Proc. Natl. Acad. Sci. USA
Vol. 77, No. 3, pp. 1408–1411, March 1980
Biochemistry

Additional component required for activity and reconstitution of light-activated vertebrate photoreceptor GTPase

(rhodopsin/cyclic GMP/phosphodiesterase/disc membrane proteins)

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Communicated by George E. Palade, December 17, 1979

ABSTRACT **A light-activated GTPase that functions as a component of the rhodopsin-linked, light-activated phosphodiesterase (PDEase) system in vertebrate photoreceptors has been reported. In our efforts to purify photoreceptor GTPase we encountered another component (which we call "helper" or "H" component) whose presence is required for expression of light-activated GTPase activity. We report here the characterization of this heat-labile, macromolecular factor and that the presence of helper is absolutely required for light- and rhodopsin-dependent activation of photoreceptor GTPase. Of equal importance, we find that the "G" component (which requires the presence of H for expression of GTPase activity) can bind GTP and can support light- and GTP-dependent PDEase activation in the absence of H component. These data support a model in which GTP binding to G component is a necessary condition for PDEase activation. Hydrolysis of GTP at the G activator locus (an H-dependent activity) is a regulatory event which reverses PDEase activation. The complexity of this regulatory mechanism provides opportunities for signal modulation and amplification.**

Studies of light-activated enzymes in disc membranes of vertebrate rod outer segments have been reported (1–3). These enzymes are peripheral membrane proteins and include a cyclic GMP phosphodiesterase (PDEase) (4) and GTPase (5, 6). Action spectra show that rhodopsin is the photopigment involved in these light-dependent activations (6, 7). The presence of GTPase is necessary for light-dependent activation of PDEase (8). This system shows striking sensitivity to light: bleaching only 1 of 1000 rhodopsin molecules will fully activate both enzymes (9).

A number of remarkable similarities between light-activated PDEase and hormone-activated adenylate cyclase have been noted (6, 8) including dependence upon GTP or its nonhydrolyzable analogue, guanylyl imidodiphosphate, the participation of a light- or hormone-activated GTPase activity, and the presence of a two-component activation mechanism (10, 11). Both light and GTP must be present in order to activate PDEase.

Purification and reconstitution of this GTPase/PDEase system should provide understanding of the mechanism which supports its activation by light. We have found evidence for still another component (which we call helper or "H") which participates in the light-dependent activation of GTPase. We report here some characteristics of H, demonstrate its activity by reconstituting a light-dependent GTPase system, and discuss its role in the regulation of GTPase and PDEase activities.

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MATERIALS AND METHODS

Separation of H and G Fractions. Preparation of EDTA-washed disc membranes and EDTA supernatant was as described (6, 8). Rod outer segments, prepared by sucrose flotation (4), were suspended in 1 mM EDTA/1 mM dithiothreitol adjusted to pH 8.1, and passed three times through a 25-gauge needle. The disc suspension was kept on ice for 60 min and then centrifuged for 60 min (65,000 × g; 4°C). The supernatant fraction was passed through a Millipore filter (HA; pore size, 0.45 μm) to remove fragments of disc membrane and then lyophilized (we call this the EDTA supernatant).

The disc membrane pellet was washed once in 10 vol of the same EDTA solution. The disc pellet was then washed in 50 vol of 20 mM Tris·HCl, pH 9.7/1 mM EDTA/1 mM dithiothreitol (EDTA enhances the elution of peripheral disc membrane proteins; dithiothreitol stabilizes the activating functions of disc membrane rhodopsin). The disc pellet was finally washed in 50 vol of 200 mM Tris·HCl, pH 7.5/20 mM $MgSO_4$/1 mM dithiothreitol. (Restoration of Mg^{2+} to the disc membranes after elution of the peripheral proteins with EDTA enhances rhodopsin stability during storage at −80°C.) The discs were then resuspended in the same buffer (2–3 mg of protein per ml), quick-frozen with acetone and dry ice, and kept at −80°C (we call this preparation EDTA-washed membranes).

The lyophilized EDTA supernatant was dissolved in buffer A [10 mM Tris·HCl, pH 7.5/6 mM $MgSO_4$/1 mM EDTA/100 mM KCl and 50% (vol/vol) glycerol] and applied to a DEAE-Sephadex A50 (Pharmacia) column. The column was then washed with buffer A containing KCl at 150 mM. The proteins were eluted with a KCl gradient (150–350 mM in buffer A).

GTPase activity was measured by the method of Abrams *et al.* (12). The reaction mixture contained 100 mM Tris·HCl, 13 mM $MgSO_4$, 1 mM dithiothreitol, 0.5 mM EDTA, 75–175 mM KCl, 25% (vol/vol) glycerol, 25 μg of EDTA-washed membranes, and 0.5 μM [γ-^{32}P]GTP (1.22 × 10^5 cpm per assay in volume of 100 μl). The reaction was initiated by the addition of [γ-^{32}P]GTP. After 3 min at 37°C, the reaction was stopped by addition of a slurry of 500 μl of 10% trichloroacetic acid containing NaH_2PO_4 (5 mM) and charcoal (6 g/100 ml). This mixture was centrifuged, and 200 μl of the supernatant was assayed in a Beckman CPM 200. In this experiment, the background GTPase activity present in EDTA-washed membranes (1.4 pmol/min) was subtracted. Data obtained by this method of GTPase assay (which has a blank of 1.7 pmol/min) corresponded perfectly with those obtained by the method of Neufeld and Levy (13).

Abbreviations: PDEase, phosphodiesterase; H, helper.
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Further Purification of G Fraction. Further purification of G fraction from DEAE-Sephadex was accomplished with blue Sepharose CL-6B (Pharmacia). The G fraction was eluted from blue Sepharose with an EDTA buffer; 60% of the activity was recovered. Subsequently the G activity was chromatographed on AH-Sepharose 6B (Pharmacia) from which the G activity was eluted with a continuous KCl gradient (0.15–0.35 M) with recovery of 70% of the activity. (The assay of GTPase activity in purified G fractions is absolutely dependent on supplementation with H.)

PDEase Assay. PDEase activity was measured as described (8). We selected cyclic AMP as a more practical substrate because the slower rates observed permit more precise measurements of PDEase activity at enzyme concentrations that retain light regulation (4).

GTP Binding Studies. For GTP binding studies, components were added in 100 mM Tris-HCl, pH 7.5/13 mM $MgSO_4$/1 mM dithiothreitol/100 mM KCl/0.5 mM EDTA/25% (vol/vol) glycerol containing 0.5 μM [γ-^{32}P]GTP (4.7×10^5 cpm) and 33 μg of EDTA-washed bleached membranes in a volume of 100 μl. Mixtures were incubated for 20 min on ice, and then 80 μl of each mixture was applied to a Millipore filter (HA; pore size, 0.45 μm) and washed with 100 mM Tris-HCl, pH 7.5/5 mM $MgSO_4$/1 mM dithiothreitol. The filter was dissolved in Formula-963 (New England Nuclear) and the bound [γ-^{32}P]GTP was quantitated in a Beckman CPM 200. Binding was fully reversed by the addition of a 100-fold excess of unlabeled GTP.

RESULTS

When EDTA-solubilized GTPase fraction (6) is chromatographed on Sepharose 6B and Sephacryl S-200 (Pharmacia), more than 80% of the added GTPase activity can be readily eluted. However, chromatography on DEAE-Sephadex resulted in a striking loss of total activity. This loss of GTPase activity was explained by differential elution of a putative catalytic moiety (G fraction) and an additional essential component (H fraction). Fig. 1 illustrates the separation of the H and G fractions on DEAE-Sephadex. By combining the early-eluting H and late-eluting G fractions, we were able to recover more than 95% of the GTPase activity with a 10-fold increase in specific activity.

H fraction did not exhibit any PDEase or GTPase activity in the presence or absence of bleached, purified, reconstituted rhodopsin [this material consists of hydroxylapatite-purified (14) bovine rhodopsin reconstituted in phosphatidylcholine vesicles]. The crude G fraction (from DEAE-Sephadex) exhibited 33% of its potential GTPase activity (in the presence of

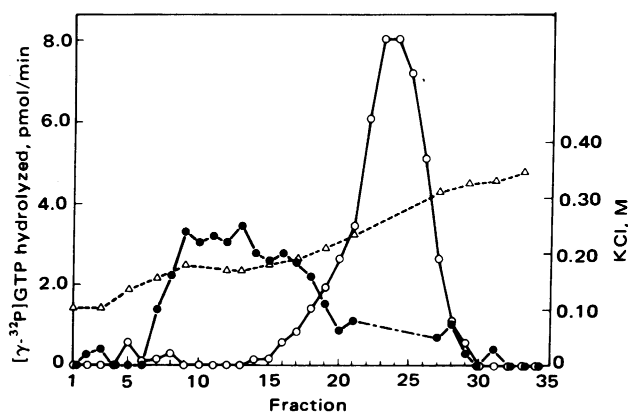

FIG. 1. Separation of H and G fractions on DEAE-Sephadex. To measure H activity (●), each fraction was analyzed for GTPase activity by adding it to the reaction mixture containing 25 μg of EDTA-washed membranes, 0.5 μM [γ-^{32}P]GTP, and G fraction (fraction 23, 0.35 μg per assay). The hydrolysis of [γ-^{32}P]GTP was determined. H activity was corrected by subtracting the hydrolysis of GTP by EDTA-washed membranes and G fraction (4.5 pmol/min) in the absence of H. Values for H activity are not shown for fractions 22–26. G fraction activity (○) was determined by measuring GTPase activity by combining EDTA-washed membranes (25 μg per assay) with each of the designated fractions. △, KCl gradient.

bleached rhodopsin), probably because of partial contamination by H (see Fig. 1 and Table 1). EDTA-washed native disc membranes exhibited only 5% of the GTPase activity found in unwashed native disc membranes. When H fraction was added to crude G fraction in the presence of bleached, EDTA-washed membranes, GTPase activity was enhanced 3-fold (Table 1). Unbleached membranes or unbleached reconstituted rhodopsin did not activate the G fraction in the presence or absence of H. More extensively purified G fraction exhibited no GTPase activity without added H, even in the presence of bleached, reconstituted rhodopsin. (Neither the reconstituted rhodopsin nor the more extensively purified G fraction contains H.) However, when H was added to the more extensively purified G fraction in the presence of bleached, reconstituted rhodopsin, the resulting GTPase activity was striking.

We tentatively assign the GTPase catalytic site—i.e., the GTP-binding site—to the G fraction because it is needed for the activation of PDEase (which also requires GTP) (Table 2). Previous data showed that GTP binding, but not GTP hydrolysis, is required for PDE activation. Activation can also be achieved with guanylyl imidodiphosphate, hydrolysis-resistant GTP analogue (5). This view is further supported by the fact that helper is necessary for GTP hydrolysis but *not* for PDEase

Table 1. Reconstitution of light-activated GTPase: H fraction is necessary for GTPase activity

G fraction	GTPase activity*					
	No addition		With EDTA-washed disc membranes†		With reconstituted rhodopsin‡	
	−	+	−	+	−	+
DEAE-Sephadex fraction§	0	0	6.6	14.4	2.7	10.9
AH-Sepharose 6B fraction¶	0	0	2.7	9.9	0	6.6

* Values (pmol of GTP hydrolyzed in 3 min) are the averages of two or more determinations that agreed within 5% (the data given are in full agreement with those obtained in four repetitions of this experiment). −, without H; +, with H (9.9 μg of H fraction from DEAE-Sephadex).

† 25 μg of protein. Hydrolysis of GTP by EDTA-washed membranes alone (2.9 pmol) was subtracted. Unextracted disc membranes with rhodopsin content equivalent to that of the EDTA-washed membranes (25 mg of membrane protein) hydrolyzed 27.7 pmol of GTP in 3 min.

‡ 25 μg of reconstituted (bleached) rhodopsin in phosphatidylcholine prepared as described (14). The hydrolysis of GTP by reconstituted rhodopsin alone was undetectable.

§ "Crude"; 0.37 μg of G fraction from DEAE-Sephadex.

¶ "More extensively purified"; G fraction from DEAE-Sephadex was further purified on blue Sepharose CL-6B (Pharmacia) and AH Sepharose 6B (Pharmacia).

Table 2. G fraction is necessary for light- and GTP-dependent PDEase activities

Addition(s)	PDEase activity* With 5.0 μM GTP	PDEase activity* No GTP
Rh†	0	0
Rh + H‡	0	0
Rh + G§	9.0	0
Rh + H + G	12.8	0

* Values are nmol of cyclic GMP hydrolyzed during a 7-min incubation at 30°C and are the averages of two or more determinations that agreed within 5%. (The data are in full agreement with those obtained in four repetitions of this experiment.) Basal PDEase activity—i.e., obtained with the PDEase fraction alone (6.6 pmol)—was subtracted in each case. PDEase (0.2 μg) partially purified by sucrose density gradient centrifugation was added to each 40-μl assay mixture.

† Purified, reconstituted (bleached) rhodopsin (8.75 μg) in phosphatidylcholine (14) was added.

‡ H fraction (0.2 μg) from DEAE-Sephadex was added.

§ G fraction (0.08 μg) from blue Sepharose CL-6B was added.

activation. In a Millipore filter-binding assay we found that the G fraction from DEAE-Sephadex is also needed for GTP binding (Table 3). Although the G fraction of GTPase is necessary for GTP binding, we do not exclude the possibility that H could enhance this binding. However, we note, that, whereas H increases GTPase activity in the G fraction 3-fold, its effect on the GTP binding of this fraction is small. Indeed, when we assayed each of the fractions from DEAE-Sephadex for GTP binding (by the Millipore filter assay) the GTP binding activity corresponded to the elution pattern G and not to that of H (data not shown).

The apparent molecular weight of H by sucrose density gradient centrifugation (15) is about 60,000. H was inactivated by heating at 60°C for 10 min, and its activity was resistant to trypsin digestion under conditions (200 μg of trypsin per ml; 1.5 min at 37°C in which the G fraction is entirely inactivated. In this experiment, trypsin action was stopped with a 4-fold molar excess of trypsin inhibitor. The PDE peak obtained by sucrose density gradient centrifugation (4) does not contain H activity.

DISCUSSION

Remarkable similarities have been noted between the light-activated photoreceptor PDEase and a number of hormone-activated adenylate cyclase systems (6, 8). The light-activated GTPase of frog rod appears to be closely analogous to the epinephrine-activated GTPase associated with turkey erythrocyte adenylate cyclase (10).

The combination of purified rhodopsin in phosphatidylcholine vesicles with the H and G fractions provides a true reconstitution of a light-activated GTPase with components which, when taken singly, are devoid of GTPase activity. These data clearly show that (in addition to bleached rhodopsin) at least two distinct components, the G and H fractions, are absolutely required for the expression of GTPase activity. Furthermore, although H is clearly necessary in order for the G fraction to demonstrate GTPase activity, the G fraction alone can function effectively as a GTP binding/PDEase activator protein.

The relative extent of H-mediated increases in GTPase activities range from very large (i.e., an absolute requirement for H) with reconstituted rhodopsin and a more extensively purified G fraction (Table 1) to much smaller (about 2-fold) increases seen with EDTA-washed membranes and DEAE-Sephadex-purified G fractions (Table 1; Fig. 1). This difference depends, for the most part, on the fact that H is to some extent already present as an impurity in both the EDTA-washed membranes and the DEAE-Sephadex-purified G fraction.

The details of the role of H in the function of other light-activated enzymes of the disc membranes is not yet fully understood. H could be needed to provide interaction of G with rhodopsin; it may be involved in binding of G or PDEase to the disc membrane; and it appears to be involved in the regulation of PDEase by promoting GTP hydrolysis at the G locus.

The data in Table 2 reveal a 40% enhancement of PDEase activity by H. This enhancement is consistently observed and is not increased by further addition of H. Indeed, if the hydrolysis of GTP is a component of the "off" step for the PDEase system, one might expect the presence of H to decrease rather than increase the observed rate of PDEase activity. This paradox is in part explained by the following. (*i*) In contrast to the epinephrine-sensitive turkey cyclase (which is a slow on and fast off system) (11), the light-activated PDE appears to be a fast on, slow off system. Thus, the ratio of activities seen with guanylyl imidodiphosphate and GTP is close to 1 for frog rod PDEase and 5 for turkey erythrocyte cyclase (11). (*ii*) If the GTP concentration is <0.5 μM, the presence of H decreases PDEase activity gradually by presiding over the hydrolysis of GTP (5, 6). At concentrations >50 μM, such an effect is not observed during the 3-min course of the PDEase assay. (*iii*) Nevertheless, and in addition, the data are compatible with the idea that the presence of H provides an allosteric conformational advantage for the PDEase reaction, perhaps by more efficient coupling of rhodopsin, G, and PDEase, in addition to its striking effects on GTP hydrolysis.

The use of pure bovine rhodopsin in the reconstituted system confirms the previous findings (7) that this photopigment participates in the light activation of PDE and that mammalian rhodopsin is fully active with frog disc peripheral proteins (1). Because bovine rhodopsin in native disc lipids or reconstituted in phosphatidylcholine exhibit (when bleached) full PDEase-activation capability, it would seem that neither composition nor degree of unsaturation of surrounding membrane lipids is critical for the GTPase and PDEase activation functions of rhodopsin. The data do not exclude the possibility that changes in the rates of activation might be altered by changes in lipid composition and membrane fluidity (or both). In addition, the efficacy of purified reconstituted rhodopsin indicates that integral disc membrane proteins (other than rhodopsin) appear to be unnecessary for the light-dependent activations studied here.

The complex nature of PDEase regulation appears to be appropriate for both of its proposed roles in rod physiology: the regulation of rod sensitivity, and the mediation of visual excitation. The abundance of regulatory elements could provide opportunities for the signal amplification essential for a process that mediates excitation and for the signal modulation essential for the regulation of rod sensitivity.

Table 3. Binding of [γ-^{32}P]GTP to disc membrane components

Addition(s)	Bound [γ-^{32}P]GTP*
None	0
H fraction†	0
G fraction‡	797
G + H fractions	957

In the absence of EDTA-washed membranes, the binding of GTP in any of the last three lines was less than 73 fmol. Under the conditions of the GTP-binding assay (20 min of incubation on ice), GTP hydrolysis was not detected.

We gratefully acknowledge the preparation of PDEase by Dr. Akio Yamazaki. This research was supported by U.S. Public Health Service Research Grants AM20179 and EY00729.

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