{342}Thr.

Rhodopsin is the photoreceptor protein of rod cells in the vertebrate retina. Upon activation by light, rhodopsin activates the G-protein transducin, which activates cGMP-phosphodiesterase. The phosphodiesterase hydrolyzes cGMP, leading to closure of cGMP-gated ion channels in the rod cell plasma membrane and hyperpolarization of the plasma membrane [reviewed in Hargrave and McDowell (1993)]. One of the mechanisms of termination of this visual transduction cascade is the inactivation of rhodopsin. This occurs by phosphorylation of rhodopsin by rhodopsin kinase and the subsequent binding of arrestin by photoactivated phosphorylated rhodopsin (Wilden et al., 1986; Miller et al., 1986).

Rhodopsin kinase uses the γ-phosphate of ATP to phosphorylate rhodopsin on multiple serines and threonines (Kühn & Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973). When rhodopsin in rod outer segments is phosphorylated in vitro, this leads to a mixture of rhodopsins phosphorylated to varying extents (Kühn & McDowell, 1977; Shichi & Somers, 1978; Sale et al., 1978). These phosphorylated rhodopsins may be separated by chromatography or chromatofocusing (Wilden & Kühn, 1982; Aton et al., 1984; Arshavsky et al., 1986). The serines and threonines that become phosphorylated are located in a compact region near rhodopsin's carboxyl terminus (Sale et al., 1978; Hargrave et al., 1980; Thompson & Findlay, 1984). There are seven such hydroxy amino acids in this region of bovine rhodopsin, and based upon the maximum extent of rhodopsin phosphorylation, all of them are likely to become phosphorylated (Wilden & Kühn, 1982). Five residues in the homologous ovine rhodopsin have been reported to become phosphorylated: serines 334, 338, and 343 and threonines 335 and 336 (Thompson & Findlay, 1984).

The site specificity of phosphorylation of proteins by several kinases has been demonstrated to depend upon the sequence surrounding the substrate hydroxy amino acid. Protein kinase A preferentially phosphorylates serines, but also threonines, that have one or more basic residues on their amino-terminal side (Edelman et al., 1987). In contrast, the phosphorylation sites for casein kinases must be flanked by acidic amino acids (Edelman et al., 1987). The preference of rhodopsin kinase for its serine and threonine substrate amino acids has been studied for peptide substrates (Palczewski et al., 1989; Brown et al., 1992) and although rhodopsin kinase prefers acidic peptides to basic ones (Palczewski et al., 1989), no general rules for sequence specificity have yet become apparent. In the present study we begin determination of the order of phosphorylation of rhodopsin as a way of elucidating the substrate specificity of rhodopsin kinase.

MATERIALS AND METHODS

11-*cis*-Retinal was kindly provided by the National Eye Institute of the National Institutes of Health. Endoproteinase Asp-N was obtained from Boehringer-Mannheim. All other chemicals were of the highest grade of purity from various commercial sources.

Preparation of Rod Outer Segments. All procedures were performed under dim red light illumination unless otherwise indicated. Rod outer segments (ROS)[1] were prepared from 200 frozen bovine retinas (Lawson, Inc., Lincoln, NB) by the method of Wilden and Kühn (1982). The ROS preparation was adjusted to 1 mg/mL rhodopsin in buffer A (100 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA), and aliquots were quick-frozen in liquid nitrogen and stored at −75 °C prior to use.

Phosphorylation of Rhodopsin. A sample of each ROS preparation (containing 3 mg of rhodopsin) was used for determination of the time course of the phosphorylation reaction in order to choose the time for maximal production of the desired species of phosphoopsin. ROS in buffer A were made 0.6 mM in ATP (final rhodopsin concentration 0.5 mg/mL), sonicated, and exposed to bright white light at 25 °C. Timed samples (500 μL) were withdrawn, made 10 mM in EDTA to stop the phosphorylation reaction, and incubated in the dark with a 3-fold molar excess of 11-*cis*-retinal to opsin at room temperature overnight to regenerate rhodopsin. ROS

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[1] Abbreviations: DHA, dehydroalanine; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; PTH, phenylthiohydantoin; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); ROS, rod cell outer segments; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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membranes were pelleted by centrifugation (48400g, 15 min) and solubilized in 100 μL of 10 mM dodecyl maltoside, and the phosphorylated rhodopsins were analyzed by isoelectric focusing (Adamus et al., 1988). On the basis of these results, reaction times of 5–20 min were generally selected for the large-scale (~170 mg of rhodopsin) batch phosphorylation reaction.

Purification of Phosphorylated Rhodopsin. The large-scale reaction was carried out under conditions identical to those for the analytical-scale phosphorylation. In some reactions the ATP included 1 mCi of [γ-^{32}P]ATP. Following regeneration of rhodopsin and pelleting of the membranes, rhodopsin was solubilized in 60 mL of 50 mM octyl glucoside in buffer B (50 mM Tris/acetate, pH 6.9, 1 mM each in Ca^{2+}, Mg^{2+}, and Mn^{2+}). Chromatography of rhodopsin was performed on concanavalin A–agarose (Litman, 1982). Purified rhodopsin was concentrated by Amicon ultrafiltration to ~4 mg/mL and dialyzed vs 10 mM imidazole hydrochloride buffer, pH 7.4. Alternatively, the buffer was exchanged by chromatography using Sephadex G-15 equilibrated with the same buffer made 6 mM in dodecyl maltoside.

Separation of Rhodopsin and Phosphorhodopsin. Phosphorhodopsins were separated from rhodopsin by chromatography on a column of Fe^{3+}-Chelex (Andersson & Porath, 1986). A 25-mL bed volume column was prepared and equilibrated in 10 mM imidazole buffer (pH 5.0) containing 6 mM dodecyl maltoside. The solution of phosphorhodopsins was carefully adjusted to pH 5.0 and applied to the column, and the column was washed with the equilibration buffer until the 498-nm absorbance of the eluate had returned to baseline. Phosphorhodopsins were eluted with the same buffer at pH 7.4. Fractions (5 mL) were collected in tubes containing EDTA to yield a final concentration of 10 mM.

Separation of Phosphorhodopsins. Phosphorhodopsins were separated by chromatography on a Mono-P column (HR 5/20, Pharmacia) by modification of previous procedures (Philippov et al., 1990; G. Adamus, A. Arendt, P. A. Hargrave, T. Heyduk, and K. Palczewski, manuscript in preparation). The column was equilibrated in buffer C (10 mM imidazole, pH 7.0, and 0.5 mM DTT) followed by 2.5 column volumes of buffer D (buffer C made 6 mM in dodecyl maltoside). A pregradient was applied of 2.5 mL of buffer PB [1:12.5 dilution of polybuffer 74 (Pharmacia), adjusted to pH 4.5 with HCl and made 0.5 mM in DTT and 6 mM in dodecyl maltoside]. Phosphorhodopsins (10 mg, concentrated to 3 mg/mL) were applied at 1 mL/min followed by 40 mL of buffer PB. Then a 40-mL linear gradient was developed from 0 to 0.4 M NaCl (from buffer D to buffer D made 0.4 M in NaCl). Fractions (1 mL) were collected into tubes containing 110 μL of 100 mM EDTA. Fractions were monitored at 500 nm and peak fractions were pooled.

Preparation of Phosphorylated Peptides from Phosphorhodopsin. Pooled fractions containing a species of phosphorhodopsin were concentrated to <2.5 mL by ultrafiltration and exchanged into buffer E (10 mM Tris-HCl, pH 7.5, 1.2 mM $ZnCl_2$, and 6 mM dodecyl maltoside) by gel filtration on a column of Sephadex G-25 (prepacked PD-10 columns, Pharmacia). The column was equilibrated with and eluted with buffer E.

Phosphorhodopsin (3–5 mg/3.5 mL) was incubated with 2 μg of endoproteinase Asp-N overnight, similar to Palczewski et al. (1991) except that the digestion was carried out in detergent solution. An aliquot of the reaction mixture was taken for SDS–polyacrylamide gel electrophoresis to monitor for digestion of rhodopsin by its decrease in molecular weight. Occasionally a second addition of enzyme was required to yield complete digestion. Subsequent procedures were performed in the light. Digested rhodopsin was precipitated by addition of solid TCA to yield 10% TCA, followed by freezing at −75 °C and thawing. Following centrifugation (39200g, 20 min), the phosphopeptide was desalted by chromatography of the supernatant on a column of Bio-Rad P4 (2.5 × 11 cm) equilibrated in 5% acetic acid. The peptide was identified by radioactivity, and fractions were pooled and lyophilized. The radioactive peptide was observed to elute in roughly the middle of the fractionation range of the column. When nonradioactive phosphopeptides were isolated, the void and included volumes of the column were determined by monitoring at 250 nm, and all fractions between the included and excluded volume were pooled. The peptide thus produced contained the carboxyl-terminal peptide from rhodopsin: ^{330}DDEASTTVSK^{340}-TETSQVAP^{348}A. Phosphopeptides were purified by reverse-phase chromatography using a Waters Novapak C_{18} cartridge (4 μm, 5 × 100 mm). The column was developed at 1 mL/min with 0–20% buffer B (0–15 min), 20–100% buffer B (15–20 min), 100% buffer B (20–25 min), 100–0% buffer B (25–30 min), and 0% buffer B (30–35 min) in buffer A, where buffer A = 0.1% TFA in water and buffer B = 0.1% TFA in acetonitrile. Column eluant was monitored at 215 nm. The entire phosphopeptide sample was applied in 5 or 6 injections. Peaks were collected, and peaks eluting at the same time were combined from each repetitive chromatography.

Characterization of Peptides. Peptides were acid-hydrolyzed (6 N HCl, 107 °C, 21 h) and their amino acid compositions were determined by analysis on a Beckman System 6300 amino acid analyzer. Chemical sequences of peptides were determined on an Applied Biosystems Model 470 peptide/protein sequencer. Peptides were sequenced while adsorbed on a Porton peptide support disk in the presence of polybrene (Figure 2, peaks 1 and 2) or following carbodiimide linking to a PVDF–arylamine membrane (Sequelon AA, Millipore), which was placed in the reaction chamber along with a Porton peptide support disk (Figure 2, peaks 4 and 5). Sequencer solvents S1, S2, and S3 were all supplemented with 13 μM DTT to enhance production of the DHA–DTT adduct. PTH-amino acids were converted automatically and analyzed on line by HPLC. For radiochemical sequencing, the peptides were linked to PVDF–arylamine membrane as above and sequenced as above except that S3 was replaced with a 25:75 mixture of CH_3CN and 0.1% TFA in water. The total product of each cycle was collected for counting. A second, smaller aliquot of the same preparation of the peptide was identically linked and the PTH-amino acids were eluted and identified normally to ensure the identity of the amino acids.

The amounts of threonine observed for threonine residues 335, 336, 340, and 342 within each peptide sequence were normalized to cycle 6 (i.e., Thr^{335}). First, the amount of threonine carried over from previous cycles was subtracted. This was determined as the fraction carryover into each of the following two cycles as functions of the cycle number. A least-squares fit of these fractions to a straight line allowed extrapolation of the fraction carryover for each of the threonine residues into the next two cycles. The fraction carryover into the second cycle was used to calculate the threonine carryover into the third and all subsequent cycles. Next, the repetitive yield was used to normalize the amount of threonine to cycle 6 (^{335}Thr). For peptides 1 and 2, the repetitive yield based on glutamic acid was used, and for peptides 3 and 4, the repetitive yield was based on alanine.

General Methods. Proteins were examined by SDS–PAGE on 10% acrylamide gels using the system of Laemmli (1970). Isoelectric focusing was performed as described previously,

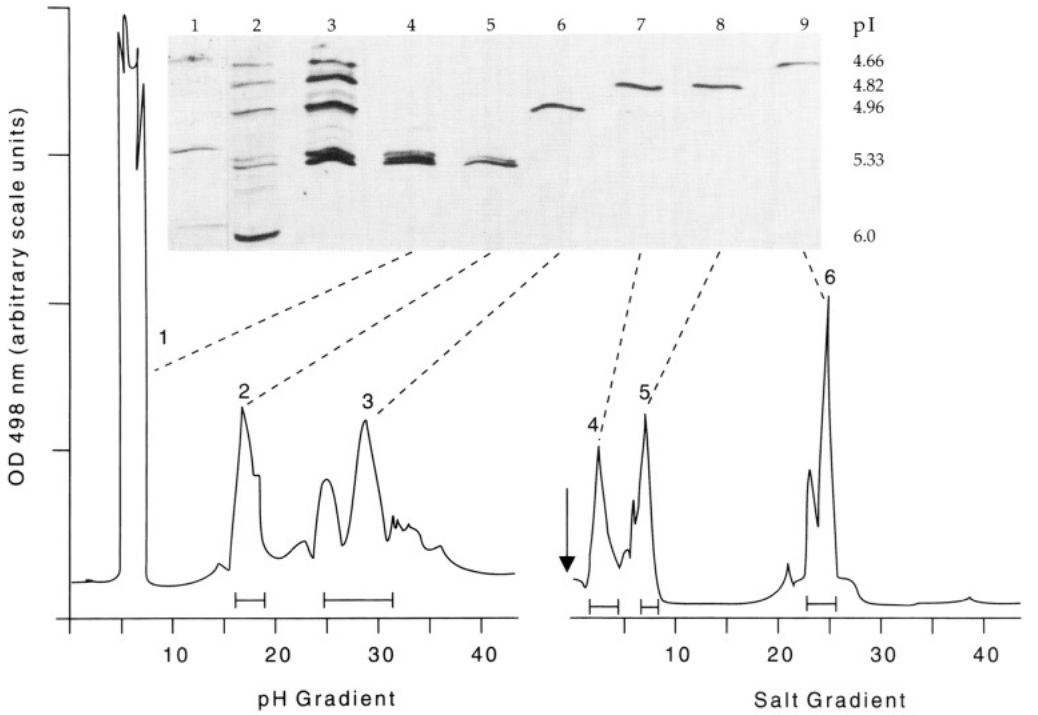

FIGURE 1: Separation of phosphorylated rhodopsins by chromatofocusing on a column of Mono P. Chromatography conditions are given in Materials and Methods. Fractions are pooled as shown by brackets. The arrow marks the start of the salt gradient. Inset: Isoelectric focusing of phosphorylated rhodopsins in polyacrylamide gel. p*I* values (based on standard proteins, lane 1, from Pharmacia) are calculated for phosphorhodopsin samples (peaks from Mono P column, this figure). Lane 1 = isoelectric focusing standards, p*I* = 5.85 (lower band), 5.20, and 4.55 (upper band); lane 2 = rhodopsin (p*I* 6.0) and phosphorylated rhodopsins purified by chromatography on concanavalin A–agarose; lane 3 = phosphorylated rhodopsins following Chelex chromatography; lane 4 = peak 1 (this figure), monophosphorhodopsins, p*I* ~ 5.33; lane 5 = peak 2, monophosphorhodopsins; lane 6 = peak 3, diphosphorhodopsin, p*I* 4.96; lane 7 = peak 4, triphosphorhodopsin, p*I* 4.82; lane 8 = peak 5, triphosphorhodopsin; lane 9 = peak 6, tetraphosphorhodopsin, p*I* 4.66.

except that dodecyl maltoside was used as the detergent (Adamus et al., 1988).

RESULTS

The phosphorylation of rhodopsin in rod cell outer segments was initiated by light in the presence of added ATP. Phosphorylation was terminated at a time when, on the basis of analytical-scale experiments, primarily mono-, di-, and triphosphoopsins predominated. In order to be able to separate and purify the different phosphorylated opsins, they were first converted to rhodopsin by the addition of 11-*cis*-retinal. The mixture of rhodopsin and phosphorylated rhodopsins was purified from other rod cell components by solubilizing in detergent and chromatographing on concanavalin A–agarose. Phosphorylated rhodopsins were separated from unphosphorylated rhodopsin by chromatography on a Fe^{3+}-Chelex resin.

Chromatofocusing on a Mono-P column was used to separate the individual phosphorylated rhodopsins (Figure 1). The presence of (phospho)rhodopsin was detected by absorbance at 500 nm. Homogeneity of protein comprising each chromatographic peak was assessed by analytical isoelectric focusing (Figure 1 inset). Rhodopsin and a number of phosphorylated rhodopsins are present in Con A-purified rhodopsin (Figure 1 inset, lane 2) but rhodopsin is efficiently removed by Fe^{3+}-Chelex chromatography (Figure 1 inset, lane 3). Each of the peaks from the Mono-P column is seen to contain essentially a single isoelectric species except for the monophosphorylated rhodopsin (peaks 1 and 2), which consists of at least two tightly focused bands. The identification of the designated phosphorhodopsins as containing one, two, or more phosphates was determined by sequence analysis (see below).

The carboxyl-terminal peptide containing rhodopsin's phosphorylation sites was prepared by endoproteinase Asp-N digestion of the protein followed by peptide purification on HPLC (Figure 2). When rhodopsin was phosphorylated using [γ-^{32}P]ATP, virtually all of the radioactivity was recovered

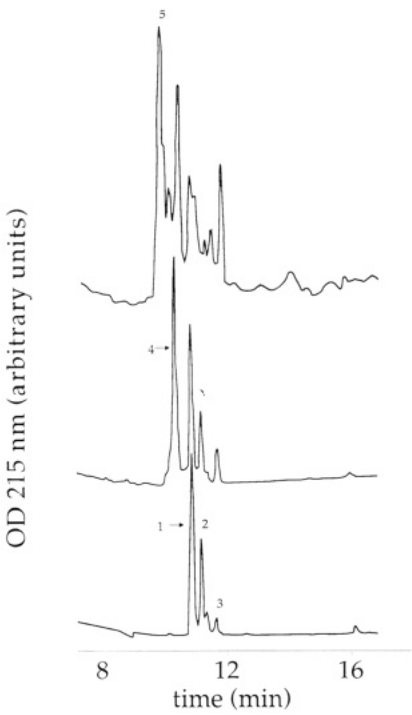

FIGURE 2: Purification of rhodopsin phosphopeptides 330–348 by HPLC. Peptides from digestion of phosphorhodopsins by endoproteinase Asp-N were purified by gel filtration and HPLC as described in Materials and Methods. The bottom trace shows HPLC separation of peptides from monophosphorhodopsin; the middle trace, peptides from diphosphorhodopsin; and the top trace, peptides from triphosphorhodopsin. Data from sequence analysis of these peptides are shown in Figure 4.

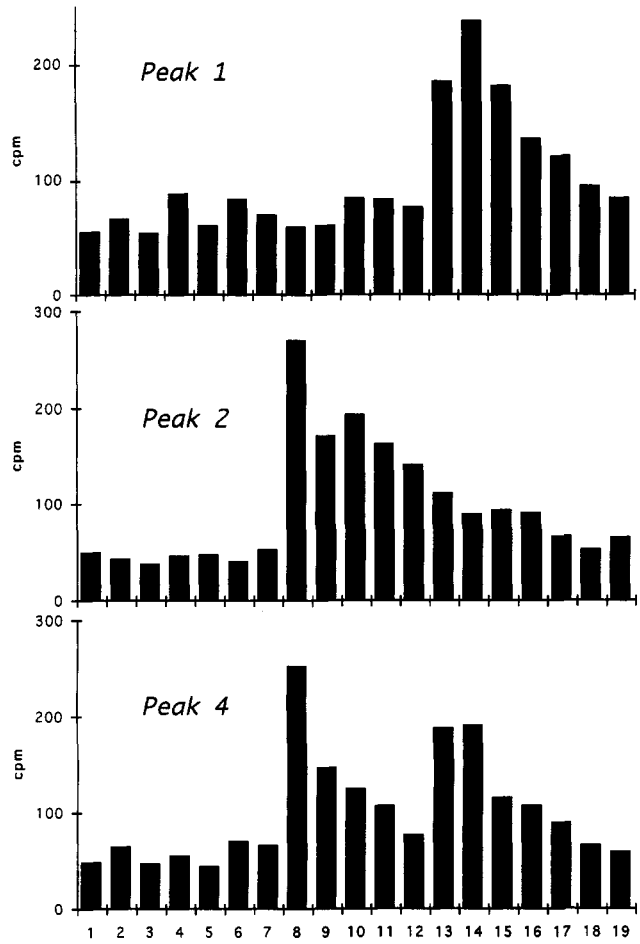

FIGURE 3: Radiochemical sequence analysis of peptides 330–348 from monophosphorhodopsin (peaks 1 and 2) and diphosphorhodopsin (peak 4). HPLC peaks from Figure 2 were submitted for radiochemical sequence analysis as described in Materials and Methods. Total counts are shown for each cycle.

in these phosphopeptides (data not shown). Monophosphorylated rhodopsin yielded peptides 1 and 2, both of which showed the amino acid composition and sequence of peptide residues 330–348 and contained ^{32}P. The radioactive peptides were submitted to radiochemical sequence analysis where the release of ^{32}P at each cycle was determined (Figure 3) as well as to standard sequence analysis where the PTH amino acid released was identified. Figure 3 shows radioactivity appearing first in cycle 13 for the peptide in peak 1, while for the peptide in peak 2, radioactivity appears first in cycle 8. For peak 4, the presumptive diphosphorylated species, radioactivity appears in both cycles 8 and 13 and appears to be essentially the sum of the radioactivity seen for sequencing peaks 1 and 2. However, cycle 8 of peak 2 is a valine residue, ^{337}Val, that precedes ^{338}Ser. Valine cannot itself become phosphorylated. This suggests that the source of the ^{32}P seen in cycle 8 is due to "preview" from the β-elimination of serine phosphate from ^{338}Ser when it becomes the new N-terminal amino acid during cycle 8. Such partial release of ^{32}P at the prior sequencing step causes us to assign the ^{32}P in cycle 8 of peptides 1 and 4 to ^{338}Ser and the ^{32}P in cycle 13 of peptides 2 and 4 to the following amino acid ^{343}Ser. We then performed chemical sequencing on the remainder of these same peptides in order to determine the amount of β-elimination of serine that had occurred at each of the serines in each peptide. The ratio of the PTH-(DHA–DTT adduct) to PTH-serine (PTH-DHA/PTH-Ser) was examined for each of the three serine residues (Figure 4). While both PTH-DHA and PTH-serine are observed for phosphorylated and unphosphorylated serine residues, phosphoserine residues yield a higher PTH-DHA/PTH-Ser ratio than unphosphorylated serine residues (Meyer, et al., 1991). Using this criterion, it is clear from Figure 4 that ^{343}Ser is phosphorylated in peak 1, ^{338}Ser is phosphorylated in peak 2, and both ^{343}Ser and ^{338}Ser are phosphorylated in peaks 4 and 5.[2]

On sequencing, phosphothreonine residues also yield higher amounts of five PTH-(DTT adducts) than do unphosphorylated residues (Dedner et al., 1988). The right column of Figure 4 shows the ratio of the sum of the areas of these five peaks to the amount of PTH-threonine observed at each of the four threonine positions. While the ratios vary somewhat for each peptide, no markedly high PTH-Thr product/PTH-Thr ratio is observed for any of the residues. Clearly, for ^{342}Thr, i.e., cycle 13 in the radiochemical sequencing shown in Figure 3, a normal, low PTH-Thr product/PTH-Thr ratio indicates that this residue is not phosphorylated and that the radioactivity observed in cycle 13 of peak 1 (Figure 3) must be due to the release of phosphate from ^{343}Ser analogous to that observed for ^{338}Ser. Finally, since no radioactivity above background was observed for the sequence ^{334}Ser^{335}Thr^{336}-Thr, we conclude that the major phosphorylation site of the peptide of peak 1 is ^{343}Ser, that of peak 2 is ^{338}Ser, and those of peak 4, the presumptive diphosphorylated rhodopsin, are both ^{343}Ser and ^{338}Ser. We cannot rule out the possibility of the presence of small amounts of other mono- and diphosphorylated species in these samples. Faint bands in the IEF gels and small side bands in the HPLC analyses suggests their presence. Since all of these residues must become phosphorylated in the most highly phosphorylated species, it is not surprising that traces of other mono- and diphosphorylated species would be present.

In the HPLC analysis of the monophosphorylated rhodopsin peptide, a minor product, peptide 3 (Figure 2, bottom trace), contains no phosphate. Since there is no evidence of unphosphorylated rhodopsin in the phosphorhodopsin from the chromatofocusing column (Figure 1 inset, lanes 4 and 5) or even in the material loaded on the chromatofocusing column (Figure 1 inset, lane 3) we conclude that this product is a dephosphorylated peptide formed during handling and storage of the phosphopeptide. This peak was observed in varying amounts from different preparations and elutes with the same retention time as the unphosphorylated synthetic peptide 330–348 (kindly provided by Dr. A. Arendt). Some of the monophosphorylated peptides are also observed in the HPLC analyses of the presumptive diphosphorylated peptide. Likewise, there is no evidence of significant amounts of the monophosphorylated species of rhodopsin in the IEF of Figure 1, suggesting that these are also breakdown products of the phosphopeptide produced during storage and handling.

The combined triphosphorylated forms of rhodopsin from peaks 4 and 5 (Figure 1) yield peptide 330–348 that shows several chromatographic species by HPLC (Figure 2, top trace). Many of the species may be assigned to un-, mono-, and diphosphorylated 330–348, presumably formed by breakdown. Sequence analysis of peak 5 and its satellites shows

[2] It should be noted that the actual values of the PTH-DHA/PTH-Ser ratios are highly dependent on the conditions of sequencing. For example, when peaks 1 and 2 (Figure 2) from a different preparation of phosphorhodopsin were linked to PVDF–arylamine membrane and sequenced *without* the addition of a Porton peptide support disk, the ratios of PTH-DHA/PTH-Ser were 0.28, 0.29, and 1.16 for serines 334, 338, and 343 of peak 1, respectively, and 0.25, 1.53, and 0.44 for these same serines of peak 2. The same pattern is observed such that residue ^{343}Ser in peak 1 and ^{338}Ser in peak 2 are still identified as sites of phosphorylation, though the values of the PTH-DHA/PTH-Ser ratios are quite different from those shown in Figure 4.

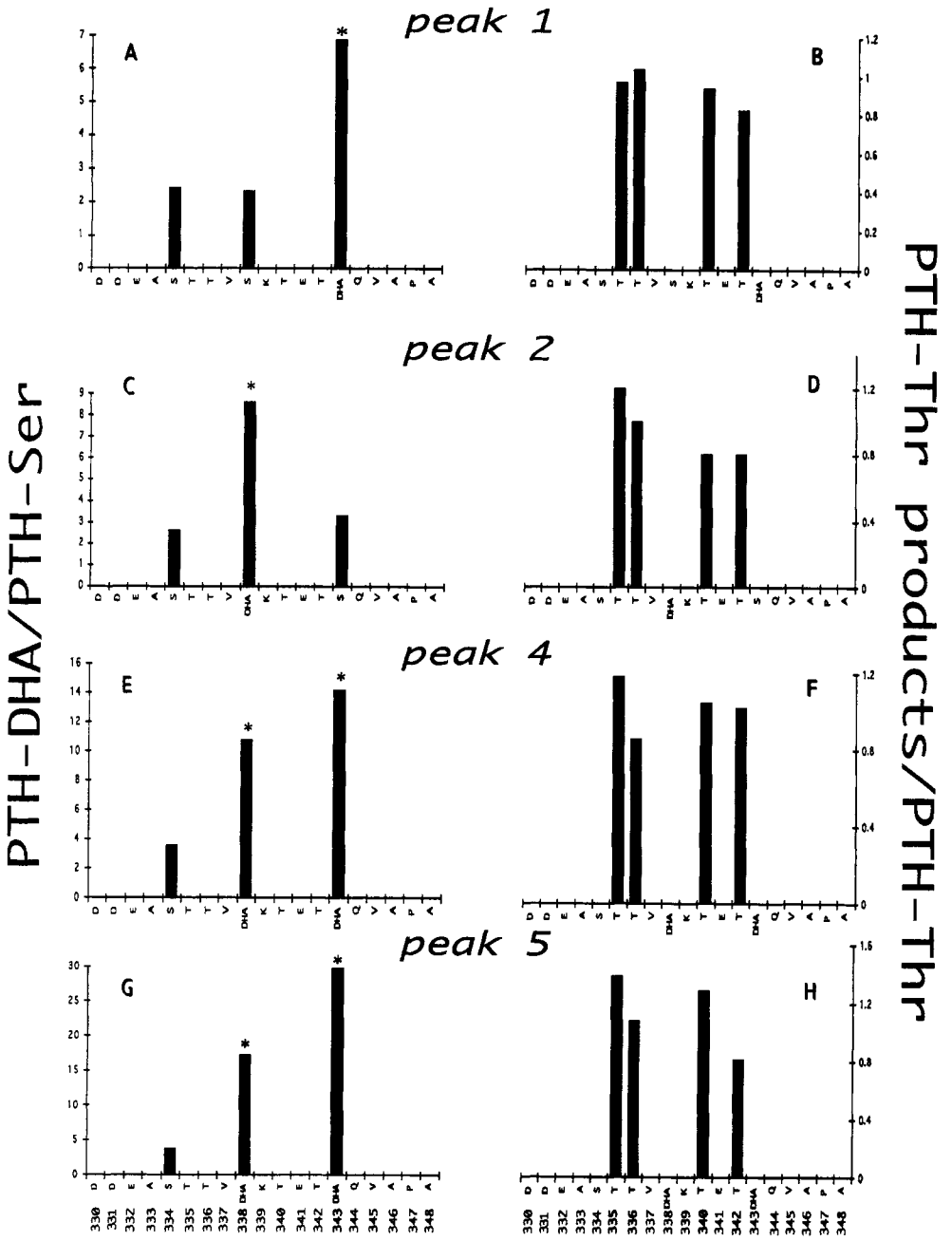

FIGURE 4: Sequence analysis of the major phosphopeptides of phosphorylated rhodopsins: ratios of products from serine and threonine sequencing. The amino acid sequence determined for peptides 330–348 is shown on the *x*-axis. Data from serine and its products of sequencing are shown in panels A, C, E, and G; data from threonine and its products of sequencing are plotted in panels B, D, F, and H. Data are plotted as a ratio of the products to the amino acid; PTH-dehydroalanine (PTH-DHA)/PTH-serine and PTH-threonine products/PTH-threonine. Small amounts of PTH-DHA are always formed by the sequence analysis of serine. Much larger quantities of PTH-DHA are formed from phosphoserine (Meyer et al., 1991). Similarly, five products of PTH-threonine β-elimination (also observed as DTT adducts) are formed in larger quantities from phosphothreonine than from threonine (Dedner et al., 1988). Panels A and B = sequencing of peptide 330–348 in peak 1, Figure 2; panels C and D = sequencing peak 2, Figure 2; panels E and F = sequencing peak 4, Figure 2; panels G and H = sequencing peak 5, Figure 2. Asterisks over the bars in panels A, C, E, and G mark those amino acids with high ratios of PTH-DHA/PTH-Ser arising from sequencing of phosphoserine residues.

that there is no difference in the PTH-DHA/PTH-Ser profile compared to that of peptide 4 from diphosphorylated rhodopsin (Figure 4, panel G). ^{334}Ser still shows the low PTH-DHA/PTH-Ser ratio characteristic of (unphosphorylated) serine; thus it is clear that ^{334}Ser did not become phosphorylated when diphosphorylated rhodopsin was converted to triphosphorylated rhodopsin. This leaves the four threonine residues as candidates for the third site of phosphorylation. Analysis of the ratio of PTH-threonine products to PTH-threonine (Figure 4) shows some differences among the four residues, but the differences are insufficient to allow a confident assignment of which residue(s) may contain phosphate. In a further attempt to determine which threonine(s) might become phosphorylated, we plotted the amount of PTH-threonine formed at each cycle (Figure 5). These amounts were normalized to the amount of PTH-threonine observed in cycle 6 (i.e., ^{335}Thr) based on the repetitive yield of either glutamic acid (peaks 1 and 2) or alanine (peaks 3 and 4), subtracting the carryover from prior threonine residues as described in Materials and Methods. The amount of PTH-^{340}Thr is somewhat lower than that of the other threonines in the triphosphorylated peptide, suggesting that ^{340}Thr may be a phosphorylation site in the triphosphorylated rhodopsin. Figure 4 also shows a pattern for peak 5 with a slightly elevated

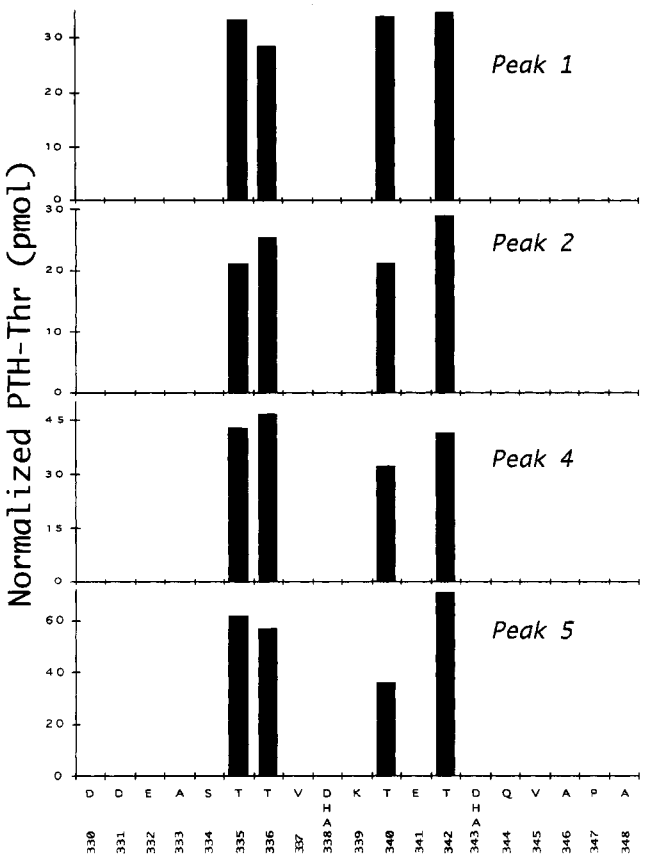

FIGURE 5: Normalized PTH-threonine amounts observed at each threonine residue for mono-, di-, and triphosphorylated rhodopsin. The amount (picomoles) of PTH-threonine observed at each cycle was corrected for carryover from previous cycles and then normalized to the expected yield at cycle 6 as described in Materials and Methods. Peak numbers refer to the peaks in Figure 2.

PTH-Thr products/PTH-Thr for ^{340}Thr consistent with this assignment. However, neither of these differences is sufficient to clearly implicate ^{340}Thr as the third preferred site.

DISCUSSION

Phosphorylation of the activated form of G-protein-linked receptors by a receptor-specific kinase has been demonstrated for rhodopsin and the adrenergic receptors [reviewed in Palczewski and Benovic (1991)] and a muscarinic acetylcholine receptor (Kwatra et al., 1989) and may also occur with other members of this receptor class. Freshly photoactivated rhodopsin becomes phosphorylated in a serine/threonine-rich block of amino acids in its carboxyl-terminal region (Sale et al., 1978; Hargrave et al., 1980; Thompson & Findlay, 1984). Because of the importance of the phosphorylation reaction in receptor turnoff, one might expect that the phosphorylation site would be highly conserved. This hydroxy amino acid rich region is regularly found in all vertebrate rhodopsins, but its sequence is highly variable (Hargrave & McDowell, 1993). As few as six and as many as 12 serine/threonine residues may be found in this compact region. In the case of chicken rhodopsin, this site contains 10 serines/threonines and has a 5 amino acid insertion and two deletions compared to bovine rhodopsin. Yet, chicken rhodopsin is phosphorylated by bovine rhodopsin kinase with a K_m and V_{max} nearly the same as that for bovine rhodopsin (Fukada et al., 1990). This raises the question of what the kinase requires in the sequence that it phosphorylates.

The question of kinase specificity has been addressed by the use of synthetic peptides as substrates (Palczewski et al., 1988, 1989; Brown et al., 1992). It seems clear that rhodopsin kinase prefers hydroxy amino acids in predominantly acidic rather than basic peptides (Palczewski et al., 1989). A recent observation that would support this is the preference, in a peptide substrate, for phosphorylation of ^{343}Ser (which is flanked by ^{341}Glu) (Brown et al., 1992). However, our finding of an equivalent preference for ^{338}Ser (which is flanked by ^{339}Lys) indicates that factors must be involved other than the charged surrounding amino acids.

One of the more striking findings from the use of peptides as substrates for rhodopsin kinase is that even the best peptides have a K_m that is 3 orders of magnitude greater than that for the intact protein (Palczewski et al., 1989). This indicates that there is less preference of the enzyme for a specific amino acid sequence than that shown by many other protein kinases. Specificity comes in part from the specificity of protein–protein interaction shown by the kinase for the freshly photoactivated rhodopsin (Kühn, 1984) which appears to involve other regions of rhodopsin's surface (Palczewski et al., 1991). Binding of rhodopsin kinase to rhodopsin's surface presumably places the carboxyl-terminal serine/threonine-rich region in close proximity so that phosphorylation is promoted.

Three serines and two threonines have been demonstrated to be phosphorylated in ovine rhodopsin (Thompson & Findlay, 1984), and it is clear that all seven sites must become phosphorylated in the most highly phosphorylated species of phosphorhodopsin (Wilden & Kühn, 1982). If there were no preference for one site over another, one might suppose that these sites would be filled randomly and that the monophosphorylated rhodopsin would consist of a mixture of the seven possible species. Alternatively, there might be a strict order of phosphorylation in which a certain serine or threonine is highly preferred because of its location in the sequence in proximity to a particular amino acid(s). From our data we can conclude that neither of these scenarios is followed. The first site phosphorylated on rhodopsin by rhodopsin kinase is either ^{338}Ser or ^{343}Ser. The second site is again either ^{338}Ser or ^{343}Ser so that diphosphoopsin is phosphorylated at both of these residues (^{338}Ser and ^{343}Ser). The third preferred phosphorylation site may be ^{340}Thr, although the present data will not allow us to confidently make this assignment. In triphosphoopsin, in spite of the generally high preference of protein kinases for serine rather than threonine residues, ^{334}Ser remains unphosphorylated.

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CORRECTIONS

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