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Solubilization and Conversion of Hepatic Adenylate Cyclase to a Form Requiring MnATP as Substrate

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A general feature of membrane-bound adenylate cyclase systems is the "lability" of the basal enzyme to dispersion by detergents. A stable form of the detergent-solubilized enzyme is obtained only if the membrane-bound enzyme is first pretreated with fluoride or Gpp(NH)p. However, we have found with the basal hepatic enzyme that the lability is evident primarily when MgATP is used as substrate; substitution of MnATP for MgATP reveals that substantial basal activity survives detergent treatment. This effect is independent of the detergent; it is seen with either Lubrol PX or with deoxycholate. In addition to the altered substrate requirement, the membrane-bound and solubilized forms of the basal enzyme exhibit other differences. In contrast to the membrane-bound form, the solubilized enzyme shows 1) weak stimulation by Gpp(NH)p; 2) little inhibition by adenosine, 3) strong inhibition by P_i or PP_i, and 4) an apparent loss of the Me^{2+}-reactive regulatory site. Such dissimilarities between membrane-bound and solubilized cyclase are not seen if the membranes are pretreated with Gpp(NH)p prior to exposure to detergents. The characteristics of the solubilized basal hepatic enzyme are similar to those of the naturally occurring soluble adenylate cyclase found in mature rat testes. It would appear that separation of adenylate cyclase from components that confer regulation by divalent cation and guanine nucleotides produces a form of the enzyme that will turnover only MnATP; this may represent the free catalytic moiety. Such preparations could be useful in reconstructing some of the regulatory functions of adenylate cyclase seen in its membrane-bound form.

Key words: adenylate cyclase, liver, solubilized, MnATP, MgATP

A long-standing problem in purifying adenylate cyclase and resolving components of the system is the marked apparent lability of the enzyme, particularly upon solubilization with detergents [1]. This property of the membrane-bound form of adenylate cyclase is in contrast to the freely soluble cyclase found in testis [2] which, in the course of purification we have found to be remarkably stable [C. Londos, unpublished]. Unlike the membrane-bound form found in most tissues, the soluble testicular enzyme is not regulated by such agents as hormones, fluoride [2], and guanine nucleotides [3]. Indeed, the testicu-

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lar enzyme uses MnATP as substrate [2], whereas membrane-bound forms use either MgATP or MnATP [1]. It occurred to us that the marked lability of the membrane enzyme may be related not to alterations in stability upon solubilization but rather to the conversion of the enzyme to a form resembling the testicular species, ie, to a form not regulated by guanine nucleotide, fluoride and which prefers MnATP as substrate.

In this report we demonstrate that so long as the liver enzyme is not pretreated with Gpp(NH)p[1] prior to solubilization, detergent treatment produces an enzyme which, like the testicular enzyme, has much higher activity with MnATP than MgATP. Moreover, the activities with MnATP in membrane bound and solubilized preparations differ kinetically, and the latter assumes some properties in common with the naturally soluble testicular enzyme.

METHODS

Rat liver membranes were isolated by the method of Neville [4], as described previously [5], and stored under liquid nitrogen. Adenylate cyclase activity was assayed by the method of Salomon et al [6] with the use of (α-^{32}P)ATP (ICN). Activity was assayed at 30°C in the presence of 0.1 mM ATP, 0.05 mM cyclic AMP, 1 mM dithiothreitol, 30 mM Tris·HCl, pH 7.4, and either 10 mM $MgCl_2$ or 1 mM $MnCl_2$.

The freely soluble adenylate cyclase from rat testis was partially purified to a specific activity of approximately 1 μmol cyclic AMP per mg prot per min at 30°C in the presence of 10 mM $MnCl_2$ and 1 mM ATP [C. Londos and T. Shinozawa, manuscript in preparation].

Solubilization and chromatography procedures are described in the legends to the figure and tables. Sodium deoxycholate (Sigma) was recrystallized from acetone:H_2O (4:1) before use.

All experiments were performed at least twice with different liver membrane preparations, and data from representative experiments are shown.

RESULTS

Lubrol Solubilization and Column Chromatography

Figure 1 (top) compares chromatographed activities with MnATP and MgATP of Lubrol-solubilized adenylate cyclase from rat liver membranes not treated with Gpp(NH)p. With MgATP as substrate three small peaks of activity were observed, one in the void volume and two included. Two peaks of activity (I and II) were seen with MnATP, both of which gave considerable more activity with Mn^{2+}than with Mg^{2+}. Throughout peaks I and II, MnATP/MgATP activity ratios were 5 to 7, whereas with liver membranes assayed under identical conditions the ratio was approximately 2. Thus, in contrast to liver membranes, the bulk of Lubrol-solubilized, untreated cyclase showed a marked preference for MnATP over MgATP as substrate. Peak III was an exception in that activity was seen only with MgATP.

The differences between MnATP and MgATP were minimal when membranes were pretreated with Gpp(NH)p and glucagon prior to solubilization (Fig. 1, bottom), and the bulk of the activity appeared in peak II with either substrate (note the different scales on the ordinates of Fig. 1).

[1] The abbreviation used is Gpp(NH)p, guanyl-5′-yl-imidodiphosphate.

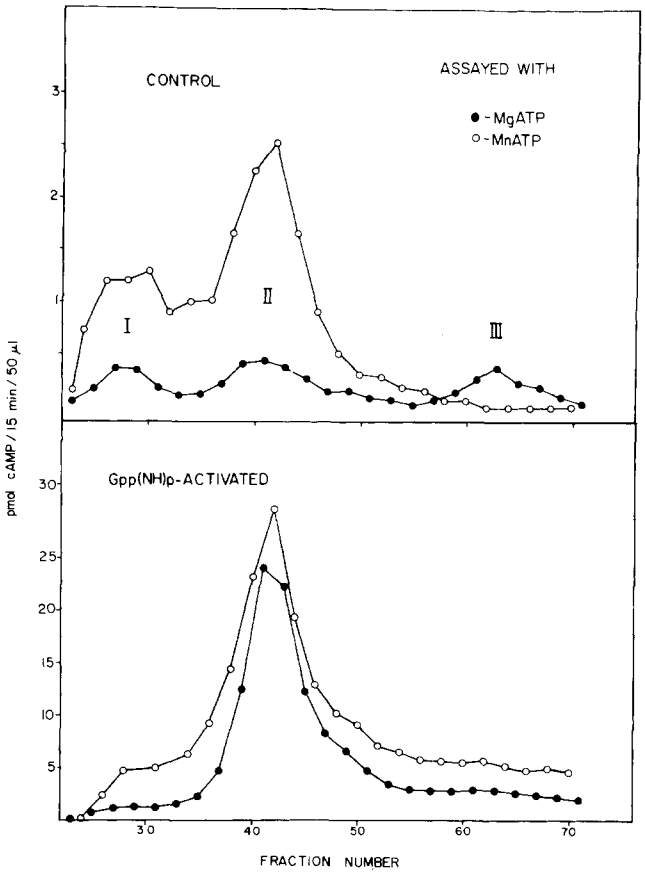

Fig. 1. Activity with MnATP and MgATP of liver membranes solubilized before and after treatment with Gpp(NH)p and glucagon. Liver membranes were solubilized and chromatographed at 0 to 4°C essentially as described by Welton et al [7]. Membranes, at 10 mg/ml protein, were mixed for 50 sec with 1% Lubrol PX (ICI) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 25% sucrose. The mixture was centrifuged for 50 min at 49,500 × g, and 7 ml of the supernate were applied to a column (2.6 × 42 cm) of Ultrogel AcA22 and eluted with the solubilization buffer containing 0.01% Lubrol PX. The flow rate was 7 ml/h and 3 ml fractions were collected. The void volume emerged at fraction 25, and is designated as Peak I. Peak II includes fractions 37 through 48, and Peak III includes fractions 58 through 66. The top panel shows material not treated prior to solubilization. The bottom panel represents membranes treated with 10 µM Gpp(NH)p and 0.1 µM glucagon prior to solubilization, as described previously [7, 16]. The distribution of protein has been published previously for numerous experiments performed under identical chromatographic conditions [7, 16]. In general, the adenylate cyclase activity in Peak II is purified three- to fourfold with respect to the material applied to the column.

The activities of all fractions in Fig. 1 were tested also in the presence of Gpp(NH)p added to the assay medium (not shown in Fig. 1). The enzyme from pretreated membranes (Fig. 1, bottom) showed a slight increase (< 10%) with the added nucleotide analog. Gpp(NH)p increased activity of the untreated material (Peak II, top panel) to only 3.5 pmol/50 µl/10 min with MgATP as substrate, and to only 4.7 pmol/50 µl/10 min with MnATP as substrate. A comparison of these activitiess with those from corresponding fractions of the pretreated enzyme (bottom panel, Fig. 1; 25 to 30 pmol/50 µl/10 min) shows that the bulk of the enzyme had lost the capacity to respond to the analog.

Stability of Solubilized Enzyme

The activity in the fractions from Fig. 1 (top) were stable for at least two months when frozen and then assayed with MnATP; the same fractions when assayed with MgATP showed a steady decline in activity to a point where the MnATP/MgATP ratios were 10 to 20. The activity in the fractions from Fig. 1 (bottom) were stable for two months when frozen and then assayed with either MgATP or MnATP.

Solubilization With Deoxycholate

It was of interest to determine whether cyclase activity measured with MnATP would survive solubilization with ionic detergents which are generally thought to destroy the enzyme [1]. As seen in Table I, the enzyme activity with MnATP was approximately six-fold greater than with MgATP, a finding similar to that with Lubrol solubilization (Fig. 1, top). Also, 50% of the activity with MnATP was recovered upon solubilization with deoxycholate, whereas only 10% of the MgATP activity survived the detergent treatment. Sucrose during solubilization [7] and subsequent dialysis to remove deoxycholate were necessary in order to detect activity in the deoxycholate-solubilized liver membranes. Thus, the increased ratio of MnATP to MgATP activity was independent of the type of detergent used to solubilize membranes. As with the Lubrol-solubilized preparation, the deoxycholate-solubilized enzyme exhibited a markedly reduced response to Gpp(NH)p. The deoxycholate-solubilized enzyme was stable at 4°C in the presence of detergent for up to 42 h under solubilization conditions described in the legend to Table I. Thus, a fraction of activity (50%) was lost immediately upon solubilization and the remaining activity was not affected by the detergent.

Kinetic Properties of Membrane-Bound and Lubrol-Solubilized Hepatic and Naturally Soluble Testicular Cyclases

We next asked whether there were kinetic differences between the membrane-bound and solubilized enzymes. One test applied was to assess the effects of combinations of Mn^{2+} and Mg^{2+} (Table II). It was shown previously [8] that the liver cyclase contains a

TABLE I. Solubilization of Liver Cyclase With Deoxycholate

		Adenylate cyclase			
		10 mM Mg^{2+}		1 mM Mn^{2+}	
Enzyme	Dialysis	pmol/mg prot/10 min	Recovery(%)	pmol/mg prot/10 min	Recovery(%)
Control membranes	+	27.8	100	33.0	100
Deoxycholate supernate	–	0.4	1.6	4.4	13.3
Deoxycholate supernate	+	2.2	7.9	13.3	40.3
Deoxycholate pellet	+	25.0	18.5	25.0	15.1

Liver membranes, at 1 mg/ml protein, were solubilized with 0.36% sodium deoxycholate at 0°C in buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, and 25% sucrose. After incubation for 10 min the material was centrifuged for 30 min at 49,000 × g, and the supernate was removed and either assayed immediately or dialyzed overnight against 500 volumes of the solubilization buffer without deoxycholate. The pellets and control membranes were dialyzed also. Ninety percent of the membrane protein was solubilized. All assays were performed with approximately 40 μg protein in the presence either of Mg^{2+} or Mn^{2+} as indicated. Note that of the activity not found in the pellet, approximately 10% was in the supernate with MgATP as substrate, but approximately 50% was in the supernate with MnATP as substrate.

TABLE II. Loss of Activation by Cations Upon Solubilization With Lubrol PX

Cations	Liver membranes		Lubrol-solubilized	
	pmol/mg prot/10 min	Relative activity	pmol/mg prot/10 min	Relative activity
10 mM Mg^{2+}	35.6 ± 1.6	1.0	19.8 ± 0.8	1.0
1 mM Mn^{2+}	80.4 ± 5.2	2.3	177.1 ± 12.6	8.9
10 mM Mg^{2+} + 0.2 mM Mn^{2+}	64.0 ± 3.2	1.8	20.0 ± 1.1	1.0
10 mM Mg^{2+} + 1 mM Mn^{2+}	92.2 ± 5.7	2.6	110.5 ± 5.1	5.1

Values for specific activities are the means of triplicate determinations ± standard deviations. Relative values are expressed as multiples of the activities with 10 mM Mg^{2+} alone. Membranes were assayed with 25 μg membrane protein, and Lubrol-solubilized activity with 19 μg protein from Peak II material from Fig. 1 (top).

cation site for activation which has an apparent affinity for Mn^{2+} far greater than for Mg^{2+}; this site is distinct from the catalytic site which exhibits an equivalent apparent K_m for MnATP and MgATP. A consequence of the difference of affinities for the two cations is that submillimolar concentrations of Mn^{2+} activate when combined with millimolar concentrations of Mg^{2+} [8]. Thus, a simple test for the presence of an activating metal site distinct from the catalytic site is the addition of a low concentration of Mn^{2+} (0.2 mM) to an assay medium containing high amounts of Mg^{2+} (10 mM). As can be seen from Table II, such an addition caused a stimulation of the membrane-bound but not the soluble form of the enzyme, indicating that the cation site for activation had been weakened or abolished upon solubilization.

The second feature of concern is the ability of the catalytic site of the membrane-bound and soluble forms of the enzymes to tolerate MgATP or MnATP as substrate. When assays were carried out with 1 mM Mn^{2+} as the only metal ion, the activity was 2.3-fold greater than with magnesium as the only cation (Table II); this, again, reflects activation by Mn^{2+} at the cation site. However, the much greater activity with manganese alone compared to magnesium alone (8.9-fold, Table II) with the solubilized enzyme reflects the preferences of this form for MnATP versus MgATP. This is substantiated further by assays with combinations of cations. With 10 mM Mg^{2+} plus 1 mM Mn^{2+} there was no reduction of the membrane-bound activity relative to that seen with 1 mM Mn^{2+} (Table II), but the activity of the soluble form was reduced (177 to 110) by the addition of magnesium with the manganese. The latter merely reflects the fact that only 35% of the ATP is in the form of MnATP with this combination of cations, the remainder being MgATP which is poorly tolerated by the soluble enzyme. These data indicate that solubilization causes 1) a loss or weakening of the cation site for activation, and 2) a conversion of the enzyme to a form which utilizes MnATP preferentially as the substrate.

Since adenosine inhibits the liver cyclase by a process somehow linked to the activation by cations [9], we examined the effects of adenosine on the solubilized preparations. The nucleoside was equally inhibitory toward the membrane-bound enzyme with either MgATP or MnATP as substrate, but was much less inhibitory toward the Lubrol-solubilized form (Table III). In separate experiments we found that 2′,5′-dideoxyadenosine, a potent inhibitor of the hepatic cyclase [9], was a weak inhibitor of both the Lubrol-solubilized hepatic enzyme and the naturally soluble testicular enzyme.

Table III also shows that the Lubrol-solubilized hepatic and the testicular cyclase were considerably more susceptible to inhibition by pyrophosphate than was the membrane-

TABLE III. Inhibition of Adenylate Cyclase Activities by Adenosine and Pyrophosphate

	Percent adenylate cyclase activity			
	Liver membranes		Lubrol-solubilized liver membranes	Testicular
Inhibitor	Mg^{2+}	Mn^{2+}	Mn^{2+}	Mn^{2+}
Adenosine				
0.1 mM	72	67	85	
0.2	61	55	83	
0.4	52	50	81	
1.0	46	39	75	
Na Pyrophosphate				
0.1 mM	98	95	86	66
0.2	92	96	63	65
0.4	71	82	29	38
1.0	50	59	3	12

The various enzyme preparations were assayed with either 10 mM Mg^{2+} or 1 mM Mn^{2+}, as indicated. The specific activity (pmol cyclic AMP/mg prot/15 min) in the absence of inhibitors was 52 for liver membranes with Mg^{2+} and 110 with Mn^{2+}, and 168 for the Lubrol-solubilized enzyme (Fig. 1, top, Peak II) in the presence of Mn^{2+}. The specific activity of the testicular enzyme was 2.6 μM cyclic AMP/mg prot/15 min, a relatively low activity for this preparation due to the suboptimal Mn^{2+} and ATP concentrations employed in this experiment.

bound enzyme tested with MgATP or MnATP. Similar observations were made with phosphate as the inhibitor (data not shown). These studies emphasize the differences between membrane-bound and soluble liver cyclase and suggest that the latter assumes properties in common with the naturally soluble testicular enzyme. However, there were some differences between the solubilized liver and testicular enzymes. The apparent K_m for MnATP was 50 μM with the Lubrol solubilized preparation, a value identical to that seen for the membrane bound enzyme with this substrate [8], but the K_m with the testicular enzyme was tenfold greater, 500 μM. Also, activity increased as the Mn^{2+} was varied from one to 10 mM with the testicular enzyme, but concentrations higher than one mM inhibited the Lubrol-solubilized preparation.

DISCUSSION

Although Pilkis and Johnson [10] noted that solubilized, as opposed to membrane-bound, hepatic adenylate cyclase exhibits higher activity with Mn^{2+} than with Mg^{2+}, these authors did not provide an explanation for this phenomenon. This study shows that the catalytic component of the hepatic adenylate system displays markedly different properties when it is solubilized with either Lubrol or with ionic detergents. These differences may be summarized as follows. The solubilized enzyme i) is less susceptible to activation by Gpp(NH)p, ii) shows no regulation by divalent cations, iii) is relatively insensitive to inhibition by adenosine which previous studies [9] have shown is functionally linked to regulation by divalent cations, and iv) preferentially utilizes MnATP as substrate. The membrane-bound adenylate cyclases from adrenal [11] and testis [12] undergo similar changes upon solubilization with detergents. Thus, it is tempting to speculate that solubilization of membrane-bound adenylate cyclases results in removal of regulatory components necessary for activation by guanine nucleotides, fluoride, and divalent cations and concomitant conversion of the enzyme to a form that utilizes MnATP preferentially as sub-

strate. In accord with this hypothesis is the report that a variant of SV49 lymphoma cells, designated AC [13], contains in its plasma membrane a form of adenylate cyclase that requires MnATP for activity [14] and which is not susceptible to activation by fluoride ion, guanine nucleotides, and magnesium ions. Moreover, it has been shown recently that the regulatory properties can be restored by combined material extracted from the parental lymphoma cell with the catalytic component of the AC⁻ variant [14, 15]. The notion that the altered characteristics of the hepatic cyclase upon detergent solubilization result from separation of regulatory components is strengthened by preliminary experiments in which we have found that combinations of fractions obtained from chromatography of deoxycholate-treated membranes results in partial restoration of the ability of Gpp(NH)p to activate the enzyme.

We have reported previously [7] that solubilization of hepatic adenylate cyclase with the nonionic detergent, Lubrol, leads to the formation of "aggregates" of proteins, lipids, and detergents and these aggregates include adenylate cyclase as well as other enzymes. For this reason we feel that the enzyme cannot be purified using nonionic detergents. Provided that MnATP is used as substrate it now seems possible to employ "harsher" detergents such as deoxycholate which have a greater dispersing power than the nonionic detergents and may therefore yield preparations of adenylate cyclase that can be purified.

Finally, it was observed in this study that the soluble adenylate cyclase from the testis has some of the properties of the detergent-solubilized hepatic enzyme. Both enzymes are dependent on MnATP for activity and are markedly inhibited by pyrophosphate and appear not to be inhibited by adenosine. It remains to be seen whether both enzymes have similar physical properties.

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REFERENCES

1. Perkins JP: Adv Cyclic Nucleotide Res 3:1, 1973.
2. Braun T, Dods RF: Proc Natl Acad Sci USA 72:1097, 1975.
3. Londos C, Rodbell M: In Roberts GCK (ed): "Drug Action at the Molecular Level." London: Macmillan Press, p 235, 1977.
4. Neville DR: Biochim Biophys Acta 154:540, 1968.
5. Pohl SL, Birnbaumer L, Rodbell M: J Biol Chem 246:1849, 1971.
6. Salomon Y, Londos C, Rodbell M: Anal Biochem 58:451, 1974.
7. Welton AF, Lad PM, Newby AC, Yamamura H, Nicosia S, Rodbell M: Biochim Biophys Acta 522:625, 1978.
8. Londos C, Preston MS: J Biol Chem 252:5957, 1977.
9. Londos C, Preston MS: J Biol Chem 252:5951, 1977.
10. Pilkis SJ, Johnson RA: Biochim Biophys Acta 341:388, 1974.
11. Mahaffee DD, Ontjes DA: J Cyclic Nucl Res 3:325, 1977.
12. Neer EJ: J Biol Chem, in press.
13. Bourne HR, Coffino P, Tomkins GM: Science 187:750, 1975.
14. Ross EM, Gilman AG: J Supramol Struct Suppl 2:266, 1978.
15. Ross EM, Gilman AG: J Biol Chem 252:6966, 1977.
16. Welton AF, Lad PM, Newby AC, Yamamura H, Nicosia S, Rodbell M: J Biol Chem 252:5947, 1977.