Light-Induced Binding of Guanosinetriphosphatase to Bovine Photoreceptor Membranes: Effect of Limited Proteolysis of the Membranes†

Hermann Kühn* and Paul A. Hargrave‡

ABSTRACT: The binding of rod cell guanosinetriphosphatase (GTPase) to rod cell disk membranes, and to disk membranes which have been modified by limited proteolysis, has been compared under different conditions of ionic strength and illumination. Disk membranes purified from bovine rod outer segments were digested with thermolysin to produce two different modified preparations. (Short-term digestion removes 12 amino acids from rhodopsin's carboxyl terminus [Hargrave, P. A., & Fong, S.-L. (1977) *J. Supramol. Struct. 6*, 99]. Long-term digestion leads to further cleavage(s) producing two large noncovalently associated membrane-bound fragments [Pober, J. S., & Stryer, L. (1975) *J. Mol. Biol. 95*, 477].) The extract from purified rod outer segments, containing the GTPase and other soluble proteins, was added to the different disk membrane preparations, and binding to the membranes was measured in the dark and following exposure to light. At *low* ionic strength the GTPase was soluble in the dark but became membrane bound upon illumination; this light-induced binding occurred to short term digested disks as well as to undigested control disks, indicating that the 12 amino acids from rhodopsin's carboxyl terminus are not involved in this light-induced binding reaction. Long term digested disks, however, exhibited a greatly diminished light-induced capacity to bind the GTPase. This suggests that the light-induced binding site is located in rhodopsin and that the site is removed as a result of digestion of an internal segment of rhodopsin's polypeptide chain. Instead of binding GTPase in response to light, long term digested disks show an increased tendency to adsorb the GTPase "nonspecifically", i.e., independently of light. GTP reverses the binding of GTPase to bleached undigested disks and bleached short term digested disks but not to long term digested disks. At *moderate* ionic strength (e.g., 100 mM Tris-HCl) the GTPase is membrane bound in both dark and light. This ionic strength dependent type of binding, in contrast to the light-induced binding, was not diminished by long-term thermolytic modification of rhodopsin. Illumination at moderate ionic strength caused the light-induced binding, superimposed on the ionic strength dependent binding, of the GTPase to control disks and to short term digested disks but not to long term digested disks. Since the GTPase exhibits activity only in the presence of rhodopsin which has been *illuminated* and since the GTPase appears to be fully active only under conditions when it is bound to the membrane at *moderate ionic strength*, this suggests that both the light-induced and ionic strength dependent binding are required for maximum GTPase activity.

Light absorption by rhodopsin in photoreceptor disk membranes leads to a sequence of spectral and probably conformational changes of rhodopsin[reviewed by Ostroy (1977)], to the activation of several enzymatic reactions, and finally to hyperpolarization of the rod cell plasma membrane and excitation of the rod cell. The light-activated enzyme reactions include a GTPase (Wheeler & Bitensky, 1977; Caretta et al., 1979; Robinson & Hagins, 1979), a cyclic GMP phosphodiesterase (Keirns et al., 1975; Baehr et al., 1979), and phosphorylation of bleached rhodopsin by a protein kinase (Kühn & Dreyer, 1972; Bownds et al., 1972). Enzyme activation is maximal by visible light at the absorption maximum of rhodopsin (Bownds et al., 1972; Keirns et al., 1975; Wheeler & Bitensky, 1977), i.e., by light which is not absorbed by the enzyme proteins themselves, suggesting that some molecular interaction between the enzymes and bleached rhodopsin is involved.

Rhodopsin is the predominant intrinsic membrane protein of ROS[1] disks (Smith et al., 1975; Krebs & Kühn, 1977; Godchaux & Zimmerman, 1979). Most of the other proteins present in ROS are either soluble or "peripherally bound" to the membranes. It has recently been observed that certain ROS proteins become strongly but reversibly bound to the disk membrane after bleaching of rhodopsin (Kühn, 1978, 1980a,b). Among these proteins are the enzymes GTPase (polypeptides of M_r = 37 000, M_r = 35 000, and $M_r \simeq$ 6000), rhodopsin kinase (M_r = 68 000), and a protein of as yet unknown function (M_r = 48 000). The GTPase is a "peripherally bound membrane protein" at moderate ionic strength but is easily extracted into aqueous buffers from *dark-adapted* ROS either at low or at very high ionic strength (Kühn, 1980a,b). After illumination, the GTPase undergoes an additional binding to the disk membranes. This "light-induced binding" is so strong that it is not disrupted by extremes of ionic strength. It may reflect the light-induced interaction between rhodopsin and the GTPase responsible for enzyme activation.

To obtain further insights into the mechanism of light-induced binding and enzyme activation, it would be of interest to know the site(s) of interaction with the disk membrane. Two modes of binding are to be distinguished: the ionic strength dependent and the light-induced binding. Chemical or enzymatic modification of the membrane is a means to perturb and thus study the mode(s) of interaction between the

† From the Institut für Neurobiologie der Kernforschungsanlage Jülich, D-5170 Jülich, West Germany. *Received July 28, 1980.* This work was supported by a grant from SFB 160 "Biologische Membranen" of Deutsche Forschungsgemeinschaft (to H.K.), National Institutes of Health Grant EY 02875 (to P.A.H.), NATO Grant RG 07680, and a grant from Deutscher Akademischer Austauschdienst (to P.A.H.).

‡ Permanent address: Department of Medical Biochemistry (School of Medicine) and Department of Chemistry and Biochemistry (College of Science), Southern Illinois University at Carbondale, Carbondale, IL 62901.

[1] Abbreviations used: des(1′–12′)rhodopsin, rhodopsin from which the first 12 carboxyl-terminal amino acids have been removed; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; P_i, inorganic phosphate; ROS, rod outer segments; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate.

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enzyme and the membrane. The solubility of the ROS proteins at low ionic strength allows modification of the membrane in the absence of the previously extracted proteins; the extract is later added back to the modified membranes for binding tests in light and darkness. In this report the protease thermolysin is used to modify rhodopsin in two distinct ways. Short-term digestion of disks leads to the loss of 12 amino acids from rhodopsin's carboxyl terminus (Hargrave & Fong, 1977), whereas long-term digestion leads to further cleavage of rhodopsin, producing two large membrane-bound fragments (Pober & Stryer, 1975). We find that the light-induced binding of the GTPase is not influenced by the short-term digestion of rhodopsin but is severely inhibited by the long-term digestion of rhodopsin, suggesting that the light-induced binding site is located in the rhodopsin molecule.

Experimental Procedures

Methods. Preparation of Rod Outer Segments (ROS) and Disk Membranes. ROS were prepared from freshly dissected bovine retinas and purified on discontinuous sucrose density gradients in 70 mM potassium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT, as previously described (Kühn, 1978). The purified ROS were stored frozen at −70 °C as pellets. For the preparation of osmotically intact disks (Smith et al., 1975), freshly purified (unfrozen) ROS pellets were suspended in an aqueous solution of 5% (w/v) Ficoll 400 (Sigma Chemical Co.) and 1 mM DTT, at ~1 mg/mL of rhodopsin, and were stored overnight at 0 °C under an argon atmosphere. The suspensions were then overlayered with 1 mM DTT in water and centrifuged for 1 h at 99000*g* in a swinging bucket rotor. The intact disks were harvested from the Ficoll–water interface and were washed by centrifugation with 5 mM Tris-HCl (pH 7.4) and 1 mM DTT. The washed disks were suspended in the same buffer and stored at 0 °C under argon. All operations were performed in the dark or in dim red light, if not otherwise stated. Rhodopsin concentrations were determined in detergent solutions from the difference in A_{500nm} before and after bleaching in the presence of 50 mM NH_2OH by assuming E_{500nm} = 40 600 and a molecular weight of 38 000.

Thermolysin Digestion of Disks. Freshly prepared disks were treated in the dark with thermolysin (Calbiochem) at room temperature (20–22 °C) either for 5 min, leading to "short term digested disks cd", or for 13 h, leading to "long term digested disks ef". The weight ratio of rhodopsin/thermolysin was always 12.5:1. A thermolysin stock solution was prepared by suspending the enzyme in 20 mM $CaCl_2$, adding 0.1 N NaOH to pH 11 to dissolve the protein, and rapidly neutralizing with Tris-HCl to pH 7.6. Between 3 and 11 mg of rhodopsin/sample was used in various digestion experiments. Final concentrations during the digestion were as follows: disks at 2.6 mg/mL rhodopsin, 0.21 mg/mL thermolysin, 5.6 mM $CaCl_2$, 7 mM Tris-HCl, 6 mM Tris–acetate, and 0.4 mM DTT (pH 7.4). The digestion was stopped by addition of neutralized EDTA to a final concentration of 17 mM. As a control, disks were incubated for 5 min with thermolysin to which the EDTA had previously been added, but otherwise under the same conditions as above. No digestion took place in the control sample ("control disks ab") indicating that inhibition of thermolysin by EDTA was complete.

After incubation and quenching, both sample and control suspensions were further diluted with 10 mM Tris–acetate containing 5 mM EDTA, centrifuged for 20 min at 50000*g*, and washed once more with this buffer; they were then washed with 10 mM Tris–acetate containing 0.6 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM DTT and finally resuspended with gentle homogenization in this latter buffer at a rhodopsin concentration of 5–8 mg/mL.

Binding of Extracted ROS Proteins to Disks and Modified Disks in Light and Darkness. Hypotonic dark extract containing the soluble ROS proteins was prepared, shortly before the binding experiment, by homogenizing a pellet of purified ROS (12–14 mg of rhodopsin) with 2 mL of 10 mM Tris-HCl (pH 7.4) and 1 mM DTT, followed by centrifugation at 50000*g* for 30–50 min. The supernatant was again centrifuged similarly to remove any residual membraneous material. Aliquots of the resulting clear extract were added to the suspensions of (modified) disks at 0 °C. Separate mixtures were prepared with the three disk preparations ab, cd, and ef (see previous section), each mixture containing 2.5 mg of rhodopsin (disks) and the extract from ROS corresponding to 2.4 mg of rhodopsin. The final volume was 870 μL, and the buffer composition was 6 mM Tris–acetate, 4 mM Tris-HCl (pH 7.4), 0.6 mM $MgCl_2$, 0.1 mM EGTA, and 1 mM DTT. The EGTA was added to quench any residual thermolysin perhaps still present.

Each suspension was divided into two equal portions (a, b, c, d, e, f), transferred to 0.6-mL cellulose nitrate centrifuge tubes, (Beckman No. 305528), and warmed to 21 °C in water baths for 2–3 min. Samples a, c, and e were kept dark, and samples b, d, and f were illuminated for 3 min with orange light ($\lambda > 540$ nm) through the side of the transparent water bath, bleaching ~80% of their rhodopsin. Then all of the tubes were cooled to 0 °C and centrifuged for 20–30 min at 50000*g*. The supernatants were termed 1a–f (first supernatants of binding experiment). The disk pellets were resuspended at 0 °C in the dark, each with 480 μL of 5 mM Tris–HCl (pH 7.4), 1 mM DTT, and 0.1 mM EDTA, and centrifuged as above, leading to the second set of supernatants termed 2a–f. Then the disk pellets were resuspended with gentle homogenization by using a plexiglass pestle fitting into the centrifuge tubes, each pellet with 250 μL of 40 μM GTP in the above buffer. The suspensions were warmed to 20 °C in the dark for 3 min and then centrifuged, leading to the third set of supernatants 3a–f. All of the supernatants were centrifuged again for 30 min at 50000*g* to make certain that all particulate material was sedimented. The supernatants, clear and free of rhodopsin, were then handled in room light. They were assayed for GTPase activity either immediately (see Table I) or after freezing in liquid nitrogen and storing at −70 °C (see Table II). About 30–50% of the activity was lost upon freezing.

The binding experiment shown in Figure 3 and Table II was performed as described above by using similar thermolysin-digested disk preparations and a similar ROS extract in 10 mM Tris-HCl, with the only exception that after mixing extract and disks, the Tris-HCl concentration was raised to 100 mM. The second (5 mM Tris-HCl, etc.) and third (40 μM GTP, etc.) sets of extracts from the disk pellets were obtained as above.

GTPase Assays. The $[^{32}P]P_i$ liberated from [γ-^{32}P]GTP (Amersham; diluted to specific activities of 500–3000 cpm/pmol) was measured by using the method of Neufeld & Levy (1969) with some modifications. To assay the GTPase activity of extracts, we added rhodopsin in the form of thoroughly washed, alum-treated ROS membranes (McDowell & Kühn, 1977) which were devoid of intrinsic GTPase activity. The extracts containing soluble GTPase were inactive in the absence of rhodopsin (Kühn, 1980a,b) but became active upon addition of the membranes in light. The apparent activity was

nearly independent of the amount of added ROS membranes if more than 3 μg of rhodopsin was added/single test sample but was strongly dependent (nearly linearly) on the amount of GTPase extract added. The assay is therefore suitable to quantitatively measure and compare the GTPase activities of different extracts.

Single samples contained 4 μg of rhodopsin (alum-treated membranes), 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 3 μM [^{32}P]GTP if not otherwise stated, and 5–15 μL of extract in a final volume of 200 μL. The incubation was started by adding prewarmed (30 °C) 6 μM [^{32}P]GTP buffer to an equal volume of a prewarmed mixture of extract and alum-treated ROS membranes. The rhodopsin was bleached a few seconds before the start of the incubation which was carried out at 30 °C in continuous white light for 2–5 min. The reaction was quenched by adding 200 μL of ice-cold 25% trichloroacetic acid containing 40 μM P_i/single sample. Normally, 650–850-μL volumes were incubated, and single samples of 200 μL were taken at timed intervals (2–5 min) for quenching. It was found unnecessary to remove the precipitated membranes by centrifugation before processing (Neufeld & Levy, 1969); instead, the ammonium molybdate solution (100 μL), reducing solution (50 μL), and 2-butanol (500 μL) were added directly to the quenched samples. After shaking for 15 s and centrifugation for 30 s in an Eppendorf centrifuge, 200 μL from the 2-butanol layer containing the ^{32}P–molybdate complex was removed and its radioactivity determined in the presence of 5 mL of Instagel (Packard).

The apparent GTPase activity of the alum-treated ROS, without added extract, was 0.4 pmol/min and was subtracted as a background value. The self-decomposition of the [^{32}P]-GTP during the incubation at 30 °C is included in this value. The GTPase activity in the presence of extracts was linear with time for at least 5 min, the standard deviation being <5%.

Gel electrophoresis was performed in 0.1% $NaDodSO_4$ on 10% polyacrylamide gels according to Laemmli (1970). Since the apparent molecular weights measured in this discontinuous electrophoretic system were often different from those measured in the more conventional continuous electrophoretic systems (Kühn, 1980b), we determined apparent molecular weights by the method of Weber & Osborn (1969).

Results

Modification of Disks by Thermolysin. Disk membranes contain rhodopsin as the predominant protein (Figure 1D) as has been documented in numerous reports [e.g., Smith et al. (1975) and Krebs & Kühn (1977)]. Most of the other polypeptides present in whole ROS are removed by repeated hypotonic treatments during the preparation of the disks. A hypotonic ROS extract containing these soluble polypeptides is shown in Figure 1E. Disk membranes treated with EDTA-inhibited thermolysin (Figure 1ab) have the same polypeptide composition as untreated disks.

When disks are treated with thermolysin for 5 min, most of the minor polypeptides are digested, and rhodopsin is converted to the slightly lower molecular weight derivative des(1′–12′)rhodopsin [see Hargrave et al. (1980)]. The conversion is nearly quantitative under the conditions used. The two proteins are clearly distinguishable by the difference in their apparent molecular weights (as shown by electrophoresis of their mixture, ab + cd, Figure 1). Long-term digestion of disk membranes with thermolysin (Figure 1ef) leads to cleavage at an exposed site in the interior of the polypeptide chain producing two large membrane-bound fragments [termed F1 and F2 by Pober & Stryer (1975)].

Light-Induced Binding of GTPase at Low Ionic Strength.

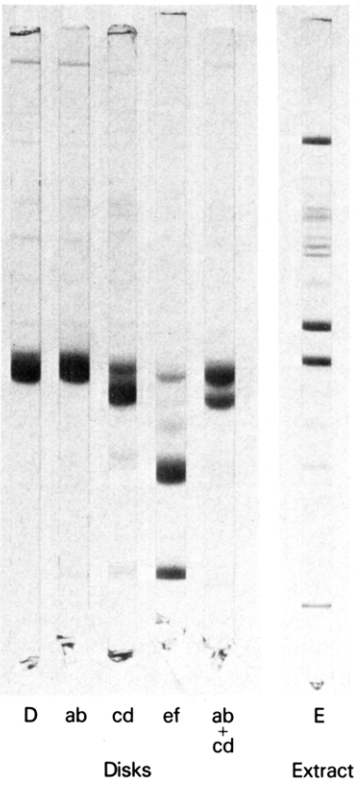

FIGURE 1: Gel electrophoresis of disk membranes prior to and after proteolytic treatment and of an extract (E) containing soluble ROS proteins. (D) Untreated disks; (ab) control disks treated with EDTA-inhibited thermolysin; (cd) short term (5 min) digested disks containing mainly des(1′–12′)rhodopsin; (ef) long term (13 h) digested disks containing proteolysis fragments F1 and F2; (ab + cd) mixture of equal amounts (4 μg each) of control disks ab and short term digested disks cd; (E) hypotonic extract from dark-adapted ROS, corresponding to 31 μg of rhodopsin in the original suspension before centrifugation. Each of the first four gels contains 10 μg of rhodopsin or its proteolysis products.

Four disk preparations which were treated differently were used to test the light-induced binding of soluble proteins: untreated disks (Figure 1D), control disks (ab) treated with quenched thermolysin, short term digested disks (cd) containing des(1′–12′)rhodopsin, and long term digested disks (ef) containing internally cleaved rhodopsin. The results obtained with untreated disks (D) were essentially the same as those obtained with control disks (ab) and are not shown. Hypotonic dark extract (see Figure 1E), containing the soluble ROS proteins, was added in equal amount to all of the disk suspensions, yielding the same concentration of rhodopsin and buffer in all suspensions. The suspensions were each divided into two equal portions for binding experiments in the dark (a, c, and e) and in light (b, d, and f).

The two supernatants obtained from the control disks (ab) show a large difference in GTPase activity (Table I), the supernatant from dark-kept membranes (1a) containing ~9 times more activity than the supernatant from illuminated membranes (1b). This difference reflects the light-induced binding of the GTPase to the bleached disks; in the dark sample, most of the GTPase is in the supernatant (1a), whereas in the bleached sample (b), most of the GTPase is in the disk pellet. Such light-induced binding has previously been observed when whole *ROS* were illuminated (Kühn, 1980a,b). We have now demonstrated that the same binding occurs when the isolated components (purified *disk membranes and ROS extract*) are mixed and illuminated. Close to 100% of the GTPase activity of the crude extract applied in the binding experiment is recovered in the dark supernatant (1a), if cor-

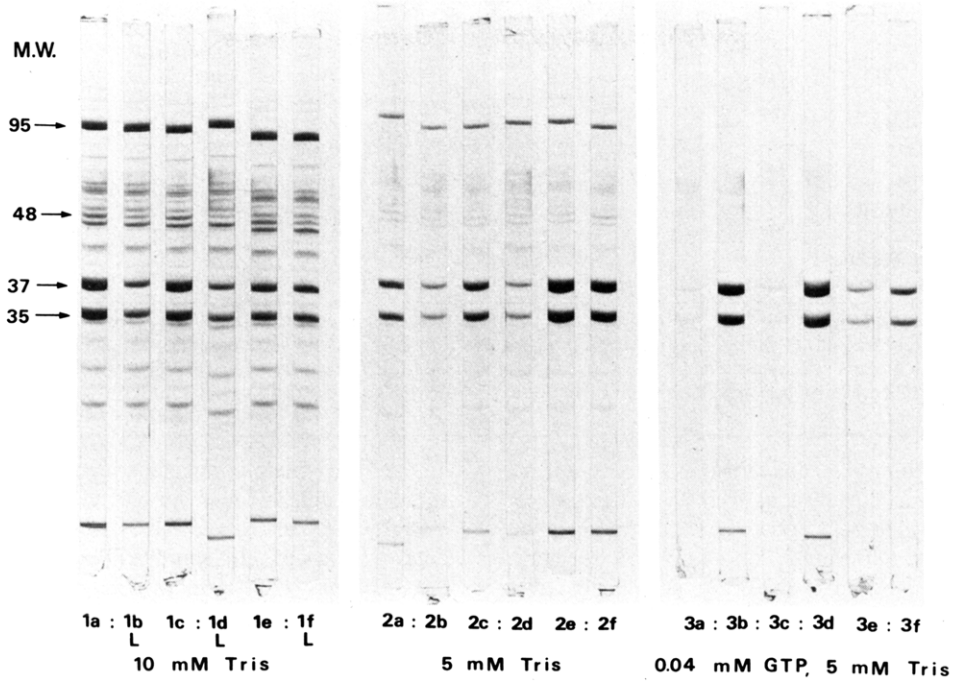

FIGURE 2: Polypeptide composition of soluble supernatants from binding experiment in 10 mM Tris. The first six supernatants (1a–f; 20 μL applied/gel) were obtained directly from the binding experiment in light (1b, 1d, and 1f) or darkness (1a, 1c, and 1e). The six membraneous pellets were then reextracted with 5 mM Tris-HCl, leading to the second set of supernatants (60 μL applied/gel). A subsequent treatment of the pellets with 40 μM GTP led to the third set of supernatants (3a–f; 20 μL applied/gel). The letters (a–f) designate the types of disks used in the binding experiment, similar to those described in Table I and Figure 1. The GTPase is represented by the polypeptides of M_r = 37 000, 35 000, and 6000. Numbers on the ordinate represent polypeptide chain molecular weight in thousands.

rections for volume losses including the volume of the pellet are made.

The short term digested disks (c and d) show the same extent of light-induced GTPase binding as the control disks, the dark supernatant (1c) containing 9 times more GTPase than the light supernatant (1d). This indicates that the removal of a small region from rhodopsin's carboxyl terminus does not influence the light-induced GTPase binding. The situation is, however, dramatically changed after long-term digestion of the disks (ef); here, the dark supernatant (1e) is only slightly (1.5 times) more active than the corresponding light supernatant (1f). Clearly, the light-induced ability of the disk membrane to bind the GTPase has been largely abolished by the internal cleavage of rhodopsin. Instead, an increased level of binding of the GTPase is observed which is *independent* of light (compare supernatants 1e and 1f with 1a; Table I).

Figure 2 shows the polypeptide components of the same supernatants whose GTPase activities are listed in Table I. Supernatants from binding experiments in the dark (1a and 1c) have the same polypeptide composition as the crude ROS extract (see Figure 1E) prior to mixing with the disks. It is clear that none of the polypeptides of the crude extract is preferentially bound to dark-adapted disks at the low ionic strength used. Supernatants obtained from binding experiments in *light* (1b and 1d), on the other hand, contain considerably reduced amounts of the GTPase polypeptides (M_r = 37 000, M_r = 35 000), corresponding to the greatly reduced GTPase activity found in these supernatants (Table I). Quantitative gel densitometry (not show) reveals that the amount of these two polypeptides quantitatively parallels the measured GTPase activity in the supernatants. The gels demonstrate that the low GTPase activities found in the light supernatants (1b and 1d) are due to the reduction in the amount of the enzyme in these supernatants since it has sedimented with the bleached membranes.

Table I: GTPase Activity in Supernatants Obtained from Binding Experiment at Low Ionic Strength[a]

disk prepn		illumination conditions	first supernatants, 1a–f (10 mM Tris)	third supernatants, 3a–f (40 μM GTP and 5 mM Tris)
control	a	dark	35.6	1.3
	b	light	4.0	30.4
short term digested	c	dark	34.8	1.2
	d	light	3.8	30.6
long term digested	e	dark	13.4	3.1
	f	light	9.3	7.5

[a] GTPase activities are expressed as picomoles of P_i liberated per minute at 30 °C per 10 μL of supernatant. In the binding experiment, ROS dark extract [activity = 88 pmol of P_i/(min 10 μL)] was added to disks and modified disks, respectively, in 10 mM Tris buffer. Mixtures b, d, and f were illuminated, and mixtures a, c, and e were kept dark. The six supernatants obtained upon centrifugation of these mixtures are the first supernatants. Their polypeptide composition is shown in Figure 2. The six disk pellets were then washed in the dark with 5 mM Tris buffer (second supernatants) and were finally treated with 40 μM GTP to elute the GTPase from its light-induced binding (third supernatants). All of the buffers contained 1 mM DTT. The supernatants were assayed for GTPase activity shortly after centrifugation (i.e., unfrozen). The GTP concentration was 2.8 μM for the assays of the first supernatants and 4.6 μM for the third supernatants. GTPase activity of the second supernatants was not assayed, but the amount of GTPase present in these supernatants can be estimated from the gels shown in Figure 2. The supernatants 2e and 2f account for most of the remaining GTPase originally applied in the binding experiment to long term digested disks.

In the two supernatants obtained from the long term digested disks (1e and 1f), the dark–light difference in the amount of the two GTPase polypeptides is nearly abolished, corresponding to the minor difference in GTPase activity.

Table II: Binding Experiment at Moderate Ionic Strength: GTPase Activity in Supernatants[a]

disk prepn		illumination conditions	first supernatants, 1a–f (100 mM Tris)	second supernatants, 2a–f (~10 mM Tris)	third supernatants, 3a–f (40 μM GTP and 5 mM Tris)
control	a	dark	3.0	13.6	0.56
	b	light	2.4	0.22	18.3
short term digested	c	dark	2.0	10.9	0.33
	d	light	1.3	0.26	19.9
long term digested	e	dark	1.9	4.4	1.5
	f	light	1.6	3.2	2.9

[a] The binding experiment was performed as described in Table I, but the Tris concentration was 100 mM. After removing the first supernatants, the pellets were reextracted by gentle homogenization with 5 mM Tris-HCl (second supernatants), and finally by 40 μM GTP (third supernatants). All of the buffers contained 1 mM DTT. The supernatants were frozen in liquid nitrogen before they were assayed for GTPase activity. The GTP concentration was 3 μM in all of the assays. Activities are expressed as picomoles of P_i per minute per 10 μL of supernatant, as described in Table I.

However, some adsorption of the GTPase to these modified disks seems to occur in darkness as well as in light since relatively less GTPase is present in both of these supernatants (1e and 1f) as compared to the crude extract (E) or to the dark supernatants (1a and 1c) (see also Table I for activities). Only the GTPase seems to be adsorbed to long term digested disks. All of the other polypeptides are present in these supernatants (1e and 1f) in their normal relative amounts.

The peptide of $M_r \simeq 6000$, which binds in a light-dependent manner and always accompanies the GTPase (Kühn, 1980a), migrated with the front band in the gels of Figure 2 but was resolved when electrophoresis was performed according to Swank & Munkres (1971) (data not shown). The M_r 48 000 polypeptide is seen to exhibit some light-induced binding to all of the three different disk preparations; the dark–light difference is nearly the same in all three cases. Note that the prominent polypeptide band at M_r = 95 000, which is in fact a doublet band and presumably represents the cyclic GMP phosphodiesterase (Baehr et al., 1979), does not exhibit light-induced binding.

Further Extraction of Disk Membrane Pellets. When the disk pellets obtained from the binding experiment are reextracted in the dark with hypotonic buffer, additional protein is removed from the membranes (Figure 2, supernatants 2a–f). This is mainly due to incomplete removal of the first supernatants from the rather voluminous pellets. An exception is the supernatants from the long term digested disk pellets (2e and 2f) which contain particularly high concentrations of GTPase; this reflects the light-*independent*, weak adsorption of the GTPase to these modified membranes in the binding experiment (see above). Lowering of the Tris buffer concentration to 5 mM for the second wash seems to favor extraction of the GTPase from these long term digested membranes.

Addition of GTP reverses the light-induced binding of the GTPase (Kühn, 1980a,b) and can be used to solubilize the enzyme following its light-induced binding. The disk pellets were therefore treated in the dark with 40 μM GTP in hypotonic buffer, and the resulting extracts (3a–f) were examined for GTPase activity (Table I) and polypeptide composition (Figure 2). The GTP extracts obtained from two of the *bleached* pellets, (b) and (d), contain the GTPase in high purity and activity. The GTP extracts (3a and 3c) obtained from the corresponding unbleached pellets, on the other hand, contain only trace amounts of GTPase since the GTPase, like the other proteins not bound to the membranes, had been washed out in the first two centrifugations. Thus, the dark–light differences of GTPase activity observed in the first supernatants (dark > light) are reversed and complemented in the third supernatants (light ≫ dark) such that the total amount of GTPase in the three supernatants is approximately equal for each of these samples (a–d).

Both GTP extracts (3e and 3f) obtained from the *long term digested* disks contain some GTPase. The extract (3f) from the bleached pellet is more active than that from the unbleached pellet (3e), indicating that some light-induced binding has occurred. However, the light–dark ratio is much smaller than in the case of the control disks (a and b) and of the peripherally modified disks (c and d). Much of the GTPase present in these samples may have been nonspecifically adsorbed and eluted. Quantitative gel densitometry showed that the combined first, second, and third supernatants contain more than 80% of the GTPase originally applied in binding experiments to both bleached and unbleached long term digested disks. A high proportion of the total GTPase (30–50%) was always found in the second supernatant, as can be seen qualitatively in Figure 2 (supernatants 2e and 2f).

Reproducibility. Four binding experiments of the type shown in Figure 2 and Table I were performed by using different preparations of disks, modified disks, and extract each time. The results in all cases were similar to those shown here in the following respects: the light-induced binding of the GTPase was always equally strong to control disks (a and b) as compared to short-term digested disks (c and d), and the dark–light difference in binding was always greatly diminished in the long term digested disks (e and f). The properties of the third supernatants, eluted in the presence of 40 μM GTP, were also always similar to those shown here. Quantitative differences were found in the extent of "nonspecific" (light-*independent*) adsorption of the GTPase to long term digested disks (e and f), but the effect was always observed.

Binding at Moderate Ionic Strength. It has been previously shown that at moderate ionic strength (e.g., 100 mM Tris-HCl, 100 mM NaCl, 100 mM KCl, or 70 mM phosphate), the GTPase is a peripherally bound membrane protein in both darkness and light; illumination in 100 mM Tris-HCl causes an additional increase in binding strength such that the GTPase cannot be extracted by a subsequent hypotonic treatment (Kühn, 1980a). We wished to investigate how the thermolytic digestion of rhodopsin influenced this ionic strength dependent binding to disk membranes. The following experiment shows that *the ionic strength dependent binding of GTPase is not diminished by thermolytic cleavage of rhodopsin*, whereas the light-induced binding is abolished, as already shown in the previous experiment.

Disks and thermolysin-digested disks were mixed with hypotonic ROS dark extract, and the Tris-HCl concentration was then raised to 100 mM. The suspensions (b), (d), and (f) were bleached, and the first set of supernatants was obtained as described above. Table II and Figure 3 (1a–f) show

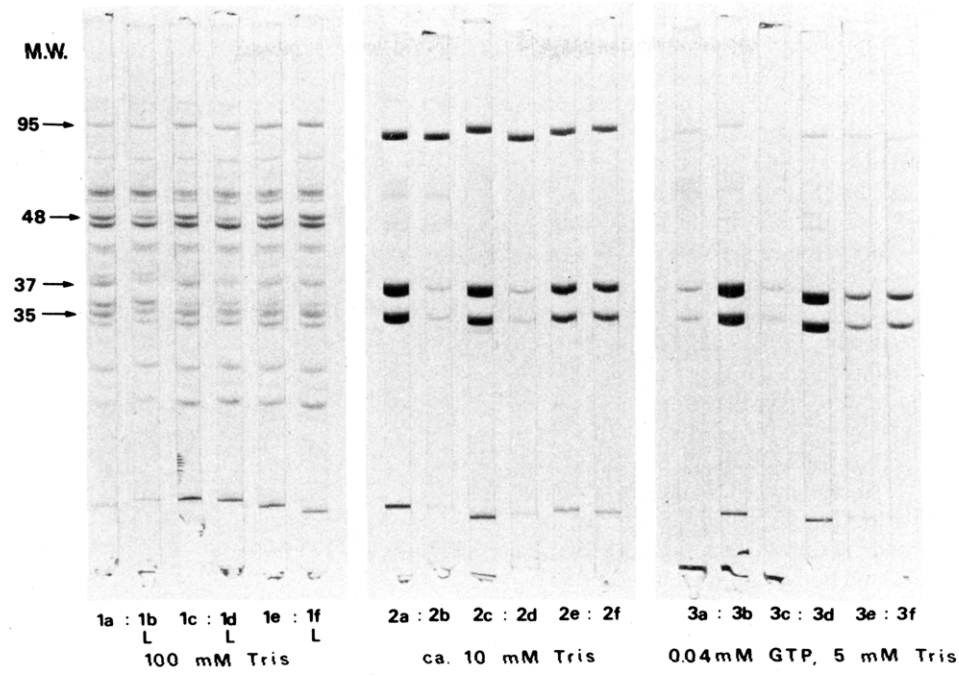

FIGURE 3: Polypeptide composition of supernatants after binding experiment in 100 mM Tris-HCl. The first set of supernatants (1a–f; 30 μL applied/gel) was obtained directly from the binding experiment. The second set (2a–f; 35 μL applied/gel) and third set (3a–f; 25 μL applied/gel) of supernatants were obtained by subsequent extractions of the six membraneous pellets, as described in Table II where the GTPase activities of the supernatants are shown.

that all of the supernatants contain only small amounts of GTPase and that the differences between dark and light supernatants are small. It seems clear that the GTPase which was soluble in 10 mM Tris has been largely membrane bound in 100 mM Tris, nearly independently of light as described for whole ROS (Kühn, 1980a) and independently of any of the proteolytic treatments of rhodopsin.[2]

When the pellets after the binding experiment were resuspended in the dark with 5 mM Tris-HCl (leading to a final Tris-HCl concentration of ~10 mM), large amounts of GTPase (activity and polypeptide chains) were eluted from the dark pellets (supernatants 2a and 2c, Table II and Figure 3) and very small amounts of GTPase from the bleached pellets (2b and 2d). This indicates that during bleaching in 100 mM Tris-HCl a strong light-induced binding of GTPase must have taken place to the control disks (b) and to the short term digested disks (d), in addition to the ionic strength dependent binding. The ionic strength dependent binding was then disrupted by the subsequent treatment with 10 mM Tris-HCl, whereas the light-induced binding was not. In the case of the *long term* digested disks, on the other hand, the second supernatants (2e and 2f) show almost no light–dark difference. This indicates that only little light-induced binding to the long term digested disks had taken place. Thus, in this same preparation (disks ef), the ionic strength dependent binding was not diminished, but the light-induced binding was strongly diminished by the long-term thermolysin treatment, demonstrating the difference between the two types of binding.

The membraneous pellets were finally treated with 40 μM GTP to elute the residual GTPase still bound. As expected from the previous experiment, the unbleached pellets contained only little residual GTPase to be extracted (supernatants 3a and 3c), whereas high GTPase activity was released from the bleached pellets (3b and 3d). Here again, the control disks (a and b) and the short term digested disks (c and d) behaved similarly, both showing large light–dark differences, whereas the light–dark difference in the GTP extracts from the long term digested disks (3e and 3f) was greatly diminished. In this experiment, a large portion (~50%) of the total GTPase could not be eluted from the *long term* digested disks by the two hypotonic extractions following the binding experiment; the GTPase probably remained associated with the modified membranes due to the same type of "nonspecific" adsorption as discussed above.

Discussion

Isolated disks, freshly purified by flotation (Smith et al., 1975), are a preparation well suited to study reactions at the cytoplasmic surface of rhodopsin. The disks are osmotically intact, all of their rhodopsin is oriented with native sideness (Adams et al., 1978), and their chemical composition is relatively well defined, rhodopsin being by far the predominant protein. The following model of rhodopsin and its orientation in the disk membrane has emerged during the past few years, based mainly on enzymatic modification of rhodopsin, amino acid sequence determination, and chemical labeling studies [for a review see Hargrave et al. (1980)]. The amino terminus bearing two oligosaccharide chains is located intradiskally; the carboxyl terminus bearing the phosphorylation sites is located at the cytoplasmic (i.e., extradiskal) surface; the polypeptide chain spans the membrane several (perhaps 5 or 7) times. Thus, the carboxyl-terminal region and several loops produced by the polypeptide chain repeatedly threading in and out of the membrane are exposed at the cytoplasmic surface.

Various proteases have been used to cleave rhodopsin at one or several of these loops, producing two or more membrane-bound fragments [e.g., Saari (1974), Pober & Stryer (1975), and Sale et al. (1977)]. Not only is the absorption spectrum of rhodopsin unaffected by these proteolytic treatments but

[2] A control binding experiment in light and darkness was performed in *10 mM Tris buffer* using the same preparation of control disks (ab) and extract. The dark supernatant contained very high GTPase activity and the light supernatant low GTPase activity as is usual in 10 mM Tris-HCl (see Figure 2 and Table I); this indicates that the binding observed in the first set of supernatants of Table II and Figure 3 is in fact due to the higher ionic strength.

also its circular dichroism in the visible and ultraviolet regions (Albert & Litman, 1978) is unaffected. Rhodopsin's capability to be regenerated after bleaching (Saari, 1974; Sale et al., 1977; Fung & Hubbell, 1978) and the equilibrium between the bleaching intermediates metarhodopsins I and II (Sale et al., 1977) are also reported to remain unchanged. This indicates that the binding site and light-induced reactions of the retinal chromophore are not affected by these proteolytic modifications of rhodopsin's cytoplasmic surface.

It now appears likely that one of the functions of rhodopsin is to activate enzymes by light-induced interactions at its cytoplasmic surface, thus changing the cytoplasmic concentrations of certain metabolites such as GTP and 3′,5′-cGMP, in response to light. This report shows that the light-induced binding of the GTPase can be influenced by proteolytic modification of rhodopsin's cytoplasmic surface. We chose to employ the protease thermolysin since it appears to cleave rhodopsin more specifically than other proteases (papain, subtilisin, chymotrypsin) used in various studies. Furthermore, its activity can be reliably and easily inhibited by addition of Ca^{2+} chelators (Matsubara, 1970). Complete inactivation was particularly important in this study since soluble proteins (GTPase, etc.), which are very susceptible to proteolysis, were added to the disks after the proteolytic treatment. As seen in Figures 2 and 3, the molecular weights of the soluble polypeptides were not changed by their contact with the thermolysin-treated disks, indicating the absence of any residual proteolytic activity.

The binding of previously extracted GTPase to enzymatically modified disks was studied as a function of illumination and ionic strength. At low ionic strength, the GTPase was soluble in the dark but underwent light-induced binding to both the control disks and the short term digested disks. This indicates that the 12 amino acids from rhodopsin's carboxyl terminus, including a large portion of the phosphorylation site, are not involved in the light-induced binding. It also suggests that the binding occurs to rhodopsin, since the minor disk proteins have been largely digested by the short-term treatment. (Binding of the GTPase to one of the minor proteins would also be unlikely for stoichiometric reasons, since the GTPase extract was added to the disks in approximately the same proportion as in native ROS and the GTPase was therefore present in large excess compared to any other protein except rhodopsin.)

Long-term digestion, on the other hand, dramatically decreases the light-induced binding of the GTPase (i.e., the difference between binding in darkness and light). This strongly suggests that the light-exposed site is located in the rhodopsin molecule, probably in or near a "loop" region which is exposed to thermolytic cleavage. Instead of the light-induced binding, these modified disks show a *light-independent* adsorption of the GTPase which is much weaker than the light-induced binding to undigested control disks. Repeated hypotonic treatment leads to nearly complete extraction of the GTPase from such long term digested disks, both bleached and unbleached (see Figure 2, supernatants 1e, 1f, 2e, and 2f). In contrast, no GTPase is eluted from *bleached control* disks by repeated washings, as long as GTP is absent (Figure 2, supernatants 1b and 2b). Another difference between the light-induced binding to control disks and the light-independent adsorption to long term digested disks is seen in the response to GTP; the GTPase bound to bleached control disks is quantitatively released by GTP (see Figures 2 and 3, supernatant 3b) whereas the GTPase adsorbed to long-term digested disks (both bleached and unbleached) is nearly unaffected by GTP (supernatants 3e and 3f). This suggests that long-term digestion of rhodopsin has not only removed a site responsible for the light-induced binding of the GTPase (in the absence of GTP) but also a site responsible for the reversal of the binding of GTPase to rhodopsin (in the presence of GTP).

At moderate ionic strength and presumably also under physiological ionic conditions, the GTPase is peripherally membrane bound in darkness as well as in light. This ionic strength dependent binding, in contrast to the light-induced binding, is not influenced by thermolytic cleavage of rhodopsin, indicating that the two types of binding are different and probably involve different sites at the surface of the disk membrane. Light induces the additional, strong interaction (which is not broken by manipulating the ionic strength) only in the case of control and short term digested disks, but not in long term digested disks.

It is well established that the GTPase requires light absorption by rhodopsin in order to be active (Wheeler & Bitensky, 1977; Caretta et al., 1979). It has therefore been suggested that the observed light-induced binding reflects the interaction between the GTPase and rhodopsin which is apparently needed for enzyme activation (Kühn, 1980a,b). On the other hand, the enzyme is found to be nearly inactive, even in light, in 5 mM Tris buffer in which the light-induced but not the ionic strength dependent binding takes place (Kühn, 1981). This suggests that *both types of binding* are needed for the GTPase to be fully active. Preliminary experiments (unpublished) with thermolysin-treated disks used for the activation of extracted GTPase show that long-term digestion reduced the capacity of the disks to activate the GTPase in light but that the residual activating capacity was still significant and higher than expected from the binding experiments. Further experiments including comparative studies of the stoichiometry of GTPase binding and GTPase activation in various modified disk membrane preparations are necessary in order to elucidate the mechanism of light activation of this enzyme.

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Topography of Rhodopsin in Retinal Rod Outer Segment Disk Membranes. Photochemical Labeling with 1-Azidopyrene†

Dennis P. Smith, Michael R. Kilbourn,‡ J. Hugh McDowell, and Paul A. Hargrave*

ABSTRACT: 1-Azido[^{3}H]pyrene ([^{3}H]AP) has been synthesized with high specific radioactivity (3 Ci/mmol) and used to photochemically label retinal rod outer segment disk membranes. The reagent reacts with rhodopsin and a $M_r \approx 240000$ protein as well as with membrane lipids. When [^{3}H]AP–rhodopsin is digested with thermolysin in the disk membrane, both membrane-bound fragments of rhodopsin, F1 and F2, are found to contain [^{3}H]AP. Reaction of the reagent appears to be restricted to the lipophilic surface of rhodopsin inasmuch as the presence of the nitrene scavenger glutathione in the aqueous medium does not significantly reduce ^{3}H incorporation into rhodopsin. Labeled F1 and F2 were prepared, their cyanogen bromide peptides partially separated, and specific radioactivities determined. A factor of 4.4-fold in specific radioactivities of peptide pools was found, which suggests that some specificity has been shown in the reaction of [^{3}H]AP toward different surfaces of rhodopsin.

Rod cells of the retina, which are responsible for dim light and black and white vision, contain disk membranes which contain the photoreceptor protein rhodopsin. Rhodopsin comprises more than 95% of the intrinsic membrane protein of this highly specialized membrane (Smith et al., 1975; Krebs & Kühn, 1977). It has been proposed that rhodopsin may fulfill its function as a visual transducer by serving as a transmembrane calcium channel (Hagins, 1972) or by interacting at its hydrophilic surface with soluble rod cell enzymes (Hubbell & Bownds, 1979). A knowledge of rhodopsin's organization and topography in the membrane may help us in understanding its mode of action. We have previously employed the hydrophilic nitrene precursor *N*-(4-azido-2-nitrophenyl)-2-aminoethane[^{35}S]sulfonate as a probe for those regions of rhodopsin which are present at an aqueous surface (Mas et al., 1980). In the present study, we report the synthesis and application of 1-azido[^{3}H]pyrene as a probe for the hydrophobic topography of rhodopsin in the rod cell disk membrane.

A variety of hydrophobic nitrene or carbene precursors has been used in attempts to label hydrophobic regions of membrane proteins [reviewed in Chowdhry & Westheimer (1979); Jori & Spikes, 1979)]. Klip & Gitler (1974) have employed 1-azido[^{3}H]naphthalene and 1-azido-4-[^{125}I]iodobenzene, and Bercovici & Gitler (1978) have introduced 5-[^{125}I]iodonaphthyl azide. Limitations of the use of many lipophilic labeling reagents have been noted by Bayley & Knowles (1978). 1-Azidopyrene has been prepared and its photolysis studied (Yamaoka et al., 1972; Sumitani et al., 1976). To our knowledge, its synthesis and purification have not been described in detail, and it has not been previously prepared in radioactive form. 1-Azidopyrene has been previously employed in a study of *Escherichia coli* membranes (Nieva-Gomez & Gennis, 1977). A similar reagent, pyrenesulfonyl azide, has been used to study protein subunits of the acetylcholine receptor (Šator et al., 1979).

A preliminary report has appeared describing the use of the reagent 1-azido[^{3}H]naphthalene to label rhodopsin in disk membranes (Klip et al., 1976). In the study reported in this paper, we have used azido[^{3}H]pyrene of high specific radioactivity to label disk membranes, and we have prepared defined proteolytic fragments of rhodopsin. Distribution of label within peptides of these fragments varies severalfold, is consistent with the specificity of labeling expected by using a lipophilic probe, and complements results previously obtained with a hydrophilic probe (Mas et al., 1980).

Experimental Section

Materials

The following were obtained from Sigma Chemical Co.: β-MSH,[1] DTE, DTT, glutathione, concanavalin A–Sepharose

† From the Department of Medical Biochemistry, School of Medicine, and the Department of Chemistry and Biochemistry, College of Science, Southern Illinois University at Carbondale, Carbondale, Illinois 62901, and the Department of Chemistry, University of Illinois, Urbana, Illinois 61801. *Received August 6, 1980.* This work was supported by Grant EY 2875 from the National Institutes of Health and Equipment Grant PCM77-17808 from the National Science Foundation.

‡ Present address: Department of Chemistry, Brookhaven National Laboratory, Upton, NY 11973.

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