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# Isolation and Identification of the Phosphorylated Species of Rhodopsin†

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ABSTRACT: Rhodopsin is phosphorylated in a light-dependent manner by a kinase intrinsic to the rod outer segment. We have used chromatofocusing to separate six phosphorylated species of rhodopsin and have recovered in the pH gradient fractions 60–80% of the initial phosphorylated sample loaded on the column. The isolated species of rhodopsin coincide with the species that are observed in isoelectric focusing gels in the pH range 6.1–4.7. Unphosphorylated rhodopsin focuses at a p*I* of 6.0. Two species having two phosphates per rhodopsin with isoelectric points of 5.45 and 5.40 have been isolated. The phosphate to rhodopsin ratios for the remaining species are 3.8, 5.0, 6.1, and 8.2 with isoelectric points of 5.16, 4.99, 4.85, and 4.73, respectively. The chromatofocusing profile suggests that there may be multiple forms of rhodopsin with the same number of phosphates among some of the other phosphorylated forms of rhodopsin.

Rhodopsin, the photosensitive pigment found in the disk membrane of the rod outer segment (ROS)[1] of the retina, is phosphorylated after photon absorption by an intrinsic rhodopsin kinase (Kuhn & Dryer, 1972; Bownds et al., 1972; Kuhn et al., 1973). There are 7 serine and threonine sites in the last 15 amino acids of the carboxyl terminus of rhodopsin as well as other sites more interior in the protein (Virmaux et al., 1975; Hargrave & Fong, 1977) which can serve as potential phosphorylation sites. The localization of the phosphorylation sites, the order of phosphorylation, and also the role of this phosphorylation in the visual process are not known. The half-times of phosphorylation and dephosphorylation are approximately 2 (Kuhn & Bader, 1976) and 13 min (Kuhn, 1974), respectively. Since these rates are much slower than those associated with the processes of visual transduction, it has been proposed that the phosphorylation is involved in light–dark adaption (Kuhn & Bader, 1976). On the other hand, when ATP is included in a continuous assay of the light activation of the ROS cGMP-specific phosphodiesterase, there is a rapid drop in the enzyme activity initiated by a flash of light (Liebman & Pugh, 1980). This observation has lead to the proposal that phosphorylation of bleached rhodopsin terminates its ability to participate in the phosphodiesterase activation cycle. Phosphorylated disks, containing a mixture of the phosphorylated species of rhodopsin, have been shown to exhibit a reduced ability to light activate the ROS phosphodiesterase as compared to unphosphorylated disks (Aton & Litman, 1983), further suggesting that the phosphorylation process is involved in the regulation of the ROS phosphodiesterase activity.

The various forms of phosphorylated rhodopsin can be differentiated by their isoelectric points. This property has been exploited in this study to separate the phosphorylated forms of rhodopsin by employing chromatofocusing, a column chromatographic method for separating proteins on the basis of differences in their isoelectric points. Using the nonionic detergent octyl glucoside, we have isolated seven species of rhodopsin with little contamination from adjacent species. Our experience suggests that this technique will be a valuable preparative technique for the separation of the phosphorylated species of rhodopsin as well as other phosphorylated proteins.

## Materials and Methods

*Rhodopsin Concentration Determination.* The rhodopsin concentration was determined throughout by the difference in absorbance at 500 nm before and after bleaching a rhodopsin sample solubilized in 70–100 mM OG using an $\epsilon_{500}$ of 42000. Aliquots of Con A purified rhodopsin solubilized in 70 mM OG, 50 mM Tris–acetate, pH 7.0, diluted 1:5 with either 100 mM OG, 50 mM Tris–acetate, pH 7.0, or 1.2% Emulphogene BC-720, 66.7 mM potassium phosphate, pH 6.5,

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[1] Abbreviations: ROS, rod outer segments; OG, octyl β-D-glucoside; Con A, concanavalin A–Sepharose 4B; IEF, isoelectric focusing; *P*/*R*, moles of phosphate per mole of rhodopsin; CB, Coomassie Brilliant Blue R250; Tris, tris(hydroxymethyl)aminomethane; $\epsilon_{500}$, molar extinction coefficient at 500 nm; EDTA, ethylenediaminetetraacetic acid.

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had identical absorbances. Therefore, we have used the published value of $\epsilon_{500}$ measured for rhodopsin solubilized in Emulphogene to determine the concentration of samples of rhodopsin (Shichi et al., 1969) solubilized in OG. The $\epsilon_{500}$ of the phosphorylated species was estimated as follows: Con A purified rhodopsin samples were prepared from phosphorylated and unphosphorylated disks. The ratio of the difference in absorbance at 500 nm to the quantity of protein determined by the modified Lowry method (Hartree, 1972) was ascertained for both samples. Since these ratios agreed within experimental error, an $\epsilon_{500}$ of 42 000 was used for each of the phosphorylated species.

*Preparation of Phosphorylated Rhodopsin.* ROS were prepared from frozen retinas (American Stores Packing Co.) according to the procedure of McDowell & Kuhn (1977). Rhodopsin was phosphorylated by incubating ROS at a rhodopsin concentration of 20 $\mu$M in 120 mM sodium phosphate buffer (pH 7.0), 1.56 mM $MgCl_2$, and 4.1 mM dithiothreitol at 30 °C for 30 min. The ATP concentration was varied from 0.6 to 3.6 mM depending on the experiment. Rhodopsin was bleached by illumination with a microscope light for approximately 10 min at the beginning of the 30-min incubation period. More than 90% of the rhodopsin was bleached after the incubation period. The banding pattern in IEF gels was the same for ROS phosphorylated immediately after isolation and ROS that had been stored frozen.

To determine the average level of phosphorylation of rhodopsin, [$\gamma$-$^{32}$P]ATP (obtained from the Diabetes Research Training Center, Analytical Core Laboratory, University of Virginia Medical Center) was added to an aliquot of ROS in the above mixture so that there were at least 500 cpm/nmol of ATP. After the appropriate incubation time, 70 $\mu$L was spotted onto filter paper, washed, and counted (Huang, 1974). When the phosphorylated rhodopsin was to be isoelectrically focused, the activity of the radioactive ATP was increased up to at least 2000 cpm/nmol of ATP.

To regenerate rhodopsin after phosphorylation, a 3-fold excess of 11-*cis*-retinal (a gift of Hoffmann-La Roche, Inc.) was added to the phosphorylation mixture, and the ROS were then incubated in the dark for 30 min at 30 °C and for at least 1.5 h at room temperature. The ROS were then centrifuged for 40 min at 28000*g*, and the pellet containing the regenerated phosphorylated rhodopsin was vortexed in 50 mM Tris–acetate buffer (pH 7.0)–75 mM OG. A yield of 85–90% of the initial rhodopsin was recovered in this supernatant. The insoluble material was removed by centrifugation for 40 min at 28000*g*. Rhodopsin was purified by affinity chromatography on a Con A column (Litman, 1982). Rhodopsin was obtained in an appropriate buffer for chromatofocusing by concentrating the column-purified rhodopsin to approximately 100 $\mu$M by using a PM 10 Amicon membrane, diluting the sample to approximately 20 $\mu$M in rhodopsin with 25 mM histidine (pH 6.4)–30 mM OG, and reconcentrating to 100 $\mu$M. This dilution and reconcentration was repeated a second time.

*Chromatofocusing Column Procedure.* The chromatofocusing column was poured and run essentially as described in the Pharmacia Fine Chemicals manual on chromatofocusing. The columns were run at 4 °C. Flow rates were maintained with a peristaltic pump. Briefly, PBE 94 column material was equilibrated with 25 mM histidine (pH 6.4) and then packed at a flow rate of 100 cm/h in a Pharmacia K9/30 or C10/40 column. This corresponds to a volume flow rate of 63.6 mL/h for the K9/30 column. The ratio of the height to the diameter of the column bed was approximately 30. One centimeter of Sephadex G-25 coarse beads was layered on top of the PBE. The column was washed with 20 column volumes of 25 mM histidine (pH 6.4) followed by 2–3 column volumes of start buffer (25 mM histidine, pH 6.4, and 50 mM OG). The flow rate was reduced to 20 cm/h for the last 20 mL of the start buffer wash and was maintained at this rate subsequently. A sample of 30 nmol of rhodopsin/mL of bed volume was loaded directly onto the Sephadex. The column was then eluted with a 1:8 dilution of Polybuffer 74 (pH 4.0) and 50 mM OG. Fractions of 1.5–2 mL were collected. The pH gradient was completed after approximately 12 column volumes. This was followed by a wash of 2 column volumes of 500 mM NaCl in the start buffer.

*Determination of Rhodopsin Phosphorylation Levels.* Rhodopsin phosphorylation levels ($P/R$) of the chromatofocusing peaks were determined as follows. Several (three to five) fractions of each species separated by chromatofocusing were pooled, the pH was adjusted to 7.0–7.5, and the samples were concentrated with an Amicon PM 10 membrane to approximately 75 $\mu$M rhodopsin. It was necessary to adjust the pH prior to concentrating, otherwise, a lower than expected $P/R$ value was determined, and little or no banding was apparent in IEF gels of the samples. The absorption spectra of these samples were taken from 600 to 270 nm; the samples were bleached and rescanned. The rhodopsin concentration was determined from the difference in absorbance at 500 nm. Aliquots were put in acid-cleaned tubes for phosphate analysis. The phosphate concentrations of the pooled samples were determined by the ultramicro modification of the method of Bartlett (1959). Standards were prepared in diluted Polybuffer (1:8) and 75 mM OG and contained 2.5–10 nmol of phosphorus. The total assay volume was 1.1 mL. The $P/R$ value was determined by dividing the rhodopsin concentration by the phosphate concentration determined for each species.

*Isoelectric Focusing Procedure.* Rhodopsin was isoelectrically focused in the dark by using a pH gradient from pH 6.5 to 3.8 which was formed by mixing LKB ampholines pH 5–7 and pH 3.5–5 in a ratio of 3:1, respectively. The gels contained 5% (w/v) acrylamide, 0.17% (w/v) *N*,*N*′-methylenebis(acrylamide), 1.25% (w/v) OG, and 2.7% (v/v) ampholine. The cathode buffer was 10 mM histidine. The anode buffer was 10 mM $H_3PO_4$. The concentrated peak fractions from the chromatofocusing column were dialyzed against at least one change of 5 mM sodium phosphate (pH 7.0) and 30 mM OG. An aliquot of 750 pmol of rhodopsin from each fraction, diluted 1:2 with 5 mM sodium phosphate buffer (pH 7.0), 40% (v/v) glycerol, 170 mM OG, 5.4% (v/v) ampholine, was loaded on a gel and was isoelectric focused for 18 h at 6 °C. The gels were stained following the procedure of Huang et al. (1973). The pH gradient was determined by cutting a blank gel run simultaneously into 24 slices and measuring the pH of each slice extracted in 1 mL of distilled water at room temperature. The isoelectric points determined in this manner agreed with the isoelectric points determined with 0.5-mm slab gels. The pH gradient of the slab gel was measured with a surface electrode at the focusing temperature, 6 °C. The agreement between the p*I*'s determined by tube gel and slab gel techniques implies that internal heating in the tube gels resulted in the actual focusing temperature being much closer to room temperature where the pHs of the tube slices were measured. Thus, the need for a temperature correction of more than 0.2 pH unit expected at pH 6 (Davies, 1969) is negated.

## Results

When the conditions described for rhodopsin phosphorylation were used, an average of four to five phosphates were

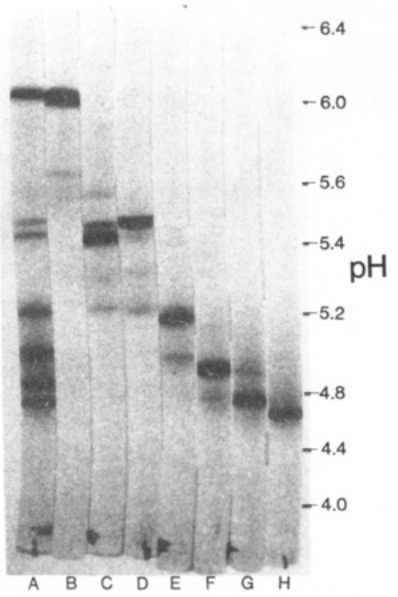


FIGURE 1: Isoelectric focusing of phosphorylated rhodopsin. ROS were phosphorylated with 3.6 mM ATP for 30 min at 30 °C. The Con A purified phosphorylated rhodopsin (24.7 mg) was applied to a chromatofocusing column (Pharmacia K9/30; bed height, 25.6 cm) and eluted with a pH 6.4–4.0 gradient, 1:8 dilution of Polybuffer 74. Fractions of 1.5 mL were collected and prepared for isoelectric focusing as described. Gel A, the phosphorylated rhodopsin sample before chromatofocusing; gel B, fractions 41–44; gel C, fractions 55–82; gel D, fractions 60–62; gel E, fractions 82–83; gel F, fractions 96–98; gel G, fractions 110–112; gel H, fractions 128–130. The $P/R$ values for gels B, C, D, E, F, G, and H are 0.2, 2.0, 2.0, 3.8, 5.0, 6.1, and 8.2, respectively.

incorporated per rhodopsin when ROS were incubated in the presence of 3.6 mM ATP. Incubation with 1.8 mM ATP yielded essentially the same level of phosphate incorporation. When the ATP concentration was 0.6 mM, there was an average of 1.5–2 $P/R$. After regeneration with 11-*cis*-retinal, IEF of phosphorylated ROS revealed eight species of rhodopsin that focused in the pH range of 4.6–6; regenerated phosphorylated rhodopsin, which was subsequently purified on a Con A column, had essentially the same banding pattern, supporting the conclusion that these bands arise from the various forms of rhodopsin. Figure 1, gel A, depicts an IEF gel of Con A purified regenerated phosphorylated rhodopsin. (The species that focuses at a pH of 4.66 was never a major species under the incubations we use and can be seen in Figure 3, gel D.) The $A_{280}/A_{500}$ spectral ratio of the Con A purified phosphorylated material was 1.7–1.8; this agrees well with values obtained for unphosphorylated rhodopsin (Litman, 1982). The molar ratio of phospholipid to rhodopsin after purification on a Con A column is approximately 1 to 10 for unphosphorylated material.

Chromatofocusing uses differences in isoelectric points of the components as its primary mechanism for separation. The elution profile, expressed as the absorbance at 500 and 280 nm of a chromatofocusing column, is shown in Figure 2. Total recovery of rhodopsin from the chromatofocusing column is approximately 80% with about 15% of this material eluting in the high-salt wash. IEF gels of the peak fractions are shown in Figure 1, gels B–H. These gels demonstrate the high resolving power of the chromatofocusing column. The species that is observed in IEF gels with a p$I$ of 4.66 is not resolved on the chromatofocusing column under our running conditions. A gel loading of 750 pmol was used for these single species samples; the weakest bands detectable under these conditions are approximately 7.5 pmol. The $P/R$ for each of these peaks

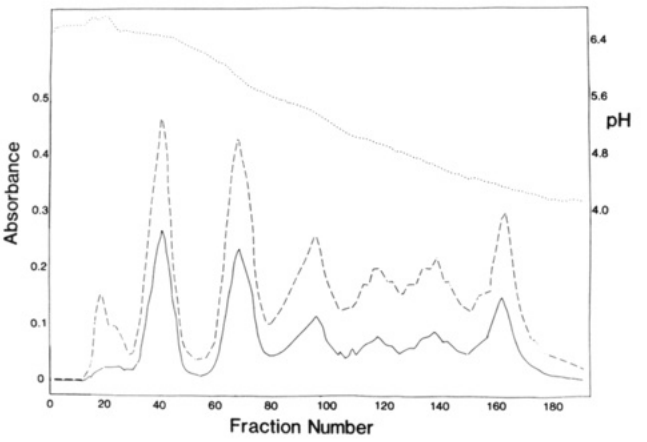


FIGURE 2: Chromatofocusing profile of phosphorylated rhodopsin. Con A purified phosphorylated rhodopsin (24 mg) was applied to a chromatofocusing column (Pharmacia K10/40; bed height, 31.5 cm) and eluted with a pH 6.4–4.0 gradient, 1:8 dilution of Polybuffer 74. Fractions of 1.6 mL were collected. The absorbance at 280 (---) and 500 nm (—) and the pH (···) of the fractions are shown.

Table I: Isoelectric Points and Phosphate to Rhodopsin Ratios for the Isolated Species of Rhodopsin

| assumed $P/R$ | measured $P/R$[a] | chromatofocusing p$I$[b] | IEF p$I$[c] | calcd p$I$ |
|---|---|---|---|---|
| 0 | 0.23 ± 0.10 | 6.32 ± 0.15 | 5.96 ± 0.09 | 5.95 |
| 2 | 1.97 ± 0.27 | 5.96 ± 0.13 | 5.45 ± 0.06 | 5.46 |
| 2 | | 5.78 ± 0.11 | 5.40 ± 0.06 | |
| 4 | 3.81 ± 0.33 | 5.29 ± 0.09 | 5.16 ± 0.07 | 5.14 |
| 5 | 4.96 ± 0.46 | 4.89 ± 0.08 | 4.99 ± 0.05 | 5.01 |
| 6 | 6.09 ± 0.46 | 4.58 ± 0.10 | 4.85 ± 0.05 | 4.90 |
| 8 | 8.21 ± 0.42 | 4.23 ± 0.10 | 4.73 ± 0.08 | 4.73 |
| 9 | | | 4.66 ± 0.04 | 4.66 |

[a] At least four determinations were made for each species. [b] The pH at which each species elutes from the chromatofocusing column was determined from the average of seven chromatofocusing runs. [c] The pH at which each species focuses in IEF gels was determined from 12 separate isoelectric focusing experiments.

is given in Table I along with the pHs at which each species elutes from the chromatofocusing column and focuses in IEF gels. If the IEF gel of a given species did not indicate a single component, then the phosphate determination was discarded.

The calculated isoelectric points (Table I) were estimated by using the following model. The isoelectric point of the species of rhodopsin containing $n$ phosphates can be fit by the expression

$$\mathrm{p}I_n = \frac{47.6 + 3.51n}{n + 8} \qquad (1)$$

This expression was derived by assuming that the isoelectric point of a species was the average of the isoelectric points of the individual charges on that species. The 47.6, 3.51, and 8 were the fitting parameters. These were determined as follows: the denominator was assumed to be a whole number. Values of 5, 6, 7, 8, and 9 were tried as fitting parameters for the denominator. The other two parameters were calculated from the equations for p$I_0$ and p$I_8$ by using the p$I$ values measured for two species. The values reported gave the best fit to the observed p$I$s. The agreement of this simple model with the observed data suggests that there is minimal interaction between the charged phosphate groups added.

The time course of the appearance of the phosphorylated forms of rhodopsin was followed by using homogenized ROS and phosphorylation incubation times of 3, 5, 10, and 30 min (Figure 3). After a 3-min incubation, the species with 2 and 4 $P/R$ values were the major phosphorylated species observed.

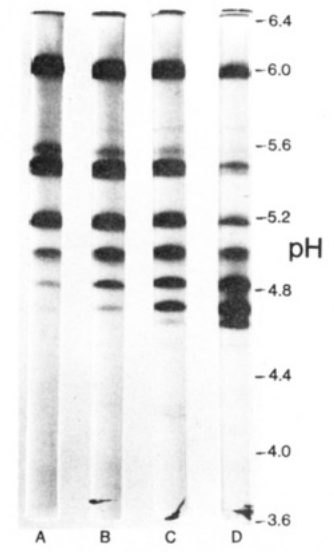


FIGURE 3: Time course of the appearance of the phosphorylated species of rhodopsin. ROS were homogenized and phosphorylated in 3.0 mM ATP. The phosphorylation was stopped by adding EDTA (final concentration, 18 mM). The incubation times are 3 min for gel A, 5 min for gel B, 10 min for gel C, and 30 min for gel D. The ROS were illuminated continuously until the reaction was stopped. The samples were regenerated with 11-*cis*-retinal and concentrated by centrifugation. The pellets were solubilized in 75 mM OG in 5 mM sodium phosphate buffer, pH 7.0, and isoelectrically focused as described.

After 10 min, all the species from 2 to 8 $P/R$ were present. After 30 min, the relative fraction of rhodopsin with 2 $P/R$ had declined, and the species with 6 and 8 $P/R$ predominated. IEF gels of these samples revealed no new band between 0 and 2 $P/R$ or between 2 and 4 $P/R$. Opsin bands stain diffusely with CB in the region between unphosphorylated rhodopsin and the peaks with 2 $P/R$ where one would expect to find the 1 $P/R$ species. No pink bands were apparent by visual inspection in this region immediately after IEF while the bands that focus at 5.96 and 5.45 are easily visible. However, using this as a criterion for detection of rhodopsin would probably require that the species be present as a major species. An upper limit on the percent of the species having 1 and 3 $P/R$ can be made. A sample of rhodopsin phosphorylated with [$\gamma$-$^{32}$P]ATP was isoelectrically focused and the gel sliced and counted. The average $P/R$ of the sample was 1.4; approximately 15% of the total rhodopsin had 2 $P/R$. Taking counts between the 0 and 2 $P/R$ species and the 2 and 4 $P/R$ species and ascribing them to the 1 and 3 $P/R$ species respectively, we estimate that the 1 and the 3 $P/R$ species are each less than 30% of the species having 2 $P/R$ or less than 5% of the total rhodopsin. CB staining of the gels suggests that there is even less, and the chromatofocusing profile gives no evidence of these species.

Heterogeneity in species of rhodopsin with the same number of $P/R$ was observed. Two species with 2 $P/R$ were resolved on both the chromatofocusing column and the IEF gels. The species that bands with a p$I$ of 5.40 on the IEF gels eluted before the species with an IEF of 5.45. In some of our chromatofocusing runs, evidence of other multiple species with approximately the same p$I$ was observed. Minor peaks on the main peaks of the species of rhodopsin having 4, 5, and 6 $P/R$ were detected. There was not enough material in these minor bands for individual analysis, but the pooled fractions of each of the major peaks containing the minor peaks appeared in IEF gels as a single species. It should be noted that the rhodopsin bands in these IEF gels are broad, whereas the bands of the proteins used for standards appeared as sharp bands. We feel that multiple forms of rhodopsin with slightly different isoelectric points of the 4, 5, and 6 $P/R$ could have been present but unresolved in the relatively broad bands of the IEF gels.

The distribution of rhodopsin in the phosphorylated species for two levels of phosphate incorporation was determined by examining IEF gels of rhodopsin labeled with [$\gamma$-$^{32}$P]ATP. The amount of rhodopsin in each species was determined by dividing the amount of phosphate in that band by the number of $P/R$ for the species. More of the highly phosphorylated species were present than in a normal distribution predicted for the random addition of two phosphates at a time to rhodopsin for samples with average $P/R$ values of 2.1 and 4.8.

## Discussion

Chromatofocusing separates six species of rhodopsin that are found in samples of phosphorylated ROS. These are the same species observed by IEF. Depending on the level of phosphorylation, at least 60% of the rhodopsin can be resolved into these components with less than 10% contamination of adjacent species. These species of rhodopsin differ in the number of phosphates covalently bound. Rhodopsins having 0, 2, 4, 5, 6, and 8 $P/R$ values have been identified. The species elute from the column in the order that would be predicted by IEF except for one case. The two species with 2 $P/R$ are reversed. The difference between their isoelectric points is estimated to be 0.05 pH unit. Even with the relatively broad Polybuffer pH gradient used, these two forms are somewhat resolved. The chromatofocusing column gradient appears to exaggerate small differences between isoelectric points of the species, yielding a better separation than expected from consideration of the p$I$s of the phosphorylated species.

Some unusual features of rhodopsin and the phosphorylation process can be noted from these results. Certain species have not been detected. Under the conditions used here, rhodopsin molecules with 1, 3, or 7 phosphates have not been observed. If the species exist, they are present only in low concentration. Wilden & Kuhn (1982) report a procedure for separating the phosphorylated species of rhodopsin by ion-exchange chromatography. In their analysis, they assume that every species between 0 and 9 is possible. There are no IEF gels of their fractions to indicate the presence of the 1 and 3 $P/R$ species nor any reported isoelectric points for these species. Although there is a 0.2–0.3 pH unit discrepancy in p$I$ with the values we report, Kuhn & McDowell (1977) also report that the first phosphorylated species of rhodopsin are ones having 2 and 4 $P/R$. Only weak justification was made by Wilden and Kuhn for including the species with 1 and 3 $P/R$ in their analysis. Furthermore, when incubation conditions are used that should result in maximal concentrations of the species having 1 and 3 $P/R$, these species are not observed by us using IEF (Figure 3).

We have compared the distribution of the phosphorylated species of rhodopsin obtained under our conditions with that reported by Wilden & Kuhn (1982). For this comparison, we have divided the rhodopsin fractions attributed to 1, 3, and 7 $P/R$ by Wilden & Kuhn (1982) between the adjacent species; i.e., half of 3 $P/R$ was added to 2 $P/R$ and half to 4 $P/R$. Approximately the same percentage of each phosphorylated species was obtained for a rhodopsin sample with an average $P/R$ of 4.8, generated under our incubation conditions, as was calculated by using the data reported by Wilden & Kuhn (1982) for a rhodopsin sample with 5 $P/R$. In the comparison of two samples, both with an average $P/R$ of 2, more of the highly phosphorylated species is present in a with

sample phosphorylated for 30 min with 0.6 mM ATP than in a sample incubated for 5 min with 3.0 mM ATP. This observation supports the conclusion of Wilden and Kuhn that there is cooperativity in the phosphorylation process; once the phosphorylation has started, there is a somewhat higher probability it will be phosphorylated than rhodopsin that is unphosphorylated. A nonuniform distribution of the kinase with the rhodopsin resulting from leaky ROS could also yield these results. Although homogenization of the ROS should alleviate this problem somewhat, further work is required to eliminate this as a factor before this conclusion can be drawn.

Both IEF and chromatofocusing rely on differences in the isoelectric points of the species for their separation. In these procedures, rhodopsin is solubilized in OG micelles. The possibility exists that more than one phosphorylated species could occupy a single micelle, giving rise in the separation to anomalous peaks. Since the rhodopsin concentration is higher in the chromatofocusing column than in the IEF gels, we have chosen those solubilization conditions for examination. The OG micelle concentration is 500 $\mu$M on the column. This was estimated by using 100 OG molecules per micelle (Mimms et al., 1981). Rosevear et al. (1980) report 30 OG molecules per micelle which would yield a micelle concentration of more than 1500 $\mu$M. The rhodopsin concentration in the column is never higher than when it is initially loaded, which is approximately 150 $\mu$M. Therefore, assuming a random distribution of rhodopsin molecules among the micelles, the percent of rhodopsin in a micelle with more than one rhodopsin molecule would be less than 3%. Furthermore, the concentration of rhodopsin is approximately an order of magnitude lower on IEF gels. The collected peak fractions when examined on gels consistently give single species peaks which would not be expected if the species isolated by chromatofocusing were made up of multiple species. Although the possibility that a particular species has a selective high affinity for another species not disrupted by the solubilization cannot be ruled out, we feel that this is highly unlikely.

Bownds et al. (1972) have reported a much higher level of phosphate incorporation (up to 50 phosphates per rhodopsin bleached) with low bleach levels. This can be satisfied either by phosphorylation of unbleached rhodopsin or by the presence of very highly phosphorylated species. No radioactive bands were detected in the 4.5–3.8 pH range in our experiments. Attainment of these levels of phosphorylation would probably require significant unfolding of rhodopsin to allow the kinase access to other serine and threonine groups or the phosphorylation of other amino acids. These are conditions under which the isoelectric points would not be expected to fit the simple pattern of eq 1. The isoelectric points of these species could then fall in the 6.0–4.6 pH range, and their concentration could be very low. Their presence would then be masked by the major species that we find.

We do not find evidence for the multiple species of purified unphosphorylated rhodopsin with p*I*s of 5.19, 5.58, and 6.14 reported by Plantner & Kean (1983). In the present studies, no new species of rhodopsin were observed in IEF gels of rhodopsin after Con A chromatography. The differences in p*I*s we observe between species with the same $P/R$ are 0.05 pH unit or less. Differences in detergent (OG vs. Emulphogene BC 720) or the separation procedure (IEF in polyacrylamide gels vs. free boundary IEF in a sucrose gradient) could be responsible for the observed discrepancy.

In summary, we have isolated six phosphorylated species of rhodopsin by chromatofocusing and determined the $P/R$ and isoelectric point of each. This column technique is shown to resolve species differing by only one phosphate group. The addition of phosphates is approximately in pairs, possibly alluding to the mechanism of the kinase action. The conditions used for phosphorylation are far from physiological, so that we cannot predict which of the phosphorylated species are the functionally significant ones or if the phosphorylation heterogeneity of a single species we observe takes place in vivo. Reconstitution of the individual species for testing in the PDE assay system is being pursued to determine their functional significance.

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