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Mechanism and Specificity of Rhodopsin Phosphorylation†

Robert N. Frank* and Sheldon M. Buzney

ABSTRACT: Partial separation of protein kinase activity from rhodopsin in isolated bovine retinal photoreceptor outer segments was accomplished by mild ultrasonic treatment followed by ultracentrifugation. Residual kinase activity in the rhodopsin-rich sediment was destroyed by chemical denaturation which did not affect the spectral properties of the rhodopsin. The retinal outer segment kinase was found to be specific for rhodopsin, since in these preparations it alone of several bovine protein kinases was capable of phosphorylating rhodopsin in the light. The phosphorylation reaction apparently requires a specific conformation of the rhodopsin molecule since it is abolished by heat denaturation of rhodopsin, and it is greatly reduced or abolished by treatment of the visual pigment protein with potassium alum after the rhodopsin has been "bleached" by light. When kinase and rhodopsin or opsin fractions were prepared from dark-adapted and bleached outer segments and the resultant fractions were mixed in various combinations of bleached and unbleached preparations, the observed pattern of light-activated phosphorylation was consistent only with the interpretation that a conformational change in the rhodopsin molecule in the light exposes a site on the visual pigment protein to the kinase and ATP. These results rule out the possibility of a direct or indirect (rhodopsin-mediated) light activation of the kinase. Finally, phosphorylation of retinal outer segment protein in monochromatic lights of various wavelengths followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that both rhodopsin and the higher molecular weight visual pigment protein reported by several laboratories have the same action spectrum for phosphorylation. This result is consistent with the suggestion that the higher molecular weight species is a rhodopsin dimer.

Rhodopsin, the visual pigment of the vertebrate retinal rod outer segments, undergoes phosphorylation by ATP or GTP in the light in a reaction that requires magnesium but is not stimulated by cyclic nucleotides (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Chader et al., 1975). Rhodopsin phosphorylation and a subsequent slow dephosphorylation have been shown to take place in vivo in the retinal rods of the frog (Kühn, 1974), but the role of these reactions in the normal physiology of the vertebrate visual cell is unknown. A previous report from our laboratory (Frank and Bensinger, 1974) and a recent communication from another group of investigators (Weller et al., 1975a) have dealt with the mechanism of the light activation of rhodopsin phosphorylation as well as the specificity of the retinal outer segment protein kinase for rhodopsin. Both reports suggested that the activation of rhodopsin phosphorylation by light results from the exposure of a phosphate-acceptor site on the visual pigment molecule to the nucleoside triphosphate and the enzyme. In addition, these studies indicated that rhodopsin is the specific substrate for a retinal outer segment protein kinase. However, as we shall discuss, the evidence thus far presented in support of these conclusions is incomplete. We now therefore describe experiments that were designed to examine these questions in further detail. Our results offer more concrete evidence in support of the conclusions of the two earlier reports, and give additional information on visual pigment phosphorylation.

Materials and Methods

Preparation of Retinal Outer Segments. Frozen, dark-adapted bovine retinas obtained from the George Hormel Co., Austin, Minn., were used in all of our experiments. Retinal outer segments were isolated by differential centrifugation on 40% sucrose in 50 m*M* Tris-HCl buffer (pH 7.4) as described previously (Frank et al., 1973). Outer seg-

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ments were prepared either under darkroom conditions in dim red light ("dark adapted") or in fluorescent room illumination with an additional fluorescent desk lamp placed on the laboratory bench within 24 in. of the work area ("bleached"). Following isolation the outer segments were stored in a freezer at −80°C until use. Protein kinase activity was dissociated from rhodopsin by mild sonication in 10 m*M* Tris-HCl buffer, containing 5 m*M* Na_2EDTA using a method similar to that of Kühn et al. (1973). The suspension was centrifuged at 98000*g* for 30 min, and the supernatant, containing most of the kinase activity, was gently removed with a Pasteur pipet. The sediment, which contained virtually all of the visual pigment protein, was either diluted with 50 m*M* Tris-HCl buffer (pH 7.4) to the appropriate protein concentration, or was washed once in distilled water prior to mixing with 7 ml of either 4% (w/v) aqueous potassium aluminum sulfate ("alum") or 2.7 *M* urea–5 m*M* Na_2EDTA in 10 m*M* Tris-HCl buffer at a final pH of 7.4. Following centrifugation at 98000*g* for 30 min the sediment was washed once with water and diluted with 50 m*M* Tris-HCl buffer (pH 7.4) to the appropriate protein concentration for use in the assays to be described.

In several experiments we prepared retinal outer segment kinase by a method similar to that of Weller et al. (1975a). Outer segments were suspended in 50 m*M* Tris-HCl buffer (pH 7.4) to a protein concentration of approximately 5 mg/ml and homogenized with ten up-and-down strokes of a Teflon pestle in a hand-held glass homogenizer (Arthur H. Thomas Co., Philadelphia). The suspension was centrifuged at 98000*g* for 30 min and the supernatant and sediment fractions were treated in a fashion similar to that of the sonicates above.

Preparation of Other Protein Kinases. Highly purified bovine cardiac muscle cAMP-dependent[1] protein kinase which had been prepared by the method of Gilman (1970) was obtained from the Sigma Chemical Co., St. Louis. Crude bovine skeletal muscle protein kinase and crude bovine brain protein kinase were prepared from tissues obtained fresh from a local slaughterhouse using the methods of Walsh et al. (1968) for the skeletal muscle enzyme and of Miyamoto et al. (1973) for the kinase from whole brain. Enzyme preparations were carried through the point of dialysis against phosphate buffer, without further chromatographic purification. Enzyme solutions were stored at −80°C until use.

Phosphorylation Assays. The assay medium consisted of 50 m*M* Tris-HCl buffer (pH 7.4), 3 m*M* $MgCl_2$, retinal outer segment protein, and kinase either from retinal outer segments or from other tissues in amounts to be indicated. In some experiments, cAMP was added in a final concentration of 5 μM. The final volume of the reaction mixture, after the addition of ATP, was 1.0 ml. The reaction was initiated by the addition of Na_2ATP (Sigma) in a final concentration of 0.5 m*M* containing 1–2 × 10^7 dpm of [γ-^{32}P]ATP (New England Nuclear, Boston), followed by agitation with a Vortex mixer. The reaction tubes were incubated for 10 min in a water bath at 37°C, since under these conditions rhodopsin phosphorylation in vitro in cattle retinal outer segments is maximal (Frank et al., 1973). The reaction was terminated with 6 ml of ice-cold 10% (w/v) trichloroacetic acid, and determination of phosphate incorporated into protein was carried out by gel electrophoresis as described below.

The activity of retinal outer segment kinase and of kinases from other bovine tissues was measured in a similar reaction mixture, but using calf thymus histone (Type II-A, Sigma) as substrate. Enzyme preparations containing 15–75 μg of protein were added to reaction mixture containing 200 μg of histone either in the presence or absence of 5 μM cAMP. Contents of the reaction tubes were emptied onto 13-mm diameter Millipore filters (Type HAWP, 0.45-μ pore size). The filters were washed once with 6 ml of 10% trichloroacetic acid and twice with 6 ml of distilled water, and were placed in liquid scintillation vials with 0.5 ml of 10% (w/v) aqueous dodecyl sulfate (Bio-Rad Laboratories, Richmond, Calif.). The vials were warmed overnight at 60°C; 5 ml of Aquasol (New England Nuclear) was added to each vial and radioactivity was determined in a liquid scintillation counter. Blanks, consisting of boiled outer segment material together with histone, were treated in a similar manner.

Polyacrylamide Gel Electrophoresis. The pattern and extent of phosphate incorporation into retinal outer segment protein was determined using electrophoresis on 7.5% polyacrylamide gels containing 0.5% dodecyl sulfate. The trichloroacetic acid denatured protein was sedimented by centrifugation and washed once with 6 ml of 10% trichloroacetic acid and twice with 6 ml of distilled water. The sediment was taken up in 0.65–1.0 ml of 10% dodecyl sulfate which had been warmed to 60°C and gently agitated overnight. Dialysis and gel electrophoresis were then performed as previously described (Frank et al., 1973), save that 15 m*M* imidazole–glycylglycine buffer (pH 7.2) was used instead of a phosphate buffer. Following electrophoresis, gels were either stained with Coomassie Brillant Blue (Mann Laboratories, Milwaukee, Wis.) or were frozen, sliced, and counted as described previously (Frank et al., 1973). The pattern of protein bands from retinal outer segment material prepared in gels with imidazole–glycylglycine buffer did not differ substantially from that described earlier using phosphate buffer (Frank et al., 1973; Frank and Bensinger, 1974). Electrophoresis of phosphorylated retinal outer segment proteins enabled us to distinguish the phosphorylation of rhodopsin, which can be precisely identified on dodecyl sulfate polyacrylamide gels, from other components of the retinal outer segment preparation.

Bleaching Experiments. We used a 150-W tungsten filament bulb placed about 50 cm above the water bath in which the assays took place. A flat-bottomed glass dish filled to a depth of 5 cm with water containing 2% (w/v) copper sulfate was interposed between the light source and the water bath to screen out infrared wavelengths. In addition, the light passed through a filter (No. 807, Dark Lemon Color Filter, Edmund Scientific Co., Barrington, N.J.) which eliminated wavelengths below 480 nm to prevent photoisomerization of near-ultraviolet absorbing late photoproducts of rhodopsin bleaching or of free retinal which had hydrolyzed from the visual pigment protein.

Action Spectrum Measurements. These were performed with the same light source and a series of 10-nm half-bandwidth interference filters (Baird Atomic, Cambridge, Mass.). The cutoff filter was removed for these experiments but the $CuSO_4$–water bath was retained, since the interference filters did not block infrared radiation. Radiant energy was measured at each of the filter wavelengths in the presence of the infrared-absorbing bath using a silicon solar cell (Type SS-10LC, Solar Systems, Skokie, Ill.) which was

[1] Abbreviation used is: cAMP, adenosine 3′,5′-monophosphate.

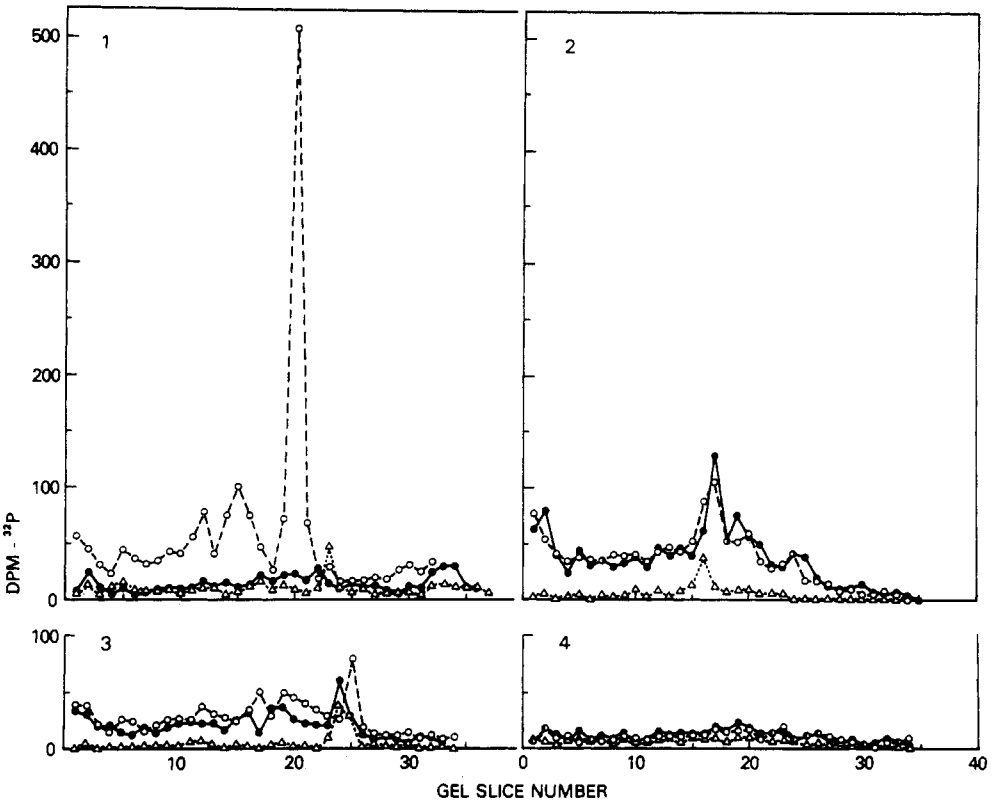

FIGURE 1: Phosphorylation of alum-treated bovine retinal outer segment membrane proteins as demonstrated by dodecyl sulfate polyacrylamide gel electrophoresis following incubation in darkness or continuous illumination with [γ-^{32}P]ATP and protein kinases from various bovine tissues. In the experiment illustrated here, all assay mixtures contained 5 μM cAMP. (1) Retinal outer segment kinase; (2) cardiac muscle kinase; (3) skeletal muscle kinase; (4) brain kinase. (●) Kinase and outer segments, dark; (○) kinase and outer segments, light; (△) kinase alone, light.

short-circuited into a low-impedance microammeter. Because the response of the cell was wavelength-dependent, meter readings were corrected from a calibration curve prepared by comparing the responses with a thermopile whose sensitivity was traceable to a National Bureau of Standards standard radiation source.[2] Based on these measurements, neutral density filters were used to produce lights of equal intensity at each of the test wavelengths. The action spectrum was then determined as the extent of phosphorylation produced by 10-min exposures to lights of equal intensity at each wavelength. It should be noted that this method is suitable for determining the wavelength at which the reaction has its peak sensitivity, but not for determining the precise shape of the spectral sensitivity curve to compare with the absorption spectrum of a visual pigment.

Spectrophotometry. Visual pigment content of the various preparations was measured in a Cary 17 recording spectrophotometer. Supernatant solutions were measured against distilled water, while retinal outer segments were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 40% sucrose and were measured against combinations of neutral density screens (Cary Instruments Division, Varian Corp., Monrovia, Calif., Parts No. 1404111-1404114). All solutions were mixed with hydroxylamine hydrochloride at a final concentration of 20 mM, titrated to pH 6.5 with NaOH. Rhodopsin content was determined from the change in absorbance at 500 nm (ΔA_{500}) of these suspensions following an exhaustive bleach in white light. Molar concentrations were calculated using a value of 40600 as the molar absorption coefficient for bovine rhodopsin (Wald and Brown, 1953).

Protein Determinations. These were carried out by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Results

Specificity of Rhodopsin Phosphorylation. Aliquots of a dark-adapted, alum-treated retinal outer segment sediment fraction were placed in reaction medium together with either the retinal outer segment kinase fraction, or kinases prepared from bovine brain, skeletal muscle, or cardiac muscle. The nonocular kinases were added to incubation mixtures containing 2000 μg of outer segment protein in quantities possessing 2–4 times the histone-phosphorylating activity (measured in the absence of cAMP) of the retinal outer segment kinase with which they were being compared. The histone-phosphorylating activity of the retinal outer segment kinase in the absence of cAMP was 940 pmol of phosphate per mg of histone per min at 37°C in the 75 μg of supernatant protein added to the incubation mixture, and the quantities of protein from the other enzyme preparations added were: brain, 150 μg; cardiac muscle, 50 μg; and skeletal muscle, 150 μg. Histone phosphorylation by all of these enzyme preparations, including that from retina, was further stimulated by a factor of 1.5–2 by 5 μM cAMP. Therefore, cAMP was also added to some experiments. Consistent with previous reports from several laboratories (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Weller et al., 1975a), we found that phosphorylation of rhodopsin by any of the protein kinases we examined was not sensitive to cAMP. Incubations were carried out in

[2] We thank Dr. William A. Hagins of the National Institutes of Health for the use of this instrument.

darkness and in light. As a further control, the enzyme preparations alone were incubated with [γ-^{32}P]ATP in the light to determine phosphorylation of endogenous proteins. The activity of ^{32}P in slices of electrophoretic gels prepared from these determinations is illustrated in Figure 1. Only the kinase present in the retinal outer segment supernatant was capable of phosphorylating the alum-treated outer segment protein during incubation in the light. There was no phosphorylation in the dark, and the retinal outer segment supernatant alone showed essentially no phosphorylation. Proteins in the other kinase preparations were themselves phosphorylated, as demonstrated by the small peaks in the appropriate curves in Figure 1. Note that the same peaks are present in the "kinase alone" curves and also in the "kinase plus retinal outer segment sediment" curves, indicating that phosphorylation in these mixtures occurred only in proteins from the enzyme preparations. As reported previously (Frank and Bensinger, 1974), when alum-treated outer segments were incubated alone with [γ-^{32}P]ATP either in darkness or in light there was no phosphorylation, demonstrating that 4% alum totally destroys the endogenous kinase activity.

In the presence of retinal outer segment kinase, two major peaks of retinal outer segment protein phosphorylation were usually observed, and a third, smaller peak was sometimes present. These correspond to the protein bands that have been identified with rhodopsin and with higher molecular weight proteins that have been considered to be either rhodopsin polymers (Heitzmann, 1972; Kühn and Dreyer, 1972; Bownds et al., 1972; Kühn et al., 1973) or, for the larger and more frequently observed of these slower moving peaks, a separate visual pigment (Cavanagh, 1970; Frank et al., 1973).

Composition of Outer Segment Supernatant Franctions. Examination of the gel electrophoresis results of Kühn et al. (1973) suggests that rhodopsin, as well as kinase activity, is present in supernatant fractions prepared by mild sonication of retinal outer segments (see their Figure 5). Such a possibility could alter the interpretation of our experiments. For example, if alum treatment denatures the phosphorylation site on the rhodopsin molecule, then only the undenatured rhodopsin in the supernatant would be capable of phosphorylation. The results of Figure 1 might then be due to phosphorylation of the rhodopsin that was present in the retinal kinase preparation but not in the kinase preparations from nonocular tissues, and the apparently increased phosphorylation observed in the presence of the alum-treated sediment might be due to coprecipitation of the rhodopsin from the relatively dilute supernatant by the high concentration of visual pigment protein in the outer segment membrane suspension.

Figure 2 presents Coomassie Blue stained gels of retinal outer segment suspensions and also of the supernatants following sonication and centrifugation. Small quantities of rhodopsin were present to varying extents in all of the supernatant fractions prepared in this way. Stained bands were not always seen following dodecyl sulfate polyacrylamide gel electrophoresis of supernatant preparations. Nevertheless, if these preparations were lyophilized and then dissolved in 10% dodecyl sulfate equivalent to 10–25% of the original volume of supernatant, gel electrophoresis of the concentrated solution always revealed a rhodopsin band. In some experiments, supernatant fractions were incubated in the light with [γ-^{32}P]ATP either alone or in the presence of 200 μg of bovine serum albumin. The latter is not phosphorylated by retinal outer segment kinase (Frank et al., 1973), and was used as a carrier protein to enhance precipitation of proteins from the dilute supernatants upon addition of trichloroacetic acid. Coomassie Blue stained gels showed no increase in the intensity of the rhodopsin band from the supernatant preparations in the presence of serum albumin (Figure 2), and counts made of slices of duplicate gels showed no enhancement of ^{32}P activity. When outer segment membrane suspensions were denatured by boiling for 10 min and then were mixed with retinal outer segment supernatant fractions, no increase in ^{32}P counts was observed in the rhodopsin band in gels prepared following incubation of the mixture with [γ-^{32}P]ATP in the light. These experiments indicate that heat-denatured rhodopsin is not a substrate for the kinase, and that coprecipitation of phosphorylated rhodopsin from the supernatant fractions cannot account for our results.

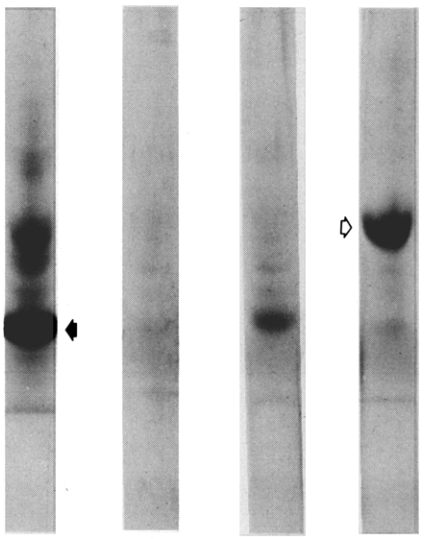

FIGURE 2: Electrophoretic patterns of bovine retinal outer segment proteins on 0.5% dodecyl sulfate–7.5% polyacrylamide gels stained with Coomassie Blue. (1) This gel shows the protein bands from an outer segment preparation following isolation by differential centrifugation. The most prominent band on the gel (solid arrow) has been identified as rhodopsin (Frank et al., 1973). Approximately 120 μg of protein was layered onto this gel. (2) The supernatant fraction prepared by sonication and ultracentrifugation of the outer segments whose protein banding pattern is illustrated in gel 1. A very faint rhodopsin band is visible. This preparation was rich in protein kinase activity. Approximately 5 μg of protein was layered onto this gel. (3) When a portion of the supernatant fraction illustrated in gel 2 was concentrated, the rhodopsin band, as well as other proteins, became more prominent. (4) When 200 μg of bovine serum albumin was added to an assay mixture containing the same supernatant fraction illustrated in gel 2 in a concentration of 75 μg of protein/ml, the retinal outer segment proteins were not precipitated to any greater extent upon addition of trichloroacetic acid. The open arrow indicates the albumin band.

Fractions prepared by mild sonication of retinal outer segments or, in a few experiments, by homogenization (Weller et al., 1975a), contained variable amounts of kinase activity. When these fractions were added back to alum- or urea-treated sediments, the resultant rhodopsin phosphorylation in the light varied from 20 to nearly 100% of that observed in an aliquot of the same outer segment preparation that had not been fractionated or incubated with denaturing agents. Sediment fractions which had not been treated with alum or urea following sonication or homogenization showed residual rhodopsin kinase activity that could reach greater than 90% of the unfractionated control. These re-

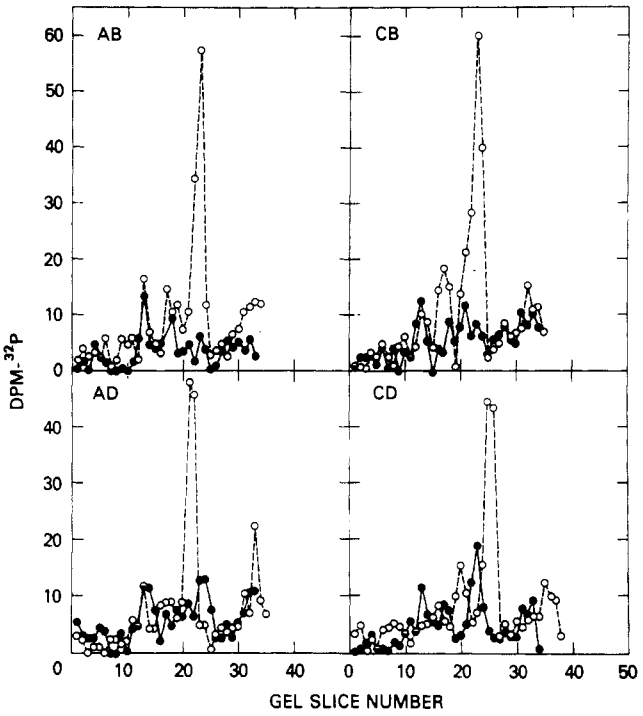

FIGURE 3: Phosphorylation of alum-treated retinal outer segment membranes which had been isolated in the darkroom or in room light. They were then recombined with dark-adapted or light-adapted supernatant fractions from the same preparations. In this and the following figures, the supernatant and sediment fractions are designated by letters as follows: (A) dark-adapted supernatant; (B) dark-adapted sediment; (C) light-adapted supernatant; (D) light-adapted sediment. (●) Phosphorylation carried out in darkness; (○) phosphorylation carried out in light.

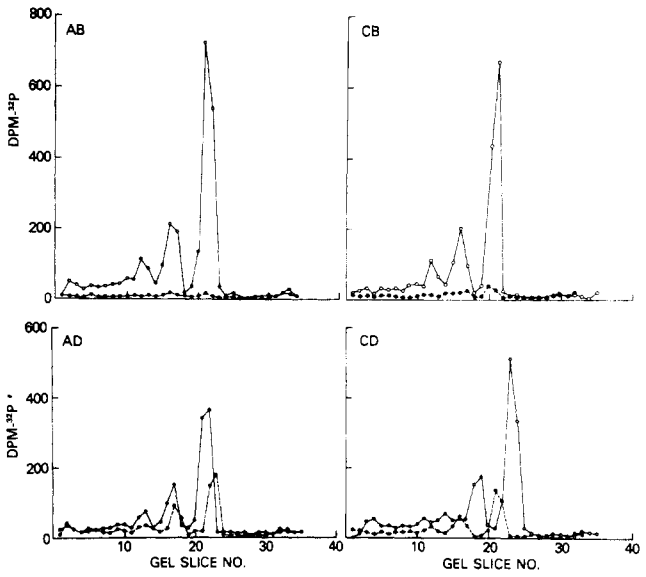

FIGURE 4: An experiment similar to that of Figure 3, but in which the sediment fractions had been treated with 2.7 *M* urea–5 m*M* EDTA instead of alum. Components of the mixtures are indicated by letters following the designations given in the caption of Figure 3. (●) Phosphorylation in dark; (○) phosphorylation in light.

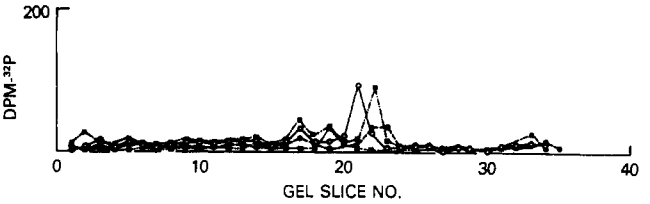

FIGURE 5: Phosphorylation of isolated supernatant and sediment fractions from the same retinal outer segment preparation used for the experiment of Figure 4. All incubations took place in the light. Similar results were obtained in other experiments in which the sediments were incubated in urea–EDTA. Note that the sediments alone show substantially less rhodopsin phosphorylation than they do following incubation with kinase-containing supernatant fractions (Figure 4). Following treatment of the sediment with alum, we have never observed even a small peak of rhodopsin phosphorylation unless a supernatant fraction is added. (●) Dark-adapted, supernatant; (○) dark-adapted, sediment; (■) light-adapted, supernatant; (□) light-adapted, sediment.

sults differ substantially from those reported by Weller et al. (1975a), who found only 20% of the total outer segment kinase activity remaining in the sediment fraction following homogenization.

Mechanism of Light-Activated Phosphorylation. Retinal outer segment supernatant and sediment fractions were isolated either in the dark-adapted or the bleached condition. The sediments were treated with either 4% alum or 2.7 *M* urea–5 m*M* EDTA and washed with water. Supernatant and sediment fractions were then added to incubation mixtures for phosphorylation in various combinations of dark-adapted and bleached samples. Amounts of protein from each fraction were kept constant in all assay tubes in each experiment and were of the order of 50–75 μg of supernatant protein and 2000 μg of sediment protein per tube. Duplicate samples of each mixture were incubated either in darkness or in continuous light for 10 min, the reaction was stopped, the samples were washed and dissolved in 10% dodecyl sulfate and dialyzed, and electrophoresis was performed.

When the outer segments used in these experiments had been treated with alum and then mixed with kinase fractions, the results of a typical experiment appeared as shown in Figure 3. Surprisingly, all four recombinant mixtures showed substantial increases in phosphorylation of the rhodopsin peak in the light, and the increases in all four were approximately equal. However, substantial phosphorylation of the rhodopsin peak observed following incubation with [γ-^{32}P]ATP in the dark was observed only in the mixture containing bleached supernatant and bleached sediment. This was not seen in all repetitions of this experiment. The total counts under the phosphorylated peaks in this experiment were low by comparison with other experiments presented in this paper. Rhodopsin phosphorylation in recombinant supernatant–sediment mixtures from different outer segment preparations varied considerably throughout this study, perhaps because of the wide variability in kinase activity recovered in the supernatants. Qualitatively, however, the results remained quite consistent.

When this experiment was repeated with urea-denatured outer segments, the results were somewhat different (Figure 4). All of the recombinant mixtures showed a consistent increase in phosphorylation of the visual pigment bands following incubation in the light. However, after urea treatment we observed substantial phosphorylation of the rhodopsin band during incubation in the dark *only in those mixtures containing bleached sediment.*

When we incubated urea-treated outer segment sediments with [γ-^{32}P]ATP in the light without addition of the kinase fraction, we observed phosphorylation of the rhodopsin band amounting to about 10% of that which occurred in mixtures of urea-treated outer segment membranes togeth-

Table I: Rhodopsin Content of Retinal Outer Segment Fractions.[a]

Fraction	ΔA_{500}	Concn (mol/l.)	Mol of Rhodopsin/ g of Protein
A	0.098 ± 0.026	$2.41 \pm 0.64 \times 10^{-6}$	$3.66 \pm 2.47 \times 10^{-6}$
C	0.000	0.000	0.000
B, alum	1.78 ± 0.82	$4.38 \pm 2.02 \times 10^{-5}$	$7.20 \pm 4.20 \times 10^{-6}$
B, urea	1.53 ± 0.65	$3.76 \pm 1.61 \times 10^{-5}$	$9.02 \pm 2.11 \times 10^{-6}$
D, alum	0.15 ± 0.20	$3.79 \pm 4.93 \times 10^{-6}$	$5.64 \pm 6.50 \times 10^{-7}$
D, urea	0.19 ± 0.13	$4.63 \pm 3.29 \times 10^{-6}$	$1.62 \pm 0.31 \times 10^{-6}$

[a] Values are means ± standard deviations for four determinations. Letters in the Fraction column are defined in the caption to Figure 3.

er with the supernatant fraction (Figure 5). Incubation with 2.7 *M* urea is therefore not as effective in destroying endogenous outer segment kinase activity as is treatment with 4% alum.

Our finding of substantial light-stimulated phosphorylation of rhodopsin in previously bleached outer segments was unexpected, and further experiments were undertaken in an attempt to provide an explanation. Spectrophotometry of supernatant and sediment fractions (Table I) revealed detectable rhodopsin in most of the dark-adapted supernatants and in the bleached sediments. Its presence in the bleached membranes suggested to us either that bleaching of rhodopsin during preparation of outer segments in room light had been incomplete, or that photoisomerization of retinal during this preparation had led to regeneration of small amounts of visual pigment. The molar concentration of rhodopsin, and its concentration per gram of protein, in the bleached membranes was about 10% of that in the dark-adapted preparations. That this small amount of unbleached rhodopsin probably was responsible for our results is suggested by measurements of the action spectrum of phosphorylation. Maximal phosphorylation in bleached as well as in dark-adapted preparations was observed with illuminations at 500 nm, both for the rhodopsin peak and of the more prominent of the smaller, higher molecular weight peaks (Figure 6).

To eliminate the effect of small amounts of unbleached rhodopsin in the light-adapted preparations, the experiments of Figures 3 and 4 were modified. The outer segment sediment fraction from 50 dark-adapted retinas was incubated for 10 min under dim red light with 6 ml of 150 m*M* hydroxylamine hydrochloride which had been titrated to pH 6.5 with NaOH. The sediment from 50 retinas prepared under fluorescent room illumination was similarly treated, but the incubation with NH_2OH was carried out in room light. The preparations were centrifuged, washed once with water, treated with either 4% alum or 2.7 *M* urea–5 m*M* EDTA, washed again, and used for phosphorylation experiments. The results for one such experiment following alum treatment are shown in Figure 7. Results with urea were identical. Light-activated phosphorylation of rhodopsin is present only in mixtures containing the dark-adapted sediment, while preparations containing totally bleached outer segments are not phosphorylated during incubation with [γ-^{32}P]ATP in darkness or in light.

Discussion

The following experiments demonstrate several important features of rhodopsin phosphorylation. (1) The phosphorylation of rhodopsin requires a specific protein kinase

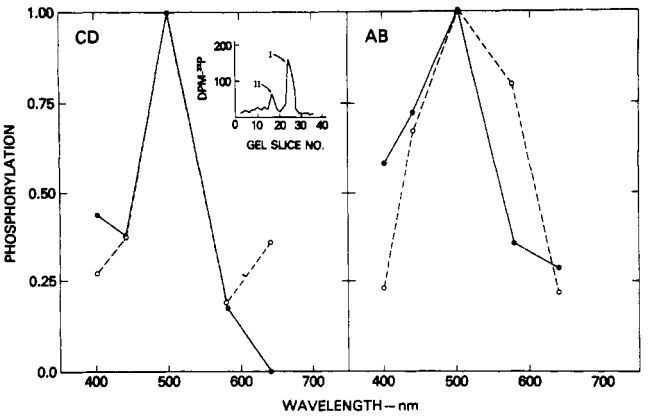

FIGURE 6: Spectral sensitivities for phosphorylation of the two major peaks (inset) observed on dodecyl sulfate-polyacrylamide gel electrophoresis of bovine retinal outer segment membranes. In this experiment, following separation of the supernatant fractions the membranes were treated with alum, however, results were similar following treatment with urea-EDTA. Totally dark-adapted recombinant mixtures (AB) as well as completely bleached ones (CD; see caption Figure 3) showed similar spectral sensitivities. Phosphorylation in this experiment was quantitated on a relative basis by tracing the peaks onto paper and weighing them. Weights were then normalized, taking the maximum value as 1.00. (○) Peak I; (●) peak II.

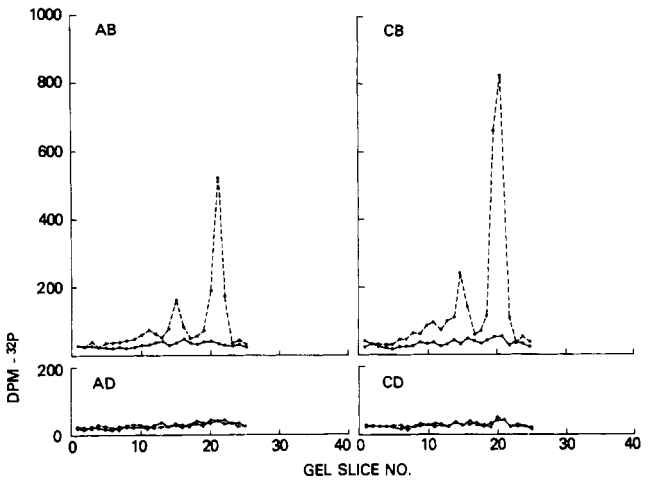

FIGURE 7: An experiment similar to that shown in Figure 3, but in which the sediment fractions were treated with NH_2OH in the dark-room or in room illumination before incubation with alum. Identical results were obtained when urea–EDTA was substituted for alum. The letters refer to the designations given in the caption to Figure 3. (●) Incubation in darkness; (○) incubation in light.

present in retinal outer segments. Kinases from other tissues are not effective. Previous studies which have suggested a similar conclusion (Frank and Bensinger, 1974; Weller et al., 1975a) were incomplete and required additional confirmation, since (a) they tested only one exogenous kinase either from bovine skeletal muscle (Frank and Bensinger, 1974) or bovine kidney (Weller et al., 1975a) and it seemed necessary to test others, particularly from additional sources in the central nervous system, and (b) Weller et al. tested their bovine kidney kinase in a system in which some of the endogenous retinal kinase remained active. In our hands, their method leaves as much as 90% or more of the kinase activity in the membrane fraction. Interpretation of their results is therefore difficult.

(2) The mechanism of activation of the reaction by light appears to involve a conformational change in the visual pigment molecule which exposes a previously masked recep-

tor site to the phosphate moiety. Direct light activation of the enzyme itself, or activation of the enzyme mediated through light absorption by rhodopsin molecules, have been ruled out. In either case, substantially increased phosphorylation would have been observed following incubation in the light in samples containing dark-adapted supernatant, which has a small amount of rhodopsin as well as kinase activity, but not in those containing bleached supernatant. Following incubation of the sediment fractions with NH_2OH to eliminate visual pigment regeneration, increased phosphorylation in the light was observed only in those mixtures with a large content of dark-adapted rhodopsin (sediment). Weller et al. (1975a) also concluded that the light effect involved rhodopsin only and not the kinase, but their evidence appears less convincing because of the substantial kinase activity remaining in their rhodopsin fractions.

If the light-activated phosphorylation of rhodopsin involves a conformational change in the visual pigment protein, at what stage in the bleaching process does this occur? It has generally been assumed that the greatest protein conformational changes during rhodopsin bleaching in detergent extracts occur after the metarhodopsin I stage (Matthews et al., 1963; Hubbard et al., 1965; Abrahamson and Ostroy, 1967). However, other investigators found no substantial changes in the circular dichroism of rhodopsin within the receptor membrane even past the metarhodopsin II stage (Shichi et al., 1969; Shichi, 1971). Although these latter studies indicate that no major conformational perturbations in the rhodopsin molecule within the receptor outer segment membrane occur during bleaching, only a very small conformational change may be necessary to make the appropriate site on the visual pigment protein available for phosphorylation.

(3) The site of rhodopsin phosphorylation is unstable and is readily denatured once it is exposed following bleaching of the visual pigment molecule. This is the most reasonable explanation for our observations of rhodopsin phosphorylation following treatment with alum or urea and following incubation with hydroxylamine. Bleached preparations which are then treated with alum show little or no phosphorylation when they are incubated with [γ-^{32}P]ATP in the dark, suggesting that the exposed phosphorylation site is no longer capable of accepting a phosphate residue. After urea treatment, bleached preparations show a small dark phosphorylation, which indicates that urea denatures the phosphorylation site less effectively, just as it less effectively destroys residual kinase activity in the outer segment membrane fraction. Since those rhodopsin molecules which have been bleached are preferentially denatured and can no longer be phosphorylated, only the small fraction of unbleached (or photoregenerated) rhodopsin is phosphorylated when the bleached sediment fractions are exposed to light in the presence of [γ-^{32}P]ATP. This appears as a surprisingly large "light–dark difference" in phosphorylation in samples that were prepared in room light. On the other hand, when we incubate bleached outer segments that have not been treated with urea or alum with [γ-^{32}P]ATP, we observe substantial phosphorylation following incubation in the dark, and very little increment following incubation in continuous light. Hydroxylamine abolishes the "light–dark difference" in phosphorylation in bleached preparations by preventing rhodopsin regeneration. Hydroxylamine appears to have a denaturing effect of its own, since urea-treated, bleached preparations that have been incubated with NH_2OH show no rhodopsin phosphorylation with [γ-^{32}P]ATP in the dark, quite different from the result observed following treatment with urea alone (Figure 4). Additional evidence that some portion of the visual pigment molecule must retain a "native" configuration to permit phosphorylation is provided by the observation that boiling of the sediment fraction prevents rhodopsin phosphorylation, even in the presence of an active retinal outer segment kinase. This requirement for a conformational specificity of the phosphate–receptor site on the rhodopsin molecule is a strong, if indirect, argument that the phosphorylation reaction is physiologically important. By contrast, sarcolemmal membrane proteins from rabbit skeletal muscle are more readily phosphorylated after denaturation by boiling than they are in the native state, and the reaction can be catalyzed by an exogenous kinase from bovine cardiac muscle (Sulakhe and Drummond, 1974).

One difficulty with the above interpretation remains. The "light–dark difference" in phosphorylation in bleached preparations that have not been treated with NH_2OH is quantitatively similar to that observed in dark-adapted membrane fractions (Figures 3 and 4), although the dark-adapted preparations contain a tenfold greater concentration of rhodopsin (Table I). We have no satisfactory explanation for this observation.

(4) Our spectral sensitivity experiments indicate that both the major rhodopsin band and the smaller, higher molecular weight visual pigment band show maximal phosphorylation with 500-nm irradiation. Although our measurements are not sufficiently detailed to rule out contributions to the phosphorylation of either of these protein bands from species with maximum absorption at other regions of the spectrum, they do provide evidence against our previous suggestion (Cavanagh, 1970; Frank et al., 1973) that the higher molecular weight band is distinct from rhodopsin and may represent a separate visual pigment.

Although our experiments provide additional arguments for the biological importance of rhodopsin phosphorylation, they give no information regarding its role in photoreceptor function. Two questions require investigation. Is the phosphorylation reaction fast enough to play a role in visual excitation, and how is it related to other physiological mechanisms in the photoreceptor such as membrane permeability, transport processes, or additional enzyme reactions? Most investigators have suggested that rhodopsin phosphorylation is too slow to be effective in visual excitation (Frank et al., 1973; Kühn et al., 1973; Weller et al., 1975a), and that it may instead be related to the dark-adaptation process (Kühn et al., 1973; Bownds et al., 1973; Kühn, 1974). However, most studies of rhodopsin phosphorylation have emphasized the *extent* of the reaction, which is maximal after 5–10 min in continuous illumination under physiological conditions in frog and cattle retinal preparations (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Weller et al., 1975a) and not the initial *rate* of the reaction, which may be more important physiologically. A recent report (Chader et al., 1975) indicates, however, that the rate of rhodopsin phosphorylation by ATP or GTP may be maximal within a few seconds following the onset of illumination. While this may still be too slow to affect visual excitation, further studies on this point are essential.

The precise biochemical steps that are controlled by rhodopsin phosphorylation are also unknown, but there are some useful clues for the design of future experiments. Phosphorylation of proteins in several systems is known to

regulate enzyme activity (Holzer and Duntze, 1971; Bownds et al., 1973). Vertebrate photoreceptor outer segments have high cyclic nucleotide phosphodiesterase activity (Pannbacker et al., 1972; Chader et al., 1973, 1974; Miki et al., 1973; Robb, 1974) which is markedly stimulated by light in the presence of ATP (Chader et al., 1973, 1974; Miki et al., 1973).[3] The role of cyclic nucleotides in the function of vertebrate visual receptors is unclear, although recent work has suggested that a hereditary visual cell degeneration in mice is associated with absence of cyclic nucleotide phosphodiesterase activity (Farber and Lolley, 1973, 1974). Since ATP and GTP are the nucleoside triphosphates that are maximally effective for the light-activated phosphorylation of rhodopsin, it is worth exploring the possibility that one important role for this reaction may be to regulate cyclic nucleotide phosphodiesterase activity in the photoreceptor outer segment and thereby to control the concentration of cyclic nucleotides in this structure.

Just after this manuscript was submitted for publication, three papers appeared that are closely related to our work. In the first, Miller and Paulsen (1975) separated protein kinase activity from rhodopsin in dark-adapted frog retinal outer segments by washing in a hypotonic buffer without homogenization or sonication. They performed recombination experiments somewhat similar to ours, with similar results. However, they did not conclude that light activates rhodopsin phosphorylation by unmasking the phosphate-receptor site on the visual pigment protein since they felt they could not rule out a catalytic stimulation of the kinase by small amounts of bleached rhodopsin. As noted in the Discussion, we believe that our experiments eliminate this mechanism precisely because—unlike the preparations of Miller and Paulsen—our kinase fractions contain small amounts of rhodopsin. If bleaching of a few rhodopsin molecules catalytically activates outer segment kinase, our results would have been different.

In the second paper, Weller et al. (1975b) have suggested that rhodopsin phosphorylation regulates the initial rate of calcium uptake and the rate of calcium release from light-adapted retinal outer segment discs. Although our experiments have no direct bearing on the conclusions of these authors, their results do not exclude the possibility that rhodopsin phosphorylation may also serve to regulate cyclic nucleotide phosphodiesterase activity, in particular since Keirns et al. (1975) have now demonstrated that the action spectrum for light stimulation of frog retinal outer segment cyclic nucleotide phosphodiesterase corresponds to the absorption spectrum of rhodopsin.

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[3] It has recently been observed that GTP supports light activation of bovine retinal outer segment phosphodiesterase equally well as ATP (G. J. Chader, personal communication).