Light-Regulated Binding of Rhodopsin Kinase and Other Proteins to Cattle Photoreceptor Membranes†

Hermann Kühn

ABSTRACT: Rhodopsin kinase, the enzymatic activity which catalyzes the light-induced phosphorylation of rhodopsin by ATP, can be readily extracted into aqueous buffers from dark-adapted, but not from illuminated, rod outer segments. Kinase activity was found to be up to 17 times higher in dark extracts than in corresponding light extracts, regardless of ionic strength and composition of the extracting buffer. The kinase binds to freshly bleached rod outer segment membranes but is released in the dark with a half-time of about 20–30 min at 20 °C. The light-induced binding is not influenced by $MgCl_2$, (ethylenedinitrilo)tetraacetic acid, or KF. This reversible binding was used to partially purify the enzyme: The kinase was bound to bleached photoreceptor disk membranes, other soluble proteins were washed out, and the kinase was then extracted in the dark. The most prominent protein in such purified extracts has a molecular weight of 48 000 and no kinase activity; its function is as yet unknown. Rhodopsin kinase, the next most prominent protein reversibly bound, has a molecular weight of 67 000–69 000, as determined both by molecular-sieve chromatography of the active enzyme and by sodium dodecyl sulfate gel electrophoresis. Gel electrophoresis also showed significantly higher amounts of these two proteins in crude dark extracts as compared with crude light extracts. Under some conditions, a third protein (ca. 37 000 daltons) was found to be present in dark extracts and absent in light extracts. The decay in the dark of the capacity of bleached membranes to bind kinase has a time course which corresponds approximately to the decay of phosphorylation activity after bleaching. It is concluded that rhodopsin or the membrane, after bleaching, *transiently* exhibits binding sites for the kinase and for the other proteins, and that the activity of the phosphate transfer, and perhaps of other reactions, is regulated by this light- and time-dependent binding.

Rhodopsin, the major protein of vertebrate rod outer segment (ROS) membranes (Heitzmann, 1972; Daemen, 1973; Papermaster and Dreyer, 1974), is phosphorylated subsequent to light absorption in a slow dark process which has been reported to take from a few minutes up to more than an hour (Kühn and Dreyer, 1972; Bownds et al., 1972; Kühn, 1974; Kühn and Bader, 1976). ATP and GTP (Chader et al., 1976) are known to be substrates; their terminal phosphate group is transferred and bound covalently to serine and threonine residue(s) in the protein moiety of rhodopsin. Unbleached cattle rhodopsin is not phosphorylated, and bleached rhodopsin can be phosphorylated only for a limited time after bleaching, suggesting that rhodopsin undergoes some changes after bleaching which make it only *transiently* available as a substrate for phosphorylation (McDowell and Kühn, 1977; Miller et al., 1977; Frank and Buzney, 1977).

The enzymatic activity which catalyzes the phosphorylation reaction, "rhodopsin kinase", has been extracted into aqueous buffers by several authors (Kühn et al., 1973; Weller et al., 1975; Frank and Buzney, 1975) and has been shown to be independent of light. Thus, the light activation of the reaction is due to the transient conversion of rhodopsin into a substrate rather than to activation of the enzyme by light (McDowell and Kühn, 1977). The phosphorylation of rhodopsin seems to occur independently of cyclic nucleotides (Kühn and Dreyer, 1972; Frank et al., 1973; Weller et al., 1975). The kinase activity present in ROS also catalyzes the phosphorylation of other substrate proteins besides rhodopsin, such as protamines and histones (Kühn et al., 1973). Not much else is known about this kinase activity. It is not even clear whether it is originally bound to the ROS disk membranes as a "peripheral membrane protein" (Singer and Nicolson, 1972) or whether it is originally soluble. So far, relatively harsh procedures involving low ionic strength, EDTA,[1] and/or sonication have been used for extraction, procedures which may solubilize peripherally bound membrane proteins.

This report shows that the kinase can exist in both water-soluble and membrane-associated forms and that binding to the membrane is dependent on the illumination history of the membrane. This reversible, selective binding can be used to separate the kinase from most of the other soluble proteins by "affinity chromatography" to the bleached membranes. Some of the properties of the kinase including the molecular weight of the active enzyme and of its polypeptide chain will also be described.

Materials and Methods

Preparation of Rod Outer Segments. ROS were prepared as described earlier (McDowell and Kühn, 1977). Briefly, freshly dissected cattle retinas were shaken in 45% sucrose. The ROS were first floated by centrifugation, then diluted to 15% sucrose, and sedimented. The resuspended crude ROS were purified by centrifugation on a stepwise sucrose gradient. The ROS fraction taken from the interface between 0.84 and 1.00 M sucrose was diluted with buffer and sedimented, and the pellet was frozen at −70 °C under argon. All solutions contained 70 mM sodium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM dithiothreitol (DTT).

† From the Institut für Neurobiologie, der Kernforschungsanlage Jülich GmbH, Postfach 1913, D-5170 Jülich, West Germany. *Received March 17, 1978; revised manuscript received July 13, 1978.* Supported by a grant from SFB 160 "Biologische Membranne" of Deutsche Forschungsgemeinschaft. Part of this work was presented in preliminary form at the Frühjahrstagung der Gesellschaft für Biologische Chemie, Frankfurt, March 1978.

[1] Abbreviations used: ROS, rod outer segments; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; cGMP, cyclic guanosine 3′,5′-monophosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

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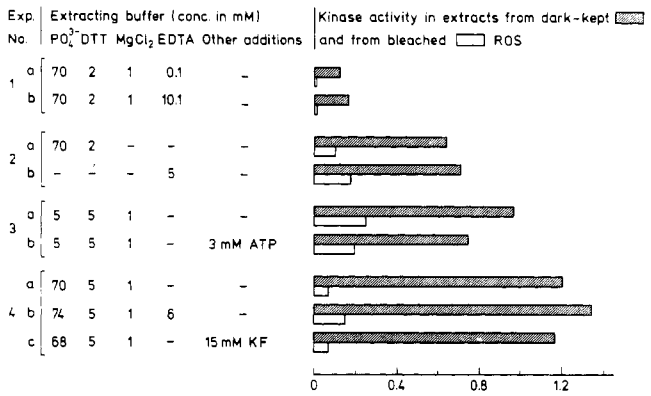

FIGURE 1: Extraction of kinase activity from ROS into various buffers in the light and in the dark. ROS were suspended in the indicated buffers and each suspension was divided into two equal parts for light and dark extraction. Both were warmed to 20 °C, one of them was illuminated for 3 min, then both were cooled in the dark and centrifuged (see Materials and Methods). The pH was 7.0 in all buffers. Kinase activity of the extracts is expressed, per 10 μL of extract, as mol of phosphate incorporated (mol of rhodopsin of alum-treated ROS)$^{-1}$ (30 min at 30 °C)$^{-1}$ (see Materials and Methods). Rhodopsin concentration in the suspensions to be extracted ranged from 150 (experiment 4) to 300 μM (experiment 2); the activity data shown have been corrected for different rhodopsin concentrations, such that they represent relative values based on a standard rhodopsin concentration of 150 μM. The activity data are averages from two to four assays for each extract. Each experiment is representative of at least two extractions performed under the same conditions. Experiments with different experimental numbers are not directly comparable because they involve different ROS preparations.

Rhodopsin concentrations were determined in detergent solutions from the difference in A_{500} before and after bleaching in the presence of NH_2OH, assuming ϵ_{500} 41 000. The spectral ratio A_{280}/A_{500} was between 2.0 and 2.4. All operations were carried out in the dark or in dim red light (Schott filter RG 665) if not stated otherwise. Alum-treated ROS, free of kinase activity, were prepared as described by McDowell and Kühn (1977).

Extraction of Kinase Activity. ROS were suspended and mildly homogenized by hand in the buffer indicated for each experiment. Rhodopsin concentrations were in the range of 120–300 μM. Sometimes the suspensions were frozen and thawed several times before extraction. All suspensions were divided into equal volumes for light and dark extraction, filled into 0.6 or 2.0-mL cellulose nitrate centrifuge tubes, and warmed up to 20 °C for 5 min in a water bath. The "light" samples were then illuminated for 3 min from the side, through the water bath, with white light from a 150-W bulb, at a distance of 20 cm. The tubes were slowly rotated during illumination, and bleaching was essentially complete after 30–60 s. After illumination, both light and dark samples were cooled in ice water in the dark for 1 min and then centrifuged at 0–4 °C for 7–10 min at 21 000 rpm (50 000g_{max}) in a Beckman JA-21 rotor fitted with adapters for the small tubes. The supernatants were removed and again centrifuged for 30–60 min to remove any residual membranous material. The resulting extracts were clear and free of rhodopsin. Sometimes, an ultracentrifuge was used at 220 000g_{max}; the properties of these extracts were similar to those of the extracts obtained at lower speed. Since the kinase activity of the extracts was not influenced by light in the absence of ROS, they could be handled in room light.

In some experiments where particularly high kinase activity was desired, the ROS were first homogenized in low ionic strength buffer [e.g., 2 mM sodium phosphate (pH 7.0), 1 mM $MgCl_2$, 2 mM DTT] to burst the plasma membrane, and then after 15 min on ice the phosphate concentration was raised to 70 mM, a concentration at which the kinase was found to be more stable.

Kinase Assay. The procedure of McDowell and Kühn (1977) was used with some modifications. Alum-treated ROS were used as substrate throughout, so as to provide a reasonably specific assay of rhodopsin kinase activity based upon the light-induced incorporation of phosphate into *rhodopsin*. In the standard procedure, 10 μL of alum-treated ROS containing 2.3 nmol of rhodopsin was mixed at 0 °C in the dark with 10 μL of the ROS kinase extract and 50 μL of phosphate buffer containing [^{32}P]ATP. Sometimes, at low kinase activities, 20 or 50 μL of the extract was used. The final concentrations were normally: 3.5 mM [^{32}P]ATP ([γ-^{32}P]ATP from Amersham Buchler, specific activity diluted to 2000–10 000 cpm/nmol), 4 mM $MgCl_2$, 5 mM DTT, 70 mM sodium phosphate buffer (pH 7.0). Slight deviation from these concentrations and partial replacement of the phosphate buffer by 20 mM Tris buffer did not change the results significantly. If EDTA was present in the extracts, an equal amount of $MgCl_2$ was added to them before the assay so as to maintain the Mg^{2+} concentration needed for the phosphorylation reaction. The samples were mildly sonicated for 1 min, warmed up to 30 °C in a thermostated water bath, and then illuminated for 30 or 60 min with white light. The light intensity (400 ft-c) was such that half of the rhodopsin was bleached within 13 s. The incubation was terminated by the addition of Cl_3CCOOH. The precipitated ROS membranes were normally washed on Millipore filters and the incorporated ^{32}P was measured by liquid scintillation counting as described previously (McDowell and Kühn, 1977).

In some more recent experiments, incubation was carried out in Eppendorf plastic vials rather than in glass tubes. The precipitated ROS membranes were then sedimented and washed by centrifugation instead of filtering. The final pellets were dissolved by shaking in a toluene-based scintillation cocktail containing 10% (v/v) 0.6 N NCS tissue solubilizer (Amersham Searle). Scintillation counting was performed in the Eppendorf vials. Washing by centrifugation was as effective as on filters, as judged by the background of unspecifically adsorbed ^{32}P which was similar for both methods.

Kinase activities will be expressed throughout as mol of ^{32}P incorporated per mol of rhodopsin per 30 min (or 60 min, if stated) of incubation at 30 °C. The following control experiments were performed (measured activities without any subtractions are given in parentheses): Alum-treated ROS without added kinase extract, incubated in light [0.012–0.020 mol of phosphate (mol of rhodopsin)$^{-1}$ (30 min)$^{-1}$] and in the dark (0.010–0.015); alum-treated ROS plus kinase extract incubated in the dark (0.015–0.020); kinase extracts without added ROS (0.035–0.040 nmol of ^{32}P/20 μL of extract in light as well as in dark). For comparison, typical values for ^{32}P incorporation into alum-treated ROS in the light with all components present were 0.5–1.5 mol of phosphate (mol of rhodopsin)$^{-1}$ (30 min)$^{-1}$. Thus, the only significant incorporation occurred when kinase and freshly bleached ROS were incubated together, and therefore only these light-induced values are presented under Results. The background of unspecifically adsorbed ^{32}P (0.020–0.030 nmol of ^{32}P/2.3 nmol of rhodopsin) was determined by adding [^{32}P]ATP buffer to previously acid-precipitated ROS. This background was subtracted from the measured light-induced values.

Gel electrophoresis on cylindrical gels containing 10% polyacrylamide and 0.1% $NaDodSO_4$ was performed according to Weber and Osborn (1969) with some modifications. Both gel and electrode buffer contained only 50 mM phosphate

instead of 100 mM. For optimum separation, the gels were electrophoresed 14–17 h at 60 V/5–6 mA per gel, which is about two to three times as long as it takes for the tracking dye to migrate through the whole gel. The gels were 12-cm long and 0.55-cm wide. For molecular weight determinations, fluorescein-labeled chymotrypsinogen (prepared from fluorescein-isothiocyanate and chymotrypsinogen) was used as an internal R_f marker for each gel. Standard proteins for molecular weight determination were phosphorylase *a* (92 500, from Boehringer), bovine serum albumin (67 000, Serva), bovine γ-globulin heavy chain (55 000, Serva), aldolase (40 000, Boehringer), pepsin (35 000, Boehringer), and chymotrypsinogen (26 000, Boehringer). Samples were denatured by incubation with 2% $NaDodSO_4$ and 2% 2-mercaptoethanol for 15–30 min at 40 °C and were applied to the gels without prior dialysis. Staining and destaining were performed according to Fairbanks et al. (1971). The position of the fluorescent marker band, visualized in UV light, was marked with India ink before staining. Stained gels were scanned at 580 nm in a densitometer connected to a Zeiss PMQ-II spectrophotometer.

Spectrophotometric measurements were made using a Cary 118 or a Cary 17 spectrophotometer. Kinetic measurements of the spectral decay reactions in ROS suspensions were performed in the scattering compartment of the Cary 118 using a thermostated (20 °C) cuvette.

Sonication was performed in a Buehler Ultramet III sonic cleaner (water bath).

Results

Extraction of Kinase in Dark and Light. In order to extract kinase activity, it is not necessary to treat ROS membranes with hypotonic shock, EDTA, or other harsh conditions, as has been done in earlier studies (Kühn et al., 1973; Weller et al., 1975; Frank and Buzney, 1975). The enzyme could be extracted in the dark by all of the buffers tested in this study, regardless of their composition and ionic strength. Some typical examples are given in Figure 1. The only requirement for extraction is that the plasma membrane which encloses the stack of disks and the cytoplasm be disrupted. This membrane appears to be easily broken, and care must be taken during the preparation of ROS to avoid serious losses of kinase activity by involuntary disruption. In fact, the supernatant obtained after the final pelleting of the ROS (see Materials and Methods, preparation of ROS) always contained significant kinase activity. In earlier experiments, disruption of the plasma membrane was attempted by repeated freezing and thawing of the suspension to be extracted (Figure 1, experiments 1–3, especially often in experiment 1). Later it was found that this was unnecessary and that each freezing of the extract causes up to a 50% loss of kinase activity. Freezing ROS *suspensions* was therefore kept to a minimum later to avoid possible damage of the kinase (Figure 1, experiment 4).

Repeated extraction in the dark removed at least 80% of the total kinase activity in most ROS preparations.[2] This indicates that in the dark most of the kinase is normally soluble or only weakly bound. In contrast, little kinase activity could be extracted from freshly bleached ROS suspensions. Kinase activity was found to be up to 17 times higher in dark extracts

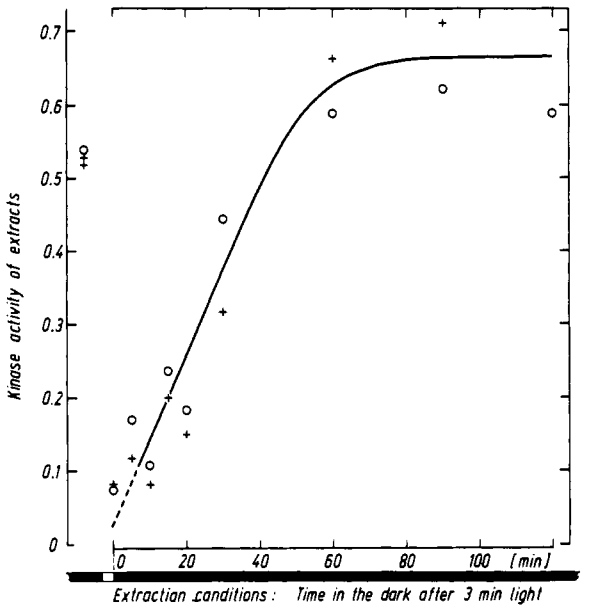

FIGURE 2: Kinase extractability as a function of the time elapsed between bleaching and centrifugation. Each of ten identical samples containing 0.5 mL of ROS suspension (110 nmol of rhodopsin) in 70 mM sodium phosphate buffer (pH 7.0), 2 mM DTT, 1 mM $MgCl_2$, and 0.1 mM EDTA was centrifuged either without bleaching (which is the dark sample before time zero), immediately after bleaching (which is time zero), or at various times after bleaching as indicated in the abscissa. Bleaching was performed for 3 min at 20 °C, as described under Materials and Methods. Kinase activity of the extracts is expressed as mol of phosphate (mol of rhodopsin)$^{-1}$ (60 min)$^{-1}$. The crosses and open circles represent two sets of experiments using two different ROS preparations. The curve was drawn by hand.

than in the corresponding light extracts (experiments 1a, 4a, and 4c in Figure 1). (In fact, this ratio is presumably even higher than 17, since at phosphate incorporations higher than one phosphate per rhodopsin, the kinase assay is no longer linear with the amount of kinase because of an insufficient excess of substrate.)

In all buffer systems tested, the dark extracts were much more active than the light extracts. For example, in an isotonic Ringer's solution (not shown in Figure 1, for composition see Kühn and Bader, 1976), the dark extract was 11 times more active than the light extract. EDTA appears to slightly increase the kinase extractability in both darkness and light (experiments 1b, 2b, and 4b), and therefore the dark/light ratio is somewhat lower in the presence of EDTA than in its absence. But otherwise, the variations in the dark/light ratio observed among different experiments are presumably due mainly to differences in the ROS preparations and/or illumination conditions rather than to specific effects of the different buffer solutions.

Reversal of Light-Induced Binding in the Dark. The light-induced decrease in kinase extractability was found to be fully reversible when the membranes were incubated in the dark for more than 1 h after bleaching. This indicates that the low activities measured in the "light extracts" are due to *transient binding* of the enzyme to the bleached membrane and not to light-induced enzyme denaturation. The time course of the return of solubility is shown in Figure 2. The data do not allow exact kinetic analysis; it is for instance not possible to judge whether or not there is an initial delay in the return of solubility at early times in the dark. However, it is clear that the kinase bound to the bleached membranes is slowly released in the dark, with a half-time in the range of 20–30 min.

Decay of Light-Induced Phosphorylation Activity in the

[2] It should be noted, however, that a few exceptions to this rule have been observed. In one ROS preparation, after extensive extraction of kinase, there was still sufficient kinase bound to the washed membranes to allow the incorporation of nearly 3 mol of phosphate/mol of rhodopsin in 60 min. Such binding of the kinase could, however, be explained, if the ROS were accidently exposed to light either before or after their preparation.

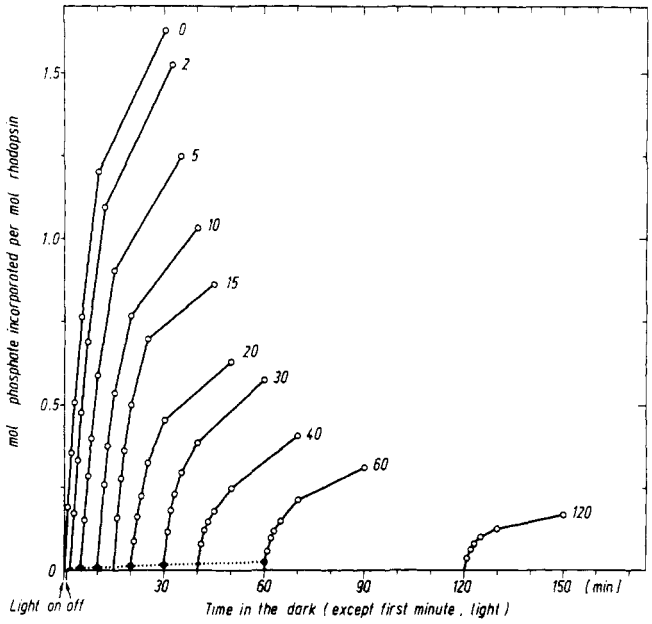

FIGURE 3: Decay of phosphorylation activity at 20 °C, determined by delayed addition of [^{32}P]ATP after bleaching whole ROS. Ten samples, each containing 0.3 mL of ROS suspension (12.2 nmol of rhodopsin), were sonicated for 1 min, prewarmed to 20 °C, bleached for 1 min with white light, and then kept in the dark at 20 °C. A 300-μL aliquot of [^{32}P]ATP buffer was then added to each at the time indicated by the number (in minutes) following each curve. Final concentrations were 3 mM [^{32}P]ATP (specific activity 13 200 cpm/nmol), 1 mM $MgCl_2$, 3.5 mM DTT, 0.05 mM EDTA, 70 mM sodium phosphate (pH 7.0). Incubation was continued in the dark at 20 °C, and 100 μL aliquots of the samples were pipetted into 200 μL of ice-cold 25% Cl_3CCOOH solution at 1, 2, 3, 5, and 10 min after addition at [^{32}P]ATP buffer. A 50-μL aliquot was taken at about 30 min (between 29 and 32 min). ^{32}P bound to rhodopsin was determined after centrifugation of the precipitated ROS as described under Materials and Methods. The ordinate represents phosphate incorporation in mol of phosphate/mol of rhodopsin. Incorporation into unbleached ROS is shown by filled circles connected with a dashed line. The ROS preparation used was the same as that from which the (+) data in Figure 2 were obtained.

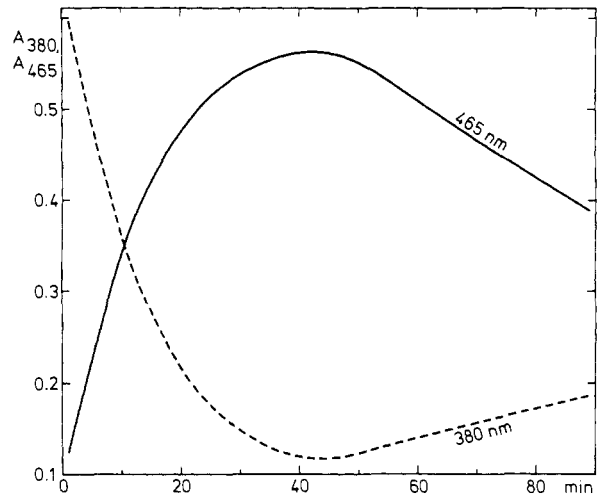

FIGURE 4: Kinetics of the late photoproducts of rhodopsin at 20 °C. The decay of meta II rhodopsin (dashed line) was recorded at 380 nm and the formation and decay of meta III rhodopsin (solid line) at 465 nm. ROS suspensions (2×10^{-5} M rhodopsin), from the same preparation and in the same buffer as used in Figure 3, were mildly sonicated for 6 min and warmed up to 20 °C in a thermostated cuvette. After illumination for about 20 s with orange light from a light pipe which bleached 85-90% of the rhodopsin, the change in A_{380}, or A_{465}, respectively, was recorded. The ordinate represents the relative absorption change.

Dark. It has previously been shown (McDowell and Kühn, 1977; Miller et al., 1977) that bleached rhodopsin is susceptible to phosphorylation only for a limited time after bleaching. If ROS suspensions are bleached in the absence of [^{32}P]ATP (or kinase), and [^{32}P]ATP (or kinase) is added later in the dark, the phosphate incorporation decreases with increasing time between bleaching and addition of [^{32}P]ATP (or kinase). The time course of this "decay of phosphorylation activity" appeared to vary considerably from one ROS preparation to another (McDowell and Kühn, 1977). Furthermore, the incubation time used to analyze the phosphate incorporation, namely, 60 min in the presence of [^{32}P]ATP and kinase, was relatively long as compared to the rate of decay of phosphorylation activity, introducing some uncertainty in the determination of this rate.

In an attempt to correlate the observed time-dependent release of kinase from bleached membranes with this decay in phosphorylation activity, the rate of the latter was measured in the same two ROS preparations as in Figure 2 and with the same buffer and temperature (20 °C). Incubation times shorter than 60 min were used to measure phosphate incorporation.

The result of one such experiment is shown in Figure 3 (the other ROS preparation gave similar results). Whole ROS still containing their intrinsic kinase activity[3] were bleached in the absence of [^{32}P]ATP and then kept in the dark for various times until [^{32}P]ATP was added. Phosphorylation was then allowed to proceed for 1, 2, 3, 5, 10, and about 30 min in each sample.

The kinetic analysis of the data is somewhat complicated by the fact that the decay of phosphorylation activity continues during the incubation with [^{32}P]ATP. Therefore, the data obtained at shorter incubation times with [^{32}P]ATP should more closely reflect the decay of phosphorylation activity than the data obtained at long incubation times with [^{32}P]ATP. Semilogarithmic plots of the data lead to a series of apparent half times which depend on the duration of the incubation after the addition of [^{32}P]ATP. These apparent half times range from 18 (obtained from the data for a 30-min incubation in the presence of [^{32}P]ATP) to about 26 min (obtained from the 2-min data). For the purpose of this paper, it is sufficient to state that in the ROS preparations used for the experiments of Figures 2 and 3 most of the phosphorylation activity decays with a "half-time" between 20 and 30 min. This is the same time range as observed for the release of kinase in the dark from previously bleached membranes (Figure 2). It should be noted that this decay of phosphorylation activity is due to *reversible* changes in the substrate molecule, rhodopsin, and not to inactivation of the enzyme; the phosphorylation activity can be restored if the bleached rhodopsin is regenerated with 11-*cis*-retinal and then rebleached (unpublished experiments).

Decay of Meta II and Formation of Meta III Rhodopsin. It was thought that the decay of phosphorylation activity might be correlated with one of the "spectral decay" reactions of rhodopsin. Spectrophotometric measurements at 380 and 465 nm using flash-bleached, sonicated ROS suspensions (Figure 4) show that both the decay of meta II and the formation of meta III rhodopsin occur with a half-time of about 9 min at 20

[3] This is the only experiment in this report measuring phosphorylation of rhodopsin by the kinase which was *originally present* in the same ROS preparation. In all other phosphorylation experiments, previously *extracted* kinase was assayed for activity using alum-treated ROS membranes as a substrate. These membranes were free of residual kinase activity (see Materials and Methods).

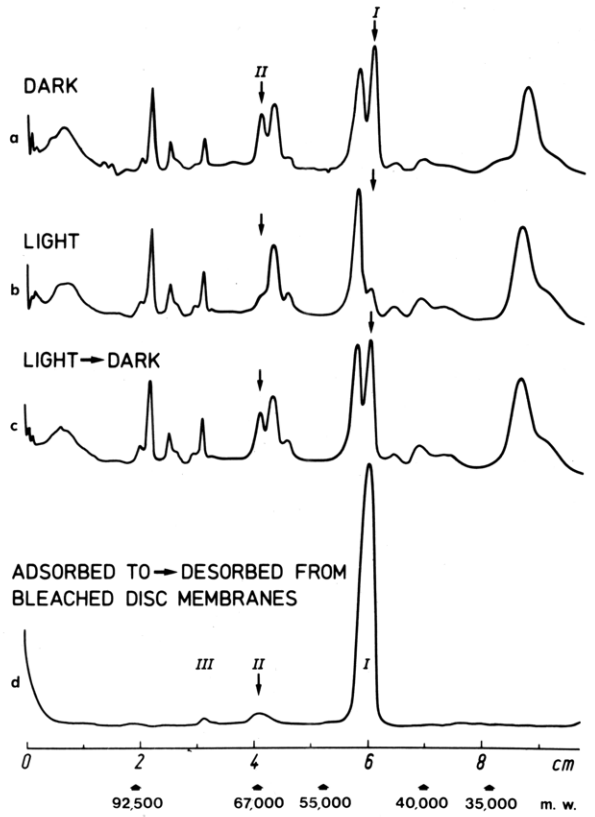

FIGURE 5: $NaDodSO_4$-gel electrophoresis of crude and purified ROS extracts. The ordinate (not shown) of the densitograms represents the relative absorbance at 580 nm: (a) extract from dark-kept ROS; (b) extract from bleached ROS, centrifuged immediately after bleaching; (c) extract from bleached ROS, centrifuged 90 min after bleaching [the extracting buffer in samples a–c contained 74 mM sodium phosphate buffer (pH 7.0), 5 mM DTT, 1 mM $MgCl_2$, 6 mM EDTA]; (d) proteins purified by specific binding to bleached disk membranes (supernatant 3 of Table I). The ROS dark extract was mixed with disk membranes in the presence of light, soluble proteins were washed out, and the proteins adsorbed to the bleached disks were eluted later in the dark.

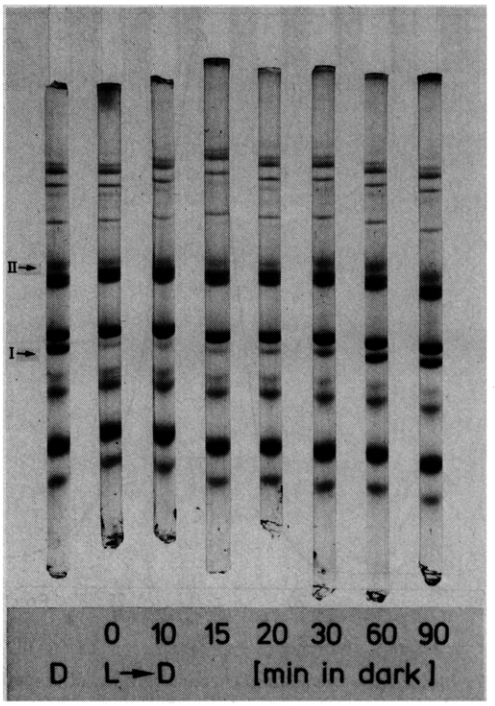

FIGURE 6: Polypeptide composition of ROS extracts as a function of time after bleaching. The extracts (O) shown in Figure 2 were electrophoresed on $NaDodSO_4$-polyacrylamide gels. The two arrows denote the light-dependent polypeptides I and II (see also Figure 5).

°C. (In five similar experiments, 9.3 ± 0.6 min was measured.) The concentration of meta III rhodopsin reached a maximum about 40 min after bleaching and then decayed in the course of several hours. These measurements show that meta II decays too fast and meta III too slowly to be correlated with the decay of kinase binding and of phosphorylation activity described above.

Protein Composition of the Extracts. After $NaDodSO_4$-polyacrylamide gel electrophoresis of the ROS extracts, about 15–20 polypeptide bands could be discerned. Comparison of dark vs. light extracts showed that two polypeptides, with apparent molecular weights of 67 000 and 48 000, were always present in dark extracts but were missing or present in reduced amount in light extracts (Figure 5a,b, polypeptide bands II and I). If ROS had been bleached and then kept in the dark for 90 min to allow release of the kinase before centrifugation, both polypeptides were found back in the soluble fraction (Figure 5c). This indicates that both polypeptides are only *transiently* bound to the bleached membranes. This dark/light difference of the protein pattern was observed in all extracts tested (see Figure 1), regardless of the buffer composition. Thus, the light-induced binding of both proteins to the photoreceptor membranes is not significantly influenced by EDTA, KF, ATP, or by ionic strength.

Analysis of the gels shown in Figure 6 indicates that both proteins I and II are released from previously bleached ROS membranes within about the same time and that this time is about the same as that in which the kinase activity reappears in the extracts (Figure 2). The dark–light difference of band I (48 000) was always large, as seen in Figure 5. The dark–light difference of band II (67 000), on the other hand, was normally less pronounced than shown in Figure 5 and was particularly low if the ROS had been frozen and thawed several times before extraction. In the example shown in Figure 5 (corresponding to experiment 4b from Figure 1), the ROS had not been frozen. Sometimes, in extracts with kinase activity ratios dark/light of more than 10, band II had a dark/light ratio of less than 2. This apparent discrepancy may be explained by the assumption that extracts from frozen ROS contain *denatured* protein II which no longer undergoes the light-dependent binding to the membranes. The presence of this denatured protein in both light and dark extracts would then mask the light dependence of band II.

In some, but not in all, experiments another light-dependent polypeptide band of molecular weight about 37 000 was found in phosphate-buffered extracts (not shown in Figure 5). Like bands I and II, it was present in dark extracts in higher amounts than in corresponding light extracts. If distilled water at pH 5.9 (Lolley and Farber, personal communication) was used instead of phosphate buffer for extraction, this 37 000 polypeptide was always the most prominent band in dark extracts and was virtually missing in corresponding light extracts.

Purification of the Light-Dependent Proteins. The light-dependent binding of some proteins to the photoreceptor membrane offers the opportunity to purify these proteins by "affinity binding chromatography", namely, to bind them transiently to the bleached membranes while washing out the other soluble proteins. Many attempts to do this starting from whole ROS with batchwise procedures as well as with column chromatographic procedures, using glass beads or concanavalin A–Sepharose as a rhodopsin-binding support, failed. The main difficulty was to quantitatively wash out the other soluble

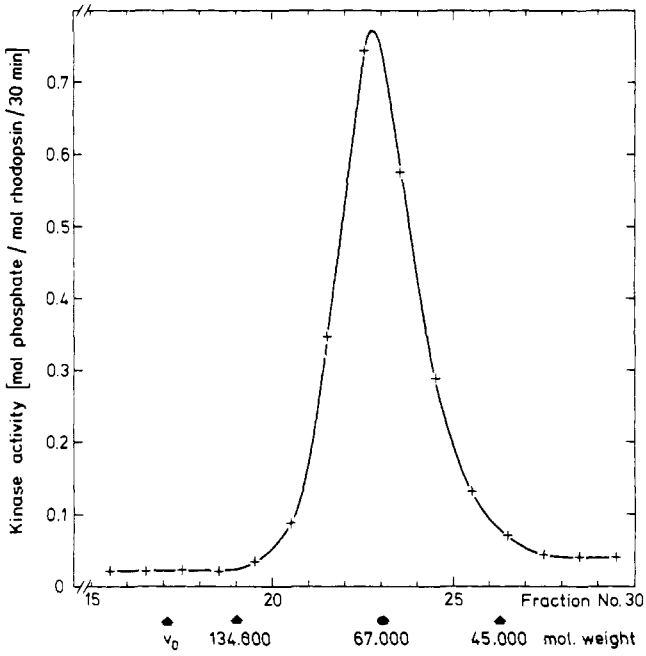

FIGURE 7: Molecular-sieve column chromatography of crude ROS extract on Sephadex G-100 Superfine. The buffer was 70 mM sodium phosphate (pH 7.0), 1 mM $MgCl_2$. The dark extract from ROS corresponding to 0.4 μmol of rhodopsin in 0.5 mL of buffer was chromatographed on a column 42-cm high and 1.0-cm wide. The column was run at 4 °C with a flow rate of 6 mL/h, and fractions of 0.65 mL were collected. Kinase assays were performed with 50 μL of each fraction. The kinase activities given for fractions 1–29 are averages of four determinations. Calibration runs with Blue Dextran (Pharmacia, for determination of void volume, v_0), bovine serum albumin (molecular weight 67 000 for the monomer and 134 000 for the dimer), ovalbumin (45 000, Serva), and myoglobin (17 000, Serva) were performed both before and after chromatography of the kinase extract. Myoglobin was eluted with its peak in fraction 35.5 (not shown); the other calibration substances were eluted at the positions indicated by the arrows. Fractions 21–26 together contained about 90% of the total kinase activity applied to the column.

proteins which stick rather tenaciously to the membranes. Finally, disks purified by the method of Smith et al. (1975) were used and washed thoroughly to remove the soluble proteins almost quantitatively.

Table I shows the results of such a purification by transient adsorption of kinase to bleached disk membranes. Supernatant 1 contained those proteins from the ROS kinase extract which were not bound to the bleached membranes. Gel electrophoresis showed this supernatant to contain all of the polypeptides normally present in ROS extracts. Supernatant 2, a second wash of the membranes after incubation with the ROS extract, contained little protein. Supernatants 3–6 were obtained after allowing sufficient time for the kinase activity to be released from the disk membranes. This repeated dark extraction led to decreasing amounts of protein corresponding to the decreasing kinase activity seen in Table I. The polypeptide pattern of supernatant 3 is shown in Figure 5d. Supernatants 3–6 each essentially consist of only the three indicated polypeptide bands, two of which correspond to bands I and II of the crude extracts. It is unclear whether polypeptide III (molecular weight about 79 000), which exhibited no light dependence in the comparison of *crude* light vs. dark extracts, is specifically bound to the bleached membranes like I and II or whether it appears only as a result of nonspecific binding. In five similar adsorption–desorption experiments using different disk preparations and ROS extracts, polypeptides I–III were always the major bands of the purified extracts, although sometimes additional bands in the molecular weight range of 50 000 to

TABLE I: Kinase Activity in Soluble ROS Fractions Adsorbed to and Desorbed from Bleached Disk Membranes.[a]

supernat fraction	total vol (mL)	act./50 μL	total act.
1	8.0	0.17	27
2	1.2	0.12	2.9
3	0.6	1.02	12.2
4	0.5	0.67	6.7
5	0.4	0.48	3.8
6[b]	1.0	0.42	8.4
crude extract	2.5	~1.55	~80

[a] Crude ROS dark extract was mixed with disk membranes at 20 °C in the dark, then illuminated for 3 min, cooled to 0 °C, and centrifuged (supernatant 1). The pellet was quickly washed with ice-cold buffer in the dark, sedimented again (supernatant 2), and finally suspended in 0.6 mL of buffer at 20 °C in the dark for 1 h. After centrifugation (supernatant 3), the pellet was extracted three more times (supernatants 4–6) in the same way. The buffer contained 70 mM sodium phosphate (pH 7.0), 5 mM DTT, and 1 mM $MgCl_2$. Kinase activity of the extracts was determined using 50 μL of extract and is expressed as mol of phosphate incorporated (mol of rhodopsin)$^{-1}$ (60 min at 30 °C)$^{-1}$. The activities given are averages of two to five assays. This experiment is representative of four similar experiments with similar results. [b] The disk pellet after the fifth extraction was frozen, thawed, and incubated for 3 h at 0 °C and for 1 h at 21 °C before centrifugation.

100 000 appeared, probably due to unspecific binding. Band I of molecular weight 48 000 was always by far the most prominent band, comprising up to 95% of the total stained material on the gels. The third "light-dependent" polypeptide (molecular weight 37 000; see previous section) was never found in such purified extracts. This may be due to the fact that 70 mM phosphate buffer and not water was used in these experiments.

Molecular Weight of the Active Kinase. Molecular-sieve chromatography of crude ROS dark extracts was used to determine which one of the bands I, II, or III seen on polyacrylamide gels may correspond to the kinase. Relatively short columns and high flow rates were used because of the instability of the kinase. Therefore, the resolution of proteins is not excellent, but it is sufficient to see (Figure 7) that the peak of kinase activity is eluted close to the position of bovine serum albumin (molecular weight 67 000) and that it is clearly separated from ovalbumin which has a molecular weight (45 000) close to polypeptide band I of Figure 5. Results from four chromatographic runs on different Sephadex G-100 columns agreed sufficiently well to give the molecular weight of the active kinase as 69 000 ± 4000. This suggests that polypeptide band II (Figure 5) represents the kinase and that the active enzyme consists of one polypeptide chain.

$NaDodSO_4$-gel electrophoretic analysis of the column fractions showed that a polypeptide of molecular weight ca. 67 000 had its maximum occurrence in fractions 22–24 corresponding to the peak of kinase activity. It is identical with polypeptide II from Figure 5. Polypeptide I is also eluted from the Sephadex column as a monomer, with its maximum occurrence in fractions 25–26; thus protein I also exists as a monomer in its native form.

Discussion

Obviously, rhodopsin kinase is a soluble enzyme, since most of it can normally be extracted from dark-kept ROS into various buffers, regardless of their ionic strength and composition. Upon illumination, however, the kinase is bound so firmly to the membranes that it sediments with them; i.e., the

kinase becomes a "peripheral membrane protein" for a limited time after bleaching. The solubility returns in the dark with a half-time of about 20–30 min.

Lolley et al. (1977) recently reported the existence of two forms of protein kinase in ROS, namely, a membrane-bound kinase which phosphorylates rhodopsin independently of cGMP and a soluble kinase which phosphorylates soluble ROS proteins and is stimulated by cGMP. It is not clear at this time whether or not both of these enzyme activities are identical with the kinase described in this report.

The decay in the dark of the capacity of bleached ROS membranes to bind the kinase (Figure 2) correlates approximately with the decay of phosphorylation activity (Figure 3). In other words, the ability of bleached ROS membranes to bind the kinase and their ability to be phosphorylated decay at about the same rate. This suggests a mechanism for light activation of the phosphorylation reaction. Upon bleaching, rhodopsin (or the membrane) undergoes some structural changes which transiently expose a site for kinase binding. Only as long as the kinase is bound to this site can it phosphorylate rhodopsin. This is an interesting way to regulate an enzymatic reaction: The light-induced change in the *substrate,* rhodopsin, permits the enzyme to be bound and the enzymatic reaction to proceed. Obviously, this light-regulated binding is independent of ATP and Mg^{2+}. This hypothetical mechanism leaves open the possibility that the serine and threonine residues are *always exposed* to the aqueous surface but can be phosphorylated only if the kinase is bound. (Another possibility is that these residues are *buried* in dark-adapted rhodopsin and opsin and are transiently exposed upon bleaching. The above model does not distinguish between the two possibilities.)

The decay of both phosphorylation activity and of kinase binding capacity does not correlate with the decay or formation of any of the "slow photoproducts" of rhodopsin: It occurs more slowly than the decay of meta II rhodopsin but faster than the decay of meta III rhodopsin. This is somewhat surprising, but one can imagine that structural changes may take place in the water-exposed part of rhodopsin independently from the spectral reactions which take place at the chromophoric center in the "hydrophobic core" of rhodopsin. For example, it is known that regeneration of opsin and of [^{32}P]opsin with 11-*cis*-retinal to rhodopsin ([^{32}P]rhodopsin) occurs *independently* of the phosphorylation state (Kühn et al., 1973; Miller et al., 1977), indicating that the chromophoric center and the phosphorylation site can act independently.

In frog ROS, the formation and decay of meta III rhodopsin at 20 °C occur considerably faster than in cattle ROS (comparison of the data of Figure 4 with the data of Miller et al., 1977). It has been shown that in frog ROS at 20 °C the phosphorylation activity decays at the same rate as meta III decays, but at lower temperature it seems to decay much more slowly than meta III (Miller et al., 1977). This again suggests that decay of phosphorylation activity and decay of the photoproducts are not closely correlated.

The transient binding of kinase activity to the bleached membranes was used to separate the kinase from most of the other proteins in ROS extracts. Surprisingly, the predominant protein in these purified fractions was not the kinase (molecular weight 67 000–69 000) but another protein with a molecular weight of about 48 000, which also consists of a single polypeptide chain. Its binding to bleached membranes and release from dark-adapting membranes appear to parallel the binding and release of kinase. This protein has been obtained in high purity, but its function is as yet undetermined.

A third protein (molecular weight ca. 37 000) was also found to be present in dark extracts and absent in light extracts, if special extracting conditions were used. The function of this light-dependent protein has also not yet been determined. It seems likely that such transient binding of soluble proteins to disk membranes after bleaching serves to regulate some enzymatic activities in the photoreceptor.

Note Added in Proof

With regard to Figure 1, recent experiments indicate that ATP can under certain circumstances influence the binding of rhodopsin kinase to disks. ATP has no effect on the binding in the dark before bleaching and has only a small effect on the binding to *freshly bleached* disks; however, ATP significantly *accelerates the release* of kinase in the dark after bleaching. Therefore, the finding in Figure 1, that ATP did not influence the ratio of kinase activity in dark vs. light extracts, should not be generalized since the kinase activity in light extracts may appear higher in the presence of ATP than in its absence due to possible differences in the release of bound kinase during the darkness which accompanies the centrifugation step.

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