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Kinetic Studies Suggest That Light-Activated Cyclic GMP Phosphodiesterase Is a Complex with G-Protein Subunits†

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ABSTRACT: Cyclic GMP phosphodiesterase (PDE) in rod disk membranes has three subunits of molecular weight 88 000 (α), 84 000 (β), and 13 000 (γ). Physiological activation of the enzyme by light is mediated by a GTP binding protein (G protein). The enzyme can also be activated by controlled digestion with trypsin, which destroys the γ subunit, leaving the activated enzyme as $PDE_{\alpha\beta}$ [Hurley, J. B., & Stryer, L. (1982) *J. Biol. Chem. 257*, 11094–11099]. Addition of purified γ subunit to $PDE_{\alpha\beta}$ inhibited the enzyme fully. This suggested the possibility that G protein could also activate PDE by removing the γ subunit and leaving the active enzyme in the form of $PDE_{\alpha\beta}$. Should this be true, the properties of light- and trypsin-activated enzymes should be comparable. We found this not to be the case. The K_m of light-activated enzyme for cyclic GMP was about 0.9–1.4 mM while that of trypsin-activated enzyme was about 140 μM. The cyclic AMP K_m was also different for the two enzymes: 6.7 mM for light-activated enzyme and 2.0 mM for trypsin-activated enzyme. The inhibition of both enzymes by the addition of purified γ subunit also differed significantly. Trypsin-activated enzyme was fully inhibited by the addition of about 200 nM γ, but light-activated enzyme could not be fully inhibited even with 2600 nM inhibitor subunit. The K_i of the trypsin-activated enzyme for γ was 15 nM and of the light-activated enzyme 440 nM. These studies suggest that unlike trypsin-activated enzyme, light-activated PDE is not $PDE_{\alpha\beta}$ but possibly a complex with G protein as $PDE_{\alpha\beta}$·G or $PDE_{\alpha\beta}$·G·γ where the γ subunit is not removed from the complex but just displaced.

Photolyzed rhodopsin in vertebrate rod disk membranes (RDM) activates GTP binding protein (Godchaux & Zimmerman, 1979) (G protein) by catalyzing the exchange of GTP for GDP on this protein (Fung & Stryer, 1980; Leibman & Pugh, 1982). Activated G protein then activates cyclic GMP phosphodiesterase (PDE) (Uchida et al., 1981; Fung et al., 1981). G protein and PDE are peripheral membrane proteins (Godchaux & Zimmerman, 1979; Baehr et al., 1979, 1982), and both are multisubunit protein complexes (Baehr et al., 1979, 1982). G protein has three subunits of M_r 39 000, 37 000, and 6000 (Godchaux & Zimmerman, 1979; Baehr et al., 1982; Wheeler et al., 1977; Kuhn, 1980), and PDE has three subunits of M_r 88 000, 84 000, and 13 000 (Baehr et al., 1979; Kohnken et al., 1981) designated respectively as α, β, and γ. The functions of the individual subunits of these two proteins and whether some of them leave the membrane and become "solubilized" during the activation process are a subject of investigation in several laboratories. The M_r 39 000 subunit of G protein carries the GTP binding site though all three subunits appear essential for the rhodopsin-catalyzed nucleotide exchange (Fung & Stryer, 1980; Fung et al., 1981; Fung, 1983). Addition of nucleotide-bound M_r 39 000 subunit alone to dark RDM activated PDE (Fung et al., 1981).

The nature of the changes in PDE subunit interactions caused by activated G protein is not clear. Hurley & Stryer (1982) showed that controlled digestion with trypsin removed the M_r 13 000 subunit from PDE, resulting in maximal activation of the enzyme. Addition of purified M_r 13 000 subunit to the trypsin-activated PDE inhibited the enzyme completely (Hurley & Stryer, 1982). If the physiological activation of

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the enzyme by G protein involves displacement of the M_r 13000 subunit, this might occur through one of the following mechanisms (eq 1–3). For convenience, the subunits of G

$$PDE_{\alpha\beta\gamma} + G \rightleftharpoons PDE_{\alpha\beta}\cdot G\cdot\gamma \quad (1)$$

$$PDE_{\alpha\beta\gamma} + G \rightleftharpoons PDE_{\alpha\beta}\cdot G + \gamma \quad (2)$$

$$PDE_{\alpha\beta\gamma} + G \rightleftharpoons PDE_{\alpha\beta} + G\cdot\gamma \quad (3)$$

protein are not represented here. In the first equation, the γ subunit is not physically removed from PDE but pushed aside by G protein. In eq 2 and 3, it is dissociated from PDE but may remain on the membrane or become solubilized. Equation 2 differs from eq 3 in that the activated PDE remains complexed with G protein. Activated PDE in eq 3 is similar to the trypsin-activated enzyme in that it has only the two higher molecular weight subunits and is free of G protein. If the physiological activation occurs as in eq 3, the light-activated enzyme should display properties similar to the trypsin-activated enzyme. In this report, we show that the kinetic properties of the light-activated and trypsin-activated PDE are quite different and that eq 3 is therefore an unlikely mechanism for light activation. We suggest that the light-activated enzyme is a complex with one or more subunits of G protein.

Materials and Methods

Preparation of Rod Disk Membranes. Dark-adapted and frozen bovine retinas were purchased from George A. Hormel Co. (Austin, MN) and stored at −40 °C until use. Preparative procedures were carried out at 0–4 °C under infrared illumination with the aid of image converters. All centrifugations utilized an SS 34 rotor in a refrigerated Sorval centrifuge. Frozen retinas were thawed in 1 mL per retina of 45% w/v sucrose in MOPS buffer [20 mM 4-morpholinepropanesulfonic acid (MOPS), 100 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and vortexed at maximum speed for 60 s. The suspension was diluted in the same medium to about 2.5 mL per retina, overlaid with 2 mL of MOPS buffer, and spun at 15000 rpm for 30 min. The carpet of rod outer segments at the interface was collected, diluted into 2 volumes of buffer, and spun at 15000 rpm for 15 min. The pellet was resuspended in buffered sucrose, floated on sucrose, and washed again. The final pellet was suspended in a small volume of MOPS buffer (1 mL/10 retinas).

This preparation was used for the estimation of native membrane PDE and G protein contents and for studies on the K_m for substrates and the K_d for the M_r 13000 subunit (also referred to as inhibitor in this report).

Intact rod outer segments were isolated from fresh bovine retinas as described earlier (Sitaramayya & Liebman, 1983b) essentially according to the method of Schnetkamp et al. (1979) and were broken open before use in phosphodiesterase assays.

Reconstitution of PDE with G Protein and Rhodopsin. Soluble and peripheral protein-depleted RDM (sRDM) were prepared as described earlier (Sitaramayya & Liebman, 1983a) and used as a source of rhodopsin. G protein and PDE were purified according to Baehr et al. (1979, 1982). Rhodopsin, G protein, and PDE were mixed in proportions estimated to be present in native RDM as determined below (Table II) and used to determine K_m and V_{max} for cAMP and cGMP in the PDE velocity assays. Velocity assays using frozen RDM preparations were done for comparison. In some preparations, velocity vs. G-protein titrations were done at saturating cGMP concentration for PDE V_{max} comparisons with the trypsin activation experiments.

Determination of PDE and G-Protein Content of RDM. The rhodopsin concentration of RDM was determined spectroscopically, and known volumes of the preparation were dissolved in electrophoresis sample buffer containing sodium dodecyl sulfate (SDS). This was then electrophoresed in a 15% polyacrylamide gel essentially according to Baehr et al. (1979). The resolved proteins were stained with Coomassie brilliant blue, and after the gel was destained, the intensities of stained PDE and G-protein bands were measured on a densitometer. The amount of protein in stained bands was determined by comparison to standard curves prepared from known amounts of G protein and PDE electrophoresed simultaneously under identical conditions.

Isolation of the M_r 13000 Subunit (γ) of PDE. The procedure described by Hurley & Stryer (1982) was followed using purified PDE, prepared according to Baehr et al. (1979). After heat treatment and centrifugation, the preparation was dialyzed against MOPS buffer containing 50% glycerol and stored at −30 °C. Electrophoresis of this preparation showed a single protein of M_r 13000.

PDE Assays. Light-activated PDE assays using either RDM or a reconstituted system were done at 37 °C in a 0.5-mL assay mixture containing MOPS buffer, 5 mM cyclic GMP, and 250 μM GTP. The reactions were started with a light flash of 1-ms duration suitably attenuated to bleach a desired fraction of rhodopsin. Unattenuated flashes bleached 22% of rhodopsin and fully activated the PDE. Hydrolysis of cyclic nucleotide was measured by the pH recording method of Liebman & Evanczuk (1982). For trypsin-activated PDE assays, reactions were started by addition of cyclic nucleotide. In assays where the K_d for inhibitor was determined, the inhibitor was added to light-activated samples 30 s after the light flash, and after an additional 1.5 min, cyclic GMP was added to start the reaction. Trypsin-activated enzyme was similarly incubated with the inhibitor for 1.5 min before the assays were initiated with cyclic GMP.

Preparation of Trypsin-Activated PDE. RDM at a rhodopsin concentration of 1.3 μM or an equivalent amount of purified PDE (0.02 μM) was incubated at 22 or 38 °C in MOPS buffer. Cyclic GMP was added to a final concentration of 5 mM. One or two microliters (depending upon the age of the frozen solution) of a 10 mg/mL solution of trypsin was then added, and the PDE activity rate as measured by a drop in pH was recorded. Usually the rate reached a maximum in 25–40 s after the addition of trypsin and remained constant for about 20 s, falling after that. Once this approximate time for maximum activity was determined, the process was repeated without cyclic GMP. Incubation with trypsin was carried out for different periods of time around the approximate maximum; the trypsin was then inactivated with a 4-fold excess of trypsin inhibitor. The optimum length of incubation was determined by the preparations that showed the highest rate of PDE activity. A larger batch of RDM or PDE sufficient for the day's experiments was then treated with trypsin for this length of time, trypsin-inactivated with trypsin inhibitor, and held on ice. Aliquots were used for assays.

Rhodopsin and Protein Determinations. The rhodopsin content of RDM or sRDM was measured spectrophotometrically by using suitable dilutions of the preparations in 25 mM NH_2OH. The absorption spectrum before and after complete bleaching was recorded and rhodopsin concentration determined from the difference spectrum at 500 nm by using an extinction coefficient of 40000 L/(mol·cm). Protein concentrations of purified PDE, G protein, and PDE γ-subunit

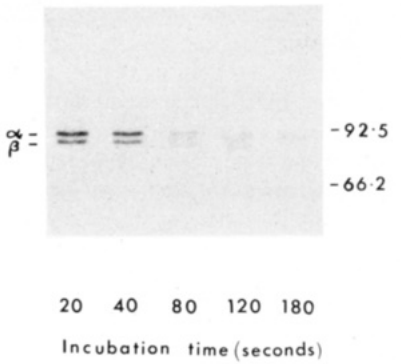

FIGURE 1: One microgram of PDE and 5 μg of trypsin were incubated at 22 °C for different periods of time, and the reaction was terminated with the addition of electrophoresis sample buffer containing SDS. The samples were electrophoresed according to Baehr et al. (1979). The α and β subunits of PDE are marked. The position and molecular weight ($\times 10^{-3}$) of marker proteins are indicated on the right. Only the top portion of the gel containing the α and β subunits of PDE is shown. The mobility and intensity of α and β subunits of PDE treated with trypsin for periods of time shorter than 20 s (not shown) were identical with those of control PDE and in lane 1.

Table I: Michaelis Constants and Turnover Numbers[a] of Light- and Trypsin-Activated PDE in Rod Disk Membranes[b]

	cyclic AMP		cyclic GMP	
prepn	K_m (mM)	turnover no. (s^{-1})	K_m (mM)	turnover no. (s^{-1})
RDM				
saturating bleach	6.67	222	1.11	303
weak bleach			0.87	55
broken ROS				
saturating bleach			1.05	586
reconstituted RDM				
saturating bleach			1.42	617
trypsin-activated RDM	2.00	420	0.14	1194
trypsin-activated purified PDE			0.16	1266

[a] Velocities are presented as V_{max} divided by PDE content because this permits the clearest mole for mole comparison of light-activated with trypsin-activated PDE. [b] The reconstituted RDM contained 4 μM rhodopsin in sRDM, 0.06 μM PDE, and 0.3 μM G protein. PDE was either fully activated by a bright flash bleaching 0.22 fraction of rhodopsin or partially activated by a weak flash bleaching 1.5×10^{-5} fraction. Michaelis constants were obtained from double-reciprocal plots. Turnover numbers are calculated by dividing the V_{max} obtained from reciprocal plots by the PDE concentration in the assay. The PDE concentration is 1/64.5 of the rhodopsin concentration in the RDM as shown in this report.

preparations were determined by the method of Sedmak & Grossberg (1977) using bovine serum albumin as standard. The molarities of purified proteins were determined by using molecular weights of 80000 for G protein, 180000 for PDE, and 13000 for the PDE γ subunit.

RESULTS

Trypsin Hydrolysis of PDE Subunits. The influence of digestion with trypsin on the PDE subunits is shown in Figure 1. Short-term digestion that activated PDE maximally did not appear to cause visible changes in the mobility of α and β subunits (lane 2). Continuing the digestion for longer periods resulted in digestion of most α and β subunits also, with the residual fraction showing a small decrease in the molecular weights of both these subunits. It was not possible to show that the PDE γ subunit was hydrolyzed without purifying the trypsinized PDE. However, Hurley & Stryer (1982) purified the trypsin-activated PDE on a high-pressure liquid chromatography (HPLC) gel filtration column and showed that the activated enzyme was depleted of the γ subunit.

Table II: G-Protein and PDE Content of Rod Disk Membranes

expt	copies of G protein/100 rhodopsins	copies of PDE/100 rhodopsins
1	5.2	1.89
2	9.62	1.56
3	7.47	1.42
4	5.83	1.32

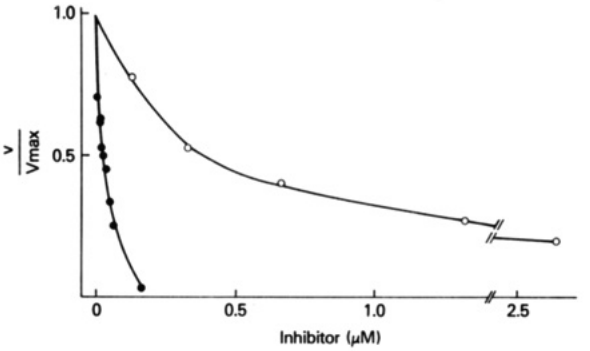

FIGURE 2: Inhibition of light- and trypsin-activated cyclic GMP phosphodiesterase activity in RDM by the purified M_r 13000 subunit (inhibitor) of PDE. The effective PDE concentration was 20.7 nM (○) in the light-activated preparation and 20 nM (●) in the trypsin-activated preparation.

Michaelis Constants of PDE for Cyclic GMP and Cyclic AMP. K_m's were compared for light- and trypsin-activated PDE (Table I). For PDE activated by a saturating bleach, the K_m for cyclic AMP was 6.67 mM and for cyclic GMP 1.1 mM. PDE activated by a very dim flash that bleached 1 in 150000 rhodopsins showed a similarly high K_m for cyclic GMP at 0.87 mM. The K_m for cyclic GMP of the enzyme in rod outer segments isolated according to Schnetkamp et al. (1979) and activated by a saturating bleach was 1.05 mM. These relatively intact outer segments were broken open only for PDE assays by passing repeatedly through a 22-gauge needle. sRDM reconstituted with purified PDE and G protein in proportions comparable to those in isolated rod disk membranes and activated by a bright flash gave a K_m for cyclic GMP of 1.42 mM. In summary, the K_m for cyclic GMP for the light-activated enzyme in the different types of preparations and conditions used ranged from 0.87 to 1.42 mM.

For trypsin-activated RDM, the K_m for cyclic GMP was 137 μM and for cyclic AMP 2.0 mM. For trypsin-activated purified PDE, the K_m for cyclic GMP was 155 μM (Table I).

G-Protein and PDE Content of Isolated Rod Disk Membranes. Reconstitution studies on PDE inhibitor as well as those described previously using a reconstituted system required preliminary estimates of the amounts of PDE and G protein normally present on the disk membranes. Analysis of four such preparations by gel electrophoresis showed that for every 100 rhodopsins there were 7.03 ± 1.97 G proteins and 1.55 ± 0.25 PDEs (Table II). The choice of protein concentrations used in our studies was based on these estimates.

PDE Inhibition by the M_r 13000 Subunit (Inhibitor). The influence of different amounts of inhibitor on the initial velocity of light- and trypsin-activated PDE in RDM was investigated. As shown in Figure 2, the trypsin-activated enzyme was inhibited at a much lower concentration of the inhibitor than the light-activated enzyme. Concentrations of inhibitor that totally inhibited the trypsin-activated enzyme yielded only weak inhibition of the light-activated enzyme. To determine the dissociation constant between inhibitor and activated PDE, free and bound inhibitor was estimated in the following way. The concentration of PDE activated by a bright flash was

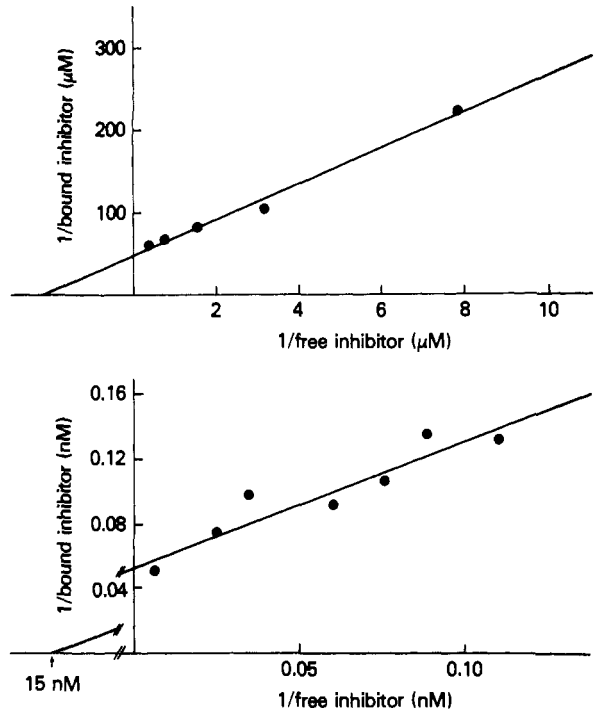

FIGURE 3: Estimation of the dissociation constant (K_d) of the inhibitor for light-activated (upper curve) and trypsin-activated (lower curve) PDE in RDM. Free and bound inhibitor concentrations were calculated as described in the text using the data shown in Figure 2.

found to be 20.7 nM in our assays. A decrease in activity on addition of inhibitor was considered proportional to the enzyme-inhibitor complex formed. Assuming that inhibitor binds activated PDE on an equimolar basis, the free inhibitor concentration was determined by subtracting the concentration of the PDE–inhibitor complex from the total inhibitor added. The assumption that the inhibited PDE complex is equimolar in its subunits is consistent with the 1:1:1 subunit ratio found for isolated (inhibited) PDE. Reciprocal plots of [free inhibitor] vs. [PDE-bound inhibitor] (Figure 3) showed that the K_d of the inhibitor was 0.44 μM for light-activated enzyme and 0.015 μM for trypsin-activated enzyme. Similar results were obtained for different batches of inhibitor preparation. Dissociation constants calculated from plots of [enzyme–inhibitor] vs. [enzyme–inhibitor]/[inhibitor] for the data shown in Figure 3 were 377 nM for light-activated enzyme and 15 nM for trypsin-activated enzyme, very similar to those obtained from reciprocal plots.

Discussion

PDE of dark-adapted RDM is thought to be inactive because of inhibition that is relieved by light activation (Miki et al., 1975; Sitaramayya et al., 1977). While several proteins are shown to have an inhibitory influence on PDE (Baehr et al., 1979; Hurley & Stryer, 1982; Dumler & Etingof, 1976; Yamazaki et al., 1983), the M_r 13 000 subunit of PDE is most likely to be the inhibitor protein coupled with the enzyme in the dark state. Purified PDE has this subunit in an equimolar ratio with the higher molecular weight subunits. Whether this subunit remains with or near its PDE of origin upon activation or is released into solution (Yamazaki et al., 1983) is not yet established.

Fung et al. (1981) have shown that the guanosine 5′-(β,γ-imidotriphosphate) (GMPPNP) complex of the M_r 39 000 subunit of G protein can alone activate PDE. The active form of PDE could then be $PDE_{\alpha\beta}$·39 kDa·γ, $PDE_{\alpha\beta}$·39 kDa, or $PDE_{\alpha\beta}$. Yamazaki et al. (1983) have reported that an inhibitor of PDE binds to the M_r 39 000 subunit of G protein and leaves the membranes. The molecular weight of the inhibitor in this study was not reported. If it was the M_r 13 000 subunit of PDE, the activated PDE on the membrane might be either $PDE_{\alpha\beta}$ or $PDE_{\alpha\beta}$ coupled with the remaining M_r 37 000 and 6000 subunits of G protein. Both of these reports are therefore consistent with the idea that light-activated PDE is $PDE_{\alpha\beta}$ either alone or coupled with subunits of G protein. We have attempted to distinguish between these two forms by comparing kinetic properties with those of $PDE_{\alpha\beta}$ known to be generated by trypsinization. Controlled digestion with trypsin is known to activate PDE maximally (Baehr et al., 1979; Miki et al., 1975). Hurley & Stryer (1982) have shown that trypsin digestion removes the M_r 13 000 subunit from PDE, leaving the active enzyme in the form of $PDE_{\alpha\beta}$. We argued that if light activation also generates such a form, the kinetic properties of the two enzymes should be similar, and if they differ, then the light-activated enzyme must be a complex with one or more subunits of G protein.

The Michaelis constants of RDM PDE for both cyclic nucleotides have been reported by several laboratories (Baehr et al., 1979; Miki et al., 1973, 1975; Manthorpe & McConnell, 1975; Farber & Lolley, 1977; Yee & Liebman, 1978; Pannbaker et al., 1972). They varied from 80 to 360 μM for cyclic GMP and from 2.3 to 8.0 mM for cyclic AMP. In all cases cited above, the assays were done in darkness or in continuous room light. In our experiments, the K_m was determined on preparations made in the dark and activated by a light flash. For the different preparations we used, the K_m for cyclic GMP varied from 0.87 to 1.42 mM. These values are in good agreement with the 0.5–1.5 mM K_m values for cyclic GMP reported by Robinson et al. (1980). The K_m for cyclic GMP for trypsin-activated enzyme was 137 μM, similar to the value of 78 μM reported by Hurley & Stryer (1982). It was unlikely that proteolysis of an RDM protein other than PDE influenced the K_m of PDE since trypsin-treated RDM as well as purified PDE had similar K_m's for cyclic GMP. In comparing the light- and trypsin-activated enzymes, we are assuming that α and β subunits of both enzymes are identical. Electrophoresis of trypsin-activated PDE showed that the mobilities of α and β subunits are not different from those of untreated enzyme. However, we are aware that, due to the limitations of the system, we cannot detect removal of one to a few amino acids from these subunits if that did not cause a material difference in their mobility.

We considered the possibility that residual structure in our RDM preparations such as disk stacks etc. might reduce the diffusional access speed of cyclic nucleotide to its enzymic active site compared to access in trypsinized preparations, thus accounting for the reduced velocity and high K_m for the former. We regard such an explanation as extremely unlikely since reconstituted preparations had the same K_m as regular membranes and the cGMP K_m for a weak bleach activation, where the local rate of cGMP consumption is low, is essentially the same as that for a strong bleach where local cGMP consumption is high. A substrate diffusional access problem would also be expected to produce nonlinearities both in cyclic nucleotide–velocity reciprocal plots and in plots that titrate PDE velocity with G protein at a fixed initial cyclic GMP concentration. Our experimental results showed no nonlinearities in either of such plots.

It may be significant that the previously reported cyclic GMP K_m's for assays done in room light and for trypsin-activated enzyme are quite similar while that for flash-activated enzyme as reported here is much higher. Possible dissociation

Table III: Maximal Activity of Purified PDE: Comparison of Trypsin Activation with Light Activation at Saturating G-Protein Concentration[a]

prepn	PDE act. [μM cyclic GMP hydrolyzed (μM PDE)$^{-1}$ s^{-1}]	
	trypsin activation	light activation at saturating [G protein][b]
1	743	666
2	2051	1905
3	4044	4166
4	503	438

[a] As evident from the table, the different PDE preparations varied in their activity, but for a given preparation, the trypsin-activated and light-activated velocities are similar. [b] Mixtures of 4 μM sRDM, 0.06 or 0.08 μM purified PDE, 5 mM cyclic GMP, and 250 μM GTP were activated by bright flashes in the presence of various concentrations of G protein at 38 °C or at 22 °C (preparation 4). The reciprocal of the initial velocity was plotted against the reciprocal of the G-protein concentration, and the V_{max} was obtained from intercepts at the y axis.

of the M_r 13 000 protein from PDE assayed after prolonged light exposure can be ruled out since the enzyme isolated from bovine retinas in room light contains this subunit in equimolar ratio with the other subunits.

The relative proportion of rhodopsin to G protein and PDE in RDM used in the present experiments was 100:7.03:1.55, within the range reported by other investigators. We found 1 G protein for every 14 rhodopsins, while the previously reported number varies from 1 in 10 to 1 in 15 rhodopsins (Godchaux & Zimmerman, 1979; Kuhn, 1980; Kuhn et al., 1981; Bennett, 1982). Baehr et al. (1979) gave the lower and upper limits of PDE content of RDM as 0.6–2.5 per 100 rhodopsins. Our estimate of PDE content at 1.55 ± 0.25 per 100 rhodopsins is within this range.

The light-activated PDE activity of RDM varied considerably from preparation to preparation and averaged 33.51 ± 13.85 μM cyclic GMP hydrolyzed per 4 μM rhodopsin per second. Since the PDE:rhodopsin ratio is 0.0155, this gives a PDE light-activated turnover number of about 560 s^{-1}. The trypsin-activated PDE activity of a given preparation was always about 3 times higher than the light-activated activity. In other words, only a third of the PDE appears to be activated by light in spite of an almost 5-fold excess of G protein over PDE in RDM. This suggests that whatever G protein binds in light-activated preparations to activate the PDE, it does so with poor affinity and randomly activates only about a third of the PDE present. Reconstitution with a large excess of G protein can allow light activation of essentially all the PDE, giving a V_{max} very close to that resulting from trypsin activation of the same PDE (Table III).

In spite of the similarity in maximal activity, we found the trypsin-activated enzyme to have a 30-fold lower K_d for inhibitor (1.51×10^{-8} M) than the light-activated one ($K_d = 4.4 \times 10^{-7}$ M). Since G protein is activated in light-activated preparations, we considered the possibility that the inhibitor titration might be dominated by weak binding to the much more plentiful G protein in competition with a sparse but tightly binding population of PDE's. However, the PDE vs. inhibitor reciprocal plots were linear over inhibitor concentrations ranging from 0.12 to 1.2 μM (or inhibitor to G protein ratio of 0.4 to 4.0), making such a dual binding site model virtually impossible. Thus, assuming inhibitor binding to G protein with a K_d of 0.44 μM, 0.44 μM inhibitor would provide more than enough free inhibitor to completely inhibit the activated PDE should its inhibitor constant be similar to that of trypsin-activated enzyme. Since this is not the case, it is apparent that light- and trypsin-activated enzymes are structurally dissimilar. It should be pointed out here that the K_d for trypsinized enzyme measured in our experiments is about 100-fold higher than that reported by Hurley & Stryer (1982). Though we cannot explain the reason for this difference, it should not influence our results since the same inhibitor preparation was tested on both trypsin- and light-activated PDE and the two enzymes showed different levels of affinity to the inhibitor.

In conclusion, the two observations, that the K_m for cyclic GMP and the K_d for inhibitor are distinctly different for light- and trypsin-activated enzymes, strongly suggest that light-activated enzyme is not $PDE_{\alpha\beta}$ as the trypsin-activated enzyme is, but instead is a complex with G protein as $PDE_{\alpha\beta}$·G·γ or $PDE_{\alpha\beta}$·G.

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Registry No. PDE, 9068-52-4; cyclic AMP, 60-92-4; cyclic GMP, 7665-99-8; trypsin, 9002-07-7.

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Autophosphorylation of Glyceraldehydephosphate Dehydrogenase and Phosphorylation of Protein from Skeletal Muscle Microsomes†

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ABSTRACT: Glyceraldehydephosphate dehydrogenase purified from rabbit skeletal muscle is autophosphorylated with MgATP. Half-maximal phosphorylation is achieved around 0.3 mM. The phosphorylation is Ca^{2+} independent. The phosphoenzyme complex is labile in alkaline conditions and stable in moderately acid media. The complex is readily hydrolyzed by 0.1 M neutral hydroxylamine, indicating the complex formed is a high-energy acyl phosphate. The phosphorylation is reduced by nicotinamide adenine dinucleotides, reduced form (NADH), glyceraldehyde 3-phosphate, and nicotinamide adenine dinucleotide (NAD^+). The enzyme is also dephosphorylated by these metabolites although to a lesser extent by NAD^+. Calsequestrin isolated from rabbit skeletal muscle inhibits the phosphorylation of the enzyme. The phosphoenzyme behaves as a kinase catalyzing the phosphorylation of proteins of M_r 80 000 and 72 000 found in the skeletal muscle terminal cisternae/triad preparation. This reaction is enhanced by NADH. The phosphate found in the protein substrate has been shown to be the same phosphate initially involved in the phosphorylation of glyceraldehydephosphate dehydrogenase.

Glyceraldehydephosphate dehydrogenase (glyceraldehyde-P dehydrogenase) has been exhaustively investigated as a key enzyme in glycolysis (Harris & Waters, 1976). The enzyme is responsible for the oxidative phosphorylation of glyceraldehyde 3-phosphate (GAP)[1] by NAD^+ and inorganic phosphate. It has been isolated from a variety of sources including yeast (Krebs et al., 1953), lobster (Allison & Kaplan, 1964), and rabbit (Caputto & Dixon, 1945). Amino acid sequencing has revealed that glyceraldehyde-P dehydrogenase is a highly conserved protein among different organisms.

In general, glycolytic enzymes have been described as soluble proteins found in the cytosolic milieu. However, glyceraldehyde-P dehydrogenase has been shown to exhibit specific binding to erythrocyte membranes (Kant & Steck, 1975). The interaction is of an ionic nature, and elution of glyceraldehyde-P dehydrogenase can be effected by physiological concentrations of salt. Moreover, ATP, NADH, and GAP can also cause specific dissociation of glyceraldehyde-P dehydrogenase from erythrocyte membranes. The site of interaction has been identified as the anion channel (band 3) of erythrocytes (Yu & Steck, 1975). As yet, the physiological significance of the interaction has not been determined.

Recently, glyceraldehyde-P dehydrogenase has been identified as a component involved in the formation of triad junctions of rabbit skeletal muscle. The purified protein has been demonstrated to promote re-formation of triad junctions previously disrupted mechanically by French press treatment (Corbett et al., 1985; Caswell & Corbett, 1985). The catalytic function is specific for T tubules and heavy terminal cisternae components as glyceraldehyde-P dehydrogenase fails to promote association of transverse tubules with nonjunctional light terminal cisternae SR or longitudinal SR. The mechanism by which glyceraldehyde-P dehydrogenase re-forms triad junctions is uncertain. However, it is possible that the enzyme is involved in organizing the membrane-bound "anchoring" protein to facilitate the interaction with the "spanning" protein to form the junctional complex.

The discovery of a glycolytic enzyme involved in an entirely unrelated catalytic function suggests the possibility that this protein participates in functions separate from glycolysis. We now report the autophosphorylation of glyceraldehyde-P dehydrogenase by MgATP which is apparently independent of glycolysis. Evidence is presented indicating that the phos-

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[1] Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GAP, glyceraldehyde 3-phosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NH_2OH, hydroxylamine; $(NH_4)_2SO_4$, ammonium sulfate; PCA, perchloric acid; SDS, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SR, sarcoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; TC, terminal cisternae; GAPD, glyceraldehyde-3-phosphate dehydrogenase.

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