the pD scale to the loss of SO_4^{2-} ion that they may well be a consequence of this loss. Thus we conclude that (1) a sulfate anion is strongly hydrogen bonded to the imidazolium N-H(D) of His-12 located in the active site in a specific way, (2) deprotonation (dedeuteronation) of the imidazolium ring results in the desorption of the sulfate anion, and (3) the surrounding structure in the active site plays an important role in maintaining the sulfate anion at the optimum position for such hydrogen bonding and desorption of the sulfate anion results in a slight relaxation of this structure as shown by its effect on the amide I′ band and the tyrosine doublet.

Acknowledgments

We are grateful to Professor Tatsuo Miyazawa of Tokyo University who kindly supplied *N*-acetylphenylalanine methylamide.

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Soluble 5′-Nucleotidase: Purification and Reversible Binding to Photoreceptor Membranes†

Hiroyuki Fukui and Hitoshi Shichi*

ABSTRACT: Bovine rod outer segment membranes contain two types of 5′-nucleotidase, an integral enzyme and a peripheral enzyme. The peripheral enzyme, which accounts for about 25% of the total 5′-nucleotidase activity, was extracted with ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) buffer in the dark. The soluble enzyme was purified to apparent homogeneity by column chromatography on Blue Sepharose, 5′-AMP–Sepharose, and Sephacryl S-200. The purified enzyme had a molecular weight of about 67 000 and an isoelectric point of 6.5 and did not seem to consist of subunits. The enzyme hydrolyzed both 5′-AMP (K_m = 1.3 μM) and 5′-GMP (K_m = 2.3 μM). Adenosine 5′-(α,β-methylenediphosphate) (K_i = 4.2 μM), adenosine 5′-diphosphate (ADP) (K_i = 0.4 μM), adenosine 5′-triphosphate (ATP) (K_i = 3.3 μM), and rabbit G-actin were competitive inhibitors. Concanavalin A (K_i = 4.6 μg/mL) inhibited the enzyme noncompetitively. EGTA extracted 5–10 times as much enzyme in the dark as in the light. $CaCl_2$ and $MgCl_2$ at 5 mM inhibited the extraction of enzyme both in the dark and in the light. $CaCl_2$ (5 mM) facilitated the rebinding of enzyme to the membrane. Light had little effect on the rebinding of enzyme whether in the presence or absence of Ca^{2+}. The enzyme rebound to the bleached membrane in the presence of Ca^{2+} became readily extractable again in the dark if the chromophore of rhodopsin was regenerated and excess Ca^{2+} was removed. The amount of enzyme bound at Ca^{2+}/rhodopsin ≃ 20 in the dark was about 50% of the total peripheral enzyme associated with intact rod membranes. The binding of 5′-nucleotidase to rod membranes was specific; malate dehydrogenase and cytochrome *c* were not bound to the membranes at any Ca^{2+} concentration tested. In the presence of Ca^{2+} the enzyme was bound to membranes that had been treated with urea but not to rhodopsin–phosphatidylcholine vesicles. Hence, the nature of the membrane component involved in the binding of peripheral 5′-nucleotidase remains unknown.

A rapid decrease in 3′,5′-cGMP[1] concentration occurs during the light-induced hyperpolarization of vertebrate rod photoreceptors [see, for a review, Hubbell & Bownds (1979)]. This is catalyzed by a light-activated cyclic nucleotide phosphodiesterase (Bitensky et al., 1978). There is electrophysiological evidence implicating a role for 3′,5′-cGMP in the depolarization of photoreceptor membranes (Nicol & Miller, 1978). In spite of the rapid hydrolysis of 3′,5′-cGMP, guanylic

† From the Laboratory of Vision Research, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20205 (H.F.), and the Institute of Biological Sciences, Oakland University, Rochester, Michigan 48063 (H.S.). *Received January 4, 1982.* This work was supported in part by a National Institutes of Health grant (EY-03807-01) to H.S.

[1] Abbreviations: 3′,5′-cGMP, guanosine cyclic 3′,5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5′-AMP, adenosine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate.

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acid does not accumulate appreciably in the irradiated photoreceptor (Robinson & Hagins, 1979). This suggests the presence of active 5′-nucleotidase (EC 3.1.3.5) in rod photoreceptors. We have previously shown that two 5′-nucleotidases are indeed associated with photoreceptor membranes, an integral enzyme tightly bound to membrane and a peripheral enzyme extractable with buffer (Fukui & Shichi, 1981). Studies on disk membrane preparations suggest that the integral enzyme does not expose its catalytic site on the cytoplasmic side of the membrane and may not be involved in the hydrolysis of intracellular 3′,5′-cGMP (Shichi & Somers, 1980; Fukui & Shichi, 1981). On the other hand, the peripheral enzyme is probably localized on the cytoplasmic side of the membrane (Fukui & Shichi, 1981). In this investigation we have purified the peripheral enzyme to apparent homogeneity and studied its interaction with rod membranes. We report that the reversible binding of the purified enzyme to rod membranes is regulated by light and calcium ion.

Materials and Methods

Extraction and Purification of 5′-Nucleotidase from Rod Membrane. Rod outer segments prepared from 2000 fresh bovine eyes (Shichi et al., 1969) were extracted by homogenization in the dark with 300 mL of 5 mM Tris-HCl, pH 8.0, containing 1 mM EGTA and 1% poly(ethylene glycol) (Sigma, M_r 400) (EGTA buffer). After centrifugation of the homogenate, the supernatant was collected and passed through a column of Blue Sepharose 4B (Pharmacia, 1.6 × 8.8 cm) preequilibrated with 10 mM Tris-HCl, pH 8.0, containing 1% poly(ethylene glycol) (Tris buffer) at a flow rate of 24 mL/h. The fractions containing enzyme activity collected in the void volume of the column were pooled and loaded on a 5′-AMP–Sepharose 4B column (Pharmacia, 0.9 × 12.2 cm) preequilibrated with Tris buffer, and the column was eluted with Tris buffer containing 10 mM 5′-AMP (AMP–Tris buffer) at a flow rate of 15 mL/h. Ten-milliliter fractions were collected. Pooled fractions containing 5′-nucleotidase activity were placed in a dialysis tubing, concentrated to 3 mL on pellets of poly(ethylene glycol) (Sigma, M_r 10000), and applied for further purification to a column of Sephacryl S-200 (Pharmacia, 2.0 × 90 cm) preequilibrated with Tris buffer. The column was eluted with Tris buffer at a flow rate of 22 mL/h. Polyacrylamide gel electrophoresis of the purified enzyme was carried out in 7.8% polyacrylamide gel containing 0.1% sodium dodecyl sulfate essentially by following the method of Fairbanks et al. (1971).

Assay of 5′-Nucleotidase and Other Enzyme Activities. The assay procedure for 5′-nucleotidase was described in detail in the previous paper (Fukui & Shichi, 1981). In brief, an assay mixture containing 15 μM 5′-[^{3}H]AMP or 5′-[^{3}H]GMP, 30 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 0.6 mg/mL bovine serum albumin (Sigma), and an appropriate amount of enzyme in a final volume of 100 μL was incubated for 5–30 min at 25 °C. After the reaction was stopped with a mixture containing EDTA (200 mM), nonradiolabeled AMP or GMP (13 mM), and adenosine or guanosine (13 mM), the assay mixture was applied to an AG1 column (Bio-Rad, 0.5 × 3.5 cm, formate form). The radioactivity of both substrate and product separated on the column was determined in a Beckman scintillation counter, Model LS 9000. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 nmol of substrate/min. To confirm that 5′-AMP (or 5′-GMP) was hydrolyzed by 5′-nucleotidase, we also determined activities of alkaline phosphatase (Garen & Levinthal, 1960) and acid phosphatase (Hufstee, 1954) for enzymatically active fractions. Protein was determined with Folin reagent (Lowry et al., 1951). Malate dehydrogenase was assayed by the method of Murphey et al. (1967). Cytochrome *c* was determined spectrally at 550 nm after reduction with hydrosulfite.

Preparation of Various Rhodopsin-Containing Membranes. Urea-treated rod membranes were prepared by suspending rod outer segments from fresh bovine eyes in 0.1 M Tris buffer (pH 8.0) containing 5 M urea and 5 mM EGTA for 2.5 h at 3 °C in the dark (Shichi & Somers, 1978). Regenerated membranes were prepared by incubating photobleached rod membranes with 11-*cis*-retinal in ethanol for 60 min at 20 °C in the dark. Rhodopsin-containing phospholipid vesicles were prepared as follows: Purified rhodopsin (1 μmol) in 3 mL of 0.3% Ammonyx L0 (Hong & Hubbell, 1972) was mixed with 25 mg of bovine brain phosphatidylcholine (Applied Science) and dialyzed against 1 L of Tris buffer for 3 weeks at 3 °C in the dark with frequent changes of buffer.

Extraction and Rebinding of 5′-Nucleotidase. For experiments of Table II, rod membranes (approximately 0.6 nmol of rhodopsin) were suspended in 0.5 mL of 5 mM Tris buffer (pH 8.0) containing 1 mM EGTA, incubated for 20 min at 20 °C, and centrifuged at 110000*g* for 60min. The extent of enzyme binding to membranes was determined by assaying 5′-nucleotidase activity remaining in the supernatant. To study the effect of 5 mM Ca^{2+}, we added 0.1 mL of Tris buffer containing 30 mM Ca^{2+} to the suspension and incubated it for 10 min at 20 °C before centrifugation. For experiments with urea-treated membranes or rhodopsin–phospholipid vesicles, a 5-mL suspension containing about 5 nmol of rhodopsin and 10 units of purified enzyme was divided into 10 aliquots, incubated with different concentrations of Ca^{2+} for 20 min at 20 °C, and centrifuged at 110000*g* for 60 min. Enzyme activity of the supernatant was assayed after calcium concentrations were reduced to noninhibiting levels with EGTA.

Results

Extraction and Purification of 5′-Nucleotidase. When rod outer segment membranes were extracted in the dark repeatedly with 5 mM Tris-HCl buffer containing 1 mM EGTA, about 25% of the total 5′-nucleotidase activity of the membrane was solubilized. Of the buffer-extractable activity, 60–80% was collected in the first extract. Dissociation of the buffer-soluble activity from the membrane was essentially complete after five extractions. The remaining activity was extractable only with detergent. In the light no more than 20% of the buffer-extractable activity was collected by the first extraction, and further extraction did not increase the amount of enzyme extracted. $CaCl_2$ and $MgCl_2$ at 5 mM inhibited solubilization of enzyme both in the dark and in the light. The inhibition was not due to a depletion of effective substrate concentrations by these cations in the assay mixture. Ca^{2+} and Mg^{2+} concentrations in the extracts were reduced before assay to noninhibiting levels with EGTA and EDTA, respectively. More detailed studies on the dissociation (and rebinding) of enzyme and the effect of Ca^{2+} are presented later. Use of rod membranes prepared from fresh retinas was essential to see the effects of light and divalent cations on the extractability of enzyme; the effects were completely lost by storage of membranes at 3 °C for 1 week or were not observed with rod membranes prepared from the retinas stored in the frozen state for months. The extracted enzyme was labile and lost its activity entirely in 10 days at −20 °C. However, the enzyme activity was almost fully retained in the presence of 1% poly(ethylene glycol) (M_r 400) after storage for 10 days at 3 °C. Glycerol (10%) provided little or no protection. The enzyme extracted in 1 mM EGTA was purified by column

Table I: Summary of Purification of 5′-Nucleotidase

procedure	total protein (mg)	total units[a]	sp act. (units/mg of protein)
crude extract	86	7500	87
Blue Sepharose	60	5500	92
AMP-Sepharose	0.2	1800	9 000
Sephacryl S-200	0.08	1500	18 800

[a] One unit = 1 nmol of 5′-AMP hydrolyzed/min.

chromatography on Blue Sepharose, 5′-AMP–Sepharose, and Sephacryl S-200. A summary of purification data is shown in Table I. Affinity chromatography on AMP–Sepharose proved to be the most effective step. Blue Sepharose, which binds other rod membrane peripheral enzymes such as rhodopsin kinase (Shichi & Somers, 1978), cyclic nucleotide phosphodiesterase, and GTPase (Shichi, 1981), was effective in separating 5′-nucleotidase from these enzymes. The final yield of purified enzyme was about 40 μg of protein/1000 bovine retinas. The purified enzyme was essentially homogeneous and migrated as a single protein (M_r 67 000) in $NaDodSO_4$–polyacrylamide gel electrophoresis. Since a M_r of 69 000 was determined by gel filtration on a Sephacryl S-200 column, the enzyme did not seem to have subunits.

Properties of Purified 5′-Nucleotidase. The purified enzyme hydrolyzed both 5′-AMP (K_m = 1.3 μM) and 5′-GMP (K_m = 2.3 μM) and did not show acid and alkaline phosphatase activities. When the enzyme was assayed with 5′-AMP as the substrate and at 1 mM Mg^{2+}, adenosine 5′-(α,β-methylenediphosphate) (K_i = 4.2 μM), ADP (K_i = 0.4 μM), and ATP (K_i = 3.3 μM) were competitive inhibitors. Noncompetitive inhibition by the plant lectin concanavalin A (K_i = 4.6 μg/mL) suggested that the enzyme might be a glycoprotein. The inhibitory effects of the adenine nucleotides were reversed almost completely by 100 mM Mg^{2+}. Rabbit G-actin (50 μg/mL) inhibited 80% of activity competitively. The isoelectric point of the enzyme was determined to be pH 6.5 by isoelectric focusing in a pH range from 3.8 to 10.0 (Fawcett, 1968).

Dissociation and Rebinding of 5′-Nucleotidase. When intact rod membranes (ca. 0.6 nmol of rhodopsin) were extracted by homogenization with 1 mM EGTA (experiment 1 in Table II), more enzyme (about 1.2 units) was extracted in the dark than in the light (0.13 unit) (A and B). The bleaching of rhodopsin following extraction but preceding centrifugation had no effect on enzyme dissociation (C). However, the addition of 5 mM Ca^{2+} after extraction with 1 mM EGTA in the dark resulted in marked reduction in the enzyme activity

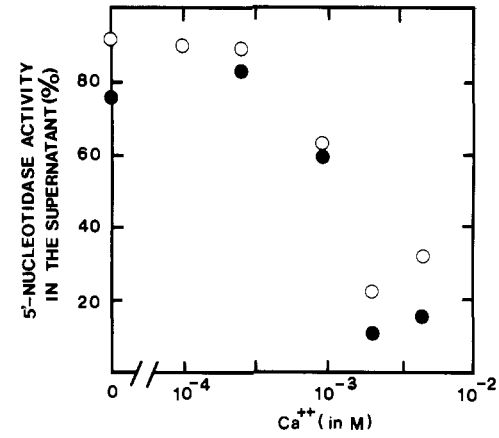

FIGURE 1: Binding of 5′-nucleotidase to urea-treated rod membranes: (○) in the light; (●) in the dark.

of the supernatant, indicating the reassociation of enzyme to the membrane (D). The bleaching of rhodopsin before the addition of Ca^{2+} in a similar experiment did not enhance the Ca^{2+} effect (E). If the added Ca^{2+} (5 mM) was removed by the subsequent addition of 2.5 mM EGTA, the rebound enzyme became extractable with 1 mM EGTA (F) in the dark but not in the light (G). In experiment 2, rod membranes were bleached by light, and 5 mM Ca^{2+} was added to rebind the enyzme to bleached membranes. The membranes were incubated with 11-*cis*-retinal in the dark to regenerate rhodopsin. Ca^{2+} was removed by the addition of 2.5 mM EGTA. As in intact membranes, more enzyme was extracted from regenerated membranes with 1 mM EGTA in the dark (H) than in the light (I). The enzyme dissociated in the dark from the membrane in the presence of EGTA was rebound if 5 mM Ca^{2+} was added, regardless of whether the membrane was bleached or not (J and K). Thus, regenerated membranes behaved like intact (i.e., unbleached) membranes in their ability to bind the enzyme in the light or in the presence of Ca^{2+} and release it into EGTA buffer in the dark. The effect of calcium concentration on enzyme binding indicated that the level of bound enzyme at Ca^{2+}/rhodopsin ≃ 20 in the dark was about 50% of the total peripheral enzyme bound to the intact membrane. Thus there must be a critical Ca^{2+} concentration required for binding of the enzyme to the membrane. Studies with urea-treated membranes seem to support this (see Figure 1). Mg^{2+} at comparable concentrations was virtually without effect for enzyme binding to the membrane. Rod membranes are known to be readily fragmented into vesicles by mechanical agitation. It was therefore possible that rod membranes were converted to vesicles during incubation

Table II: Extraction and Rebinding of Peripheral 5′-Nucleotidase to Rod Outer Segment Membranes

procedures	units of 5′-nucleotidase act. in supernatant
expt 1 (intact rod membranes)	
(A) extraction with 1 mM EGTA in the dark	1.20
(B) extraction with 1 mM EGTA in the light	0.13
(C) extraction with 1 mM EGTA in the dark and then rhodopsin bleaching	1.20
(D) extraction with 1 mM EGTA in the dark and then addition of 5 mM Ca^{2+} in the dark	0.18
(E) extraction with 1 mM EGTA in the dark, bleaching of rhodopsin, and addition of Ca^{2+}	0.15
(F) extraction with 1 mM EGTA in the dark, addition of 5 mM Ca^{2+}, removal of excess Ca^{2+}, and extraction with 1 mM EGTA in the dark	1.05
(G) same as (F) except extraction with 1 mM EGTA in the light	0.35
expt 2 (regenerated rod membranes)	
(H) extraction with 1 mM EGTA in the dark	1.00
(I) extraction with 1 mM EGTA in the light	0.37
(J) extraction with 1 mM EGTA in the dark and then addition of 5 mM Ca^{2+} in the dark	0.13
(K) extraction with 1 mM EGTA in the dark, bleaching of rhodopsin, and addition of 5 mM Ca^{2+}	0.16

and the enzyme was trapped or sequestered within the vesicles rather than bound to the membrane. However, this possibility was ruled out because [^{3}H]inulin incubated with rod membrane under similar conditions was not trapped in the membrane vesicles that were collected by centrifugation after incubation.

We have previously shown that peripheral enzymes are inactivated by washing rod membranes with 5 M urea without denaturing rhodopsin. For example, treatment of rod membranes with 5 M urea denatured almost completely membrane-associated rhodopsin kinase (Shichi & Somers, 1978) and GTPase (H. Shichi and R. L. Somers, unpublished results). When rod membranes that had been previously treated with 5 M urea were incubated with purified soluble 5′-nucleotidase at different Ca^{2+} concentrations, little enzyme binding occurred at calcium concentrations lower than 1 mM, while 80–90% binding was observed when the Ca^{2+} concentration was at 2–5 mM (Figure 1). At higher Ca^{2+} concentrations, the binding of enzyme to membrane decreased somewhat. The ratio of Ca^{2+}/rhodopsin $\simeq$ 1000 calculated at 1 mM Ca^{2+} may be a critical concentration required for enzyme binding. As was the case with intact membrane (Table II, D, E), there was no significant difference in the amount of enzyme bound to urea-treated membranes between in the light and in the dark. The results also indicate that the membrane component responsible for binding the enzyme in the presence of Ca^{2+} was not inactivated by 5 M urea. In order to determine whether or not the binding component was rhodopsin, we incorporated purified rhodopsin in phospholipid vesicles and incubated it with the purified enzyme and calcium. Little or no binding occurred at any calcium concentration tested both in the dark and in the light.

To check the specificity of binding, we examined the affinities of rod membranes for two proteins, malate dehydrogenase and cytochrome *c*. Malate dehydrogenase, whose isoelectric point [pH 6.5 (Davies & Kun, 1957)] is similar to that of 5′-nucleotidase, was not bound to rod membranes at any calcium concentration tested. Cytochrome *c* with an isoelectric point at 10.6 [from Lehninger (1970)] was bound to rod membranes in EGTA buffer. Increasing Ca^{2+} concentrations in the incubation medium resulted in the inhibition of binding.

Discussion

The buffer-extractable 5′-nucleotidase is distinct from the integral enzyme tightly associated with rod membranes in several respects: (i) No more than 25% of the total 5′-nucleotidase activity of rod membranes is extracted with EGTA buffer. (ii) The two enzymes have different molecular weights, ca. 75000 for the integral enzyme (Fukui & Shichi, 1981) and ca. 67000 for the peripheral enzyme (this study). (iii) Rabbit G-actin inhibits the integral enzyme noncompetitively and the peripheral enzyme competitively. (iv) The integral enzyme extracted in detergent is stable for weeks at 3 °C, whereas the peripheral enzyme is very unstale at 3 °C. The soluble (peripheral) rod enzyme seems to be different from similar enzymes in other tissues. The rod enzyme shows higher affinity for 5′-AMP and 5′-GMP than do soluble enzymes from pigeon heart (Gibson & Drummond, 1972), chicken liver (Naito & Tsushima, 1976), and rat liver (Van Den Berghe et al., 1977) and seems to be different from these enzymes.

Extraction of more enzyme in the dark than in the light suggests that bleached membranes retain peripheral 5′-nucleotidase more strongly than do unbleached membranes. The effect of calcium on blocking the dissociation of enzyme from rod membranes and facilitating enzyme binding to them is consistent with the effectiveness of the calcium chelating agent EGTA for enzyme extraction. The blocking effect of calcium in the dark indicates that calcium can mimic the effect of light. It is therefore possible that the bleaching of rhodopsin may increase the membrane's affinity for soluble 5′-nucleotidase through a mediation of calcium ion. Unbleached membranes probably bind the enzyme loosely because the membranes have lower affinity for calcium ion. This interpretation is consistent with the finding that regeneration of rhodopsin makes the membrane-bound enzyme extractable again with EGTA buffer. Although the state of the rhodopsin chromophore thus regulates extractability of enzyme, it does not play a major role in binding the enzyme to rod membranes. It is Ca^{2+} ion that is essential for enzyme binding both in the dark and in the light. Ca^{2+} may serve as a bridge between the negative charges of the membrane and the enzyme. The specificity of binding will then depend on the surface charge distribution of 5′-nucleotidase. This explains why malate dehydrogenase, which has a similar isoelectric point to that of 5′-nucleotidase but has different surface charge distribution, does not bind to rod membranes at any calcium concentration tested. The binding of cytochrome *c* to the membrane in the absence of added Ca^{2+} and its inhibition by Ca^{2+} suggest that this protein with highly positive charges at neutral pH binds to the membrane and competes with Ca^{2+} for binding sites.

Does rhodopsin provide the binding site of peripheral 5′-nucleotidase? The binding of enzyme to urea-treated membranes supports the hypothesis that rhodopsin is probably the binding protein because other proteins such as rhodopsin kinase (Shichi & Somers, 1978) and GTPase (H. Shichi and R. L. Somers, unpublished observation) are denatured by 5 M urea. However, the possibility of rhodopsin being the binding protein is not supported by the failure of rhodopsin–phosphatidylcholine vesicles to bind the enzyme. It is possible that rhodopsin may require a specific phospholipid for binding of 5′-nucleotidase. Phosphatidylinositol has been suggested to be involved in binding 5′-nucleotidase to the sarcolemma membrane since the enzyme is released by a phosphatidylinositol-specific phospholipase C (Panagia et al., 1981). The nature of the binding site in rod membranes for 5′-nucleotidase remains to be determined.

Rhodopsin kinase is a peripheral enzyme and readily extracted from rod membranes with buffer in the dark (Kühn, 1978; Shichi & Somers, 1978). The extracted enzyme is bound to rod membranes in the light (Kühn, 1978). Another peripheral enzyme, GTPase, is extracted by buffer in the dark and bound to rod membranes upon illumination (Kühn, 1980). Both rhodopsin kinase (Liebman & Pugh, 1979) and GTPase (Wheeler & Bitensky, 1977) have been proposed to be involved in the regulation of cyclic nucleotide phosphodiesterase, another enzyme activated by light (Wheeler & Bitensky, 1977). These enzymes probably form a functional complex in vivo. The light- and Ca^{2+}-regulated reversible binding of 5′-nucleotidase to rod membranes suggests that this enzyme also is a part of the complex on rod membranes. It is then reasonable to conclude that the peripheral 5′-nucleotidase is bound tightly to the membrane in the light and hydrolyzes 5′-GMP, which accumulates quickly on the cytoplasmic surface of the membrane during the light activation of phosphodiesterase.

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3-Bromo-2-ketoglutarate: A Substrate and Affinity Label for Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

Rodney A. Bednar,‡ Fred C. Hartman, and Roberta F. Colman*

ABSTRACT: Pig heart DPN-dependent isocitrate dehydrogenase is progressively inactivated by (*RS*)-3-bromo-2-ketoglutarate at pH 6.15 in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer containing 2 mM $MnSO_4$ and 20% glycerol. With increasing concentrations of bromoketoglutarate, the reaction exhibits rate saturation: the minimum inactivation half-time is 20 min, with a K_{inact} of 6.2 mM. Isocitrate protects against inactivation to an extent consistent with its directly determined binding constant. 2-Ketoglutarate offers no significant protection, but it does not bind, competitively with isocitrate, to free enzyme. Only weak protection against inactivation is seen with the coenzyme DPN. The allosteric activator ADP offers protection against inactivation consistent with its directly determined binding constant. Incubation of bromoketoglutarate with the enzyme results in a loss of allosteric ADP activation at the same rate as inactivation. Equal protection is afforded by isocitrate or ADP against loss of activity and loss of ADP activation, making it likely that both processes result from the same molecular event. The measured loss of ADP activation must result from a change in the influence of ADP on the catalytic activity of unmodified subunits, suggesting that there is interaction between modified and unmodified subunits. The inactivation correlates with the covalent incorporation of approximately 0.83 mol of reagent/mol of average subunit, implying that three to four of the structurally distinct subunits of the enzyme tetramer must be modified for complete loss of activity. One-half of (*RS*)-3-bromo-2-ketoglutarate is also a substrate for isocitrate dehydrogenase with a K_m of 5 mM. The product is probably 2-hydroxy-2-bromoglutarate, since there is oxidation of DPNH, no requirement for CO_2, and no release of Br^-. The agreement of K_m and K_{inact} for bromoketoglutarate suggests that the active site is the target of modification. These results indicate that 3-bromo-2-ketoglutarate functions as an affinity label of the substrate binding site of the DPN-dependent isocitrate dehydrogenase.

A continuing series of investigations have been directed at the identification of residues that are involved in the active site of pig heart DPN-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] by chemical modification. By the use of a variety of "group-specific" reagents, several amino acid residues have been determined to be critical to the function of isocitrate dehydrogenase: lysyl (Shen & Colman, 1975; Hayman & Colman, 1977), cysteinyl (Mauck & Colman, 1976), argininyl (Hayman & Colman, 1978), and glutamyl or aspartyl residues (Ramachandran & Colman, 1977).

Results of studies with group-specific reagents are frequently difficult to interpret because of reaction with more than one amino acid residue. Affinity labeling offers a viable solution to this problem. An affinity label, or an active-site-directed reagent, is structurally analogous to the substrate (and, hence, can bind to the active site) but has in addition a functional group capable of reacting irreversibly with an amino acid

† From the Department of Chemistry (R.A.B. and R.F.C.), University of Delaware, Newark, Delaware 19711, and the Biology Division (F.C.H.), Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received January 13, 1982.* This work was supported by the Pierre W. Hoge Research Foundation of the American Heart Association of Delaware, Inc., U.S. Public Health Service Grant 2-R01-AM17552 (to R.F.C.), and U.S. Department of Energy Contract W-7405-eng-26, UCND (to F.C.H.).

‡ Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, MA 02254.

0006-2960/82/0421-3681$01.25/0 © 1982 American Chemical Society