ADENYL CYCLASE

The Extent of Reversibility of the Reaction

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Received April 5, 1971

SUMMARY

The reversibility of the reaction that catalyzes the formation of cyclic AMP from ATP was studied using adenyl cyclase of *Brevibacterium liquefaciens*, which had been purified free from cyclic 3',5'-nucleotide phosphodiesterase, adenosine triphosphatase and inorganic pyrophosphatase. After a prolonged incubation, labeled ATP was essentially converted to cyclic AMP. Conversely, labeled cyclic AMP and inorganic pyrophosphate did not give rise to detectable amounts of labeled ATP, even in the presence of non-labeled ATP. These results indicate that the equilibrium of the reaction favors the formation of cyclic AMP from ATP and that the reaction is essentially not reversible.

INTRODUCTION

Adenyl cyclase catalyzes the formation of cyclic AMP from ATP and the byproduct of the reaction is inorganic pyrophosphate as depicted below:

$$\text{ATP} \xrightarrow{\text{Adenyl Cyclase}} \text{Cyclic AMP} + \text{PPi}$$

Using a purified adenyl cyclase from *B. liquefaciens*, Greengard, Hayaishi and Colowick (1) found that the enzyme catalyzed the synthesis of ATP from cyclic AMP and PPi and concluded that the reaction was readily reversible. In contrast, Tao and Lipmann (2) were unable to demonstrate reversibility with a purified adenyl cyclase preparation from *Escherichia coli*, nor could Rosen and Rosen (3) detect reversibility using purified adenyl cyclase from frog erythrocytes. However, the presence of ATPase or pyrophosphatase or both in these two preparations did not allow definitive conclusions. The apparent discrepancy concerning the reversibility of adenyl cyclase prompted us to re-examine this problem. The enzyme from *B. liquefaciens* was chosen because the purified preparation has been shown to be relatively free from interfering enzymes (4). The present investigation did not support the findings of Greengard *et al.* (1) that the reaction catalyzed by adenyl cyclase was readily reversible. The purpose of this note is to present evidence demonstrating the essential irreversibility of the reaction.

MATERIALS AND METHODS

Purification of Adenyl Cyclase

B. liquefaciens were grown and harvested according to Hirata and Hayaishi (4). Subsequent procedures were modified after them and were performed at 0-4°.

1. Cell-free extract - Washed cells, 100 gm, were suspended in 300 ml of 0.1 M Tris-HCl (pH 8.3) and were disrupted in a Sonifier Cell Disrupter for 20 min. Unbroken cells were collected by

centrifugation for 20 min at 8000 x *g*, resuspended in 300 ml of the same buffer, sonicated and centrifuged as before. The supernatant solutions from the centrifugation were combined and diluted to 1000 ml with the Tris-HCl buffer. B-mercaptoethanol was added to a final concentration of 1mM, and the solution was centrifuged for 50 min at 40,000 x *g* to remove turbidity.

2. Streptomycin sulfate treatment - The supernatant fluid was adjusted to pH 6.5 with 1 M HCl, and 65 ml of a 5% streptomycin sulfate solution was added slowly with stirring. After 60 min, the precipitate was removed by centrifugation, and the supernatant fluid was adjusted to pH 8.3 with 1 M KOH.

3. Ammonium sulfate fractionation - Solid $(NH_4)_2SO_4$ was added slowly to the supernatant fluid to 33% saturation. After 30 min, the resulting turbidity was removed by centrifugation. More $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation. The 33 to 50% sediment was collected, dissolved in 40 ml of 0.1 M Tris-HCl, pH 7.0, and dialyzed overnight against the same buffer.

4. DEAE Sephadex column chromatography - The dialyzed fraction was applied to a DEAE Sephadex (A-50) column (1.5 cm x 75 cm) which had been equilibrated with 0.1 M NaCl in 0.1 M Tris-HCl (pH 8.3). The enzyme was eluted with 0.16 M NaCl in 0.1 M Tris-Cl (pH 8.3). The fractions with the highest activity were pooled, and solid $(NH_4)_2SO_4$ was added to 50% saturation. The precipitate was dissolved in 0.1 M Tris-HCl (pH 8.3) and dialyzed against the same buffer.

5. Sephadex G-100 chromatography - The dialyzed fraction was applied to a Sephadex G-100 column (1.5 cm x 28 cm) which had been equilibrated with 30 mM NaCl in 0.1 M Tris-HCl (pH 8.3). The enzyme was eluted with the same buffer. Peak activity tubes were pooled, and the solution was concentrated under negative pressure with nitrocellulose membrane filter (Sartorius Division, Brinkmann Instruments, Inc.) to approximately 0.5 mg protein per ml. The concentrated sample was stored in small aliquots in dry ice until use.

Assay of Adenyl Cyclase

The standard reaction mixture of 0.1 ml contained 100 mM Tris-HCl (pH 9.0), 5 mM sodium pyruvate, 1 mM dithiothreitol, 10 mM $MgSO_4$, 2 mM ATP-8-C^{14} (85 mμCi/μmole), and about 10 μg purified enzyme. The reaction was incubated at 30° for 30 min, and was arrested in a boiling water bath for 2 min. Carrier ATP and cyclic AMP were added and an aliquot of the reaction mixture was spotted on Whatman No. 1 paper for descending chromatography. The solvent system contained 95% ethanol and 1 M ammonium acetate (75:30 v/v). This system clearly separated ATP from cyclic AMP. The nucleotides were located on paper by ultraviolet illumination, the spots were removed and counted in a liquid scintillation spectrometer. The counting fluid contained 3 g PPO and 100 mg POPOP per 1 liter of toluene.

Assay of Other Enzymes

Cyclic 3',5'-nucleotide phosphodiestrease was determined as previously described (5). The activity of adenosine triphosphatase and pyrophosphatase was followed by the release of inorganic phosphate according to Hirata and Hayaishi (4).

Determination of Protein

Protein was determined according to Gornall *et al.* (6) or Warburg and Christian (7).

Chemicals

ATP-8-C^{14}, cyclic AMP-8-C^{14}, and cyclic AMP-8-H^3 were purchased from Schwartz BioResearch, and were used without further purification.

RESULTS AND DISCUSSION

A soluble adenyl cyclase was purified 150-200 fold from *B. liquefaciens*. Table I summarizes

TABLE I

PURIFICATION OF ADENYL CYCLASE AND THE DISTRIBUTION OF PHOSPHODIESTERASE, ADENOSINE TRIPHOSPHATASE AND INORGANIC PYROPHOSPHATASE IN THE DIFFERENT FRACTIONS

Fraction	Protein (mg)	Adenyl Cyclase	ATPase	PDE	PPase
			(nmoles/mg protein/min)		
40,000 g supernatant of cell-free extract	4252	1.2	3	1.1	3.8
Streptomycin treatment	4147	1.4	0.7	0.06	0.22
Ammonium sulfate fractionation	258.6	10.4	0.7	0.06	0.04
DEAE-Sephadex (A-50)	8.8	132	0	0	0.03
Sephadex G-100	3	200	0	0	0

One hundred g of washed cells were used for the purification of adenyl cyclase as described in the text. An aliquot of each fraction was assayed for adenyl cyclase, phosphodiesterase (PDE), adenosine triphosphatase (ATPase), and inorganic pyrophosphatase (PPase). No PDE, ATPase, nor PPase was detected in the purified adenyl cyclase preparation when assayed with 100 μg protein after incubation at 30° for 60 min.

the purification of adenyl cyclase and presents the distribution of cyclic 3',5'-nucleotide phosphodiesterase, adenosine triphosphatase, and inorganic pyrophosphatase in the different fractions. Note that the purified enzyme was free from these interfering enzymes.

Table II shows the requirement of the adenyl cyclase reaction. Two reagents were critical for adenyl cyclase activity. Mg^{++} was an absolute requirement; no activity was observed when the divalent cation was omitted. Pyruvate stimulated the enzyme pronouncedly, but only in the presence of Mg^{++}. Dithiothreitol increased the activity slightly. Interestingly, F^- ion, which usually stimulated adenyl cyclase of mammalian tissues (9), inhibited the bacterial enzyme slightly (not shown).

The product of the adenyl cyclase reaction with ATP as a substrate was identified by its chromatographic behavior in five solvent systems: (1) 95% ethanol:1 M ammonium acetate (75:30 v/v), pH 7.5; (2) n-butanol:water (86:14 v/v), pH 7.2; (3) isopropanol:conc NH_4OH:water (70:10:20 v/v), pH 12; (4) water:conc NH_4OH (150:0.3 v/v), pH 10; and (5) isopropanol:conc NH_4OH:0.1 M boric acid (7:1:2 v/v), pH 11. In all these solvent systems the product was

TABLE II

REACTION REQUIREMENTS FOR ADENYL CYCLASE OF *BREVIBACTERIUM LIQUEFACIENS*

Reaction Mixture	Activity (nmoles/mg/min)
Complete	188
-Mg++	0
-pyruvate	5.2
-dithiothreitol	168
-dithiothreitol and pyruvate	7.3

The activity was assayed as described in the text, except for the omission of reagents as indicated. The data have been corrected for a control containing a boiled enzyme and all other reagents.

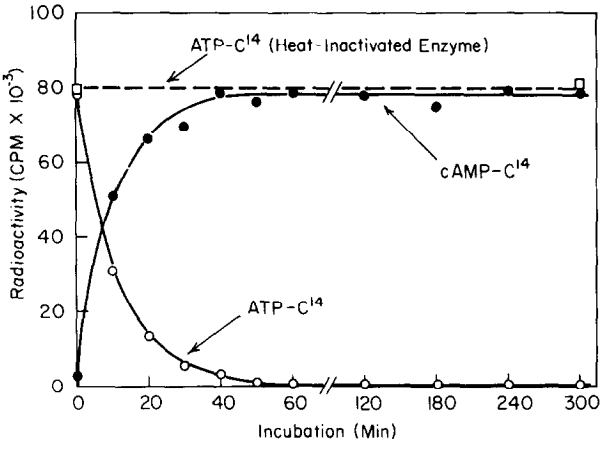

Figure 1. The reaction mixture contained 100 mM Tris-Cl, pH 9.0, 10 mM Mg++, 1 mM dithiothreitol, 5 mM pyruvate, 2 μM ATP-C^{14} (150 μCi/mmole), and 116 μg purified enzyme, in a final volume of 1.0 ml. The incubation was performed at 30°. At times indicated, 100 μl of the incubation mixture was transferred to a tube in boiling water bath. After 2 min in the water bath, the tubes were removed, cooled, and carrier ATP and cyclic AMP added. An aliquot of the reaction mixture was then put on Whatman paper for descending chromatography as described in the text.

indistinguishable from an authentic sample of cyclic AMP. Further, upon incubation with a phosphodiesterase highly purified from *Serratia marcescens* (8) the product was converted to 5'-AMP.

Figure 1 shows the disappearance of isotope ATP and the concurrent formation of cyclic AMP as a function of time. Note that the curve depicting the formation of cyclic AMP is the mirror image of that depicting the disappearance of ATP. When ATP was exhausted at 60 min, cyclic AMP had reached its maximal level. The amount of isotope associated with cyclic AMP remained constant even after 5 hrs. The control experiment in which the enzyme was heat inactivated prior to assay showed that at the end of the incubation all the isotope remained with ATP. These data suggest that the equilibrium favors the synthesis of cyclic AMP and that the reaction is essentially not reversible.

To further establish this point, we used labeled cyclic AMP and inorganic pyrophosphate as reactants, Table III. At the end of a 60-min incubation, no radioactivity was detected in the ATP spot, and all the isotope remained with cyclic AMP (Tube A). To exclude the possibility that newly

TABLE III

EXPERIMENTS TO EXAMINE POSSIBLE REVERSAL OF ADENYL CYCLASE OF *BREVIBACTERIUM LIQUEFACIENS*

Tube	Additions (mM)	Radioactivity (cpm)		Recovery of
		ATP	Cyclic AMP	Radioactivity
A	cAMP-H^3 (10), PPi (10)	0	46,036	100
B	cAMP-H^3 (10), PPi (15), ATP (5)	0	41,533	90
C	cAMP-H^3 (15), PPi (10), ATP (10)	0	46,136	100
D	cAMP-H^3 (10), PPi (10), ATP (10)	13	43,715	94

The reaction mixture of 0.1 ml contained 50 mM Tris-Cl, pH 7.3, 10 mM Mg^{++}, 3 mM F^-, 5 mM pyruvate, 7 μg purified enzyme, 10 mM or 15 mM cyclic AMP-H^3 (55,300 cpm/tube) and other additions as indicated in the table. At the end of 60 min, the reaction was terminated by heating in a boiling water bath. Carrier ATP and cyclic AMP were added and an aliquot of the reaction mixture was put on Whatman paper for descending chromatography as described in the text. The enzyme was the same preparation used in Figure 1, and it catalyzed the formation of cyclic AMP from ATP at a rate of 235 nmoles/mg protein/min under the standard condition.

synthesized ATP might be metabolized via unknown secondary reactions and hence might have escaped detection, we added non-labeled ATP to the reaction mixture of Tubes B, C, and D prior to assay. The addition of non-labeled ATP served two purposes: first, to protect any newly synthesized radioactive ATP from possible degradation via unknown secondary reactions; and second, and more important, if the reaction was reversible, to allow isotope exchange between the non-labeled ATP and the newly synthesized ATP. If the reaction were reversible, exchange of isotope would appear likely. The results in these tubes showed that even in the presence of added non-labeled ATP, no radioactive ATP was detected, confirming our earlier conclusion that the reaction was virtually irreversible.

It is important to note that whereas the tissue levels of cyclic AMP and PPi are generally in the range of 10^{-7} M (9) and 10^{-5} M (10), respectively, and are much lower than the concentrations used in Table III, the tissue concentration of ATP is in the millimolar range. It thus appears very unlikely that a reversal of the cyclase reaction takes place to any significant extent under physiological conditions.

The condition in Tube A of Table III was identical to that used by Greengard *et al.* (1), as described by Greengard and Kuo (11). The only difference between their work and ours was the technique with which ATP was assayed. Greengard *et al.* (1) used the firely luminescence technique and we used an isotope assya. It is interesting to note that Tao and Lipmann (2) and Rosen and Rosen (3) also used radioisotope assays and could not detect reversibility of the reaction.

In summary, two lines of evidence indicated that the adenyl cyclase reaction was not readily reversible. First, the conversion of ATP to cyclic AMP was virtually quantitative, and secondly, no labeled ATP was produced from labeled cyclic AMP and PPi even in the presence of added non-labeled ATP. Therefore, it is concluded that the adenyl cyclase reaction is essentially not reversible.

The question of reversibility of the adenyl cyclase is potentially important in considering the possible modes of action of cyclic AMP. The observed reversibility of the reaction has led Greengard *et al.* (1,11) to postulate that cyclic AMP may be a thermodynamically reactive compound, and that it may serve as a protein adenylylating agent. There is as yet no evidence that cyclic AMP adenylylates proteins. On the other hand, the widespread occurrence of proteins with a specific site for the reversible binding of cyclic AMP (12-19) strongly suggests that the nucleotide functions as a versatile allosteric effector (12).

ACKNOWLEDGEMENT

This work was supported by NS-08059 and CA-08480 from the U.S. Public Health Service, by ALSAC, and by a grant-in-aid from Eli Lilly and Company.

REFERENCES

1. Greengard, P., Hayaishi, O., and Colowick, S.P., Fed. Proc., 28:467, 1969.
2. Tao, M. and Lipmann, F., Proc. Nat. Acad. Sci., 63:86, 1969.
3. Rosen, O.M. and Rosen, S.M., Arch. Biochem. Biophys., 131:449, 1969.
4. Hirata, M. and Hayaishi, O., Biochim. Biophys. Acta, 149:1, 1967.
5. Cheung, W.Y., Biochim. Biophys. Acta, 191:303, 1969.
6. Gornall, A.G., Bardawill, G.S., and David, M.M., J. Biol. Chem., 177:751, 1949.
7. Warburg, O. and Christian, W., Biochem. Z., 210:384, 1941.
8. Okabayashi, T. and Ide, M., Biochim. Biophys. Acta, 220:116, 1970.
9. Robison, G.A., Butcher, R.W., and Sutherland, E.W., Ann. Rev. Biochem., 37:149, 1968.
10. Floguard, H., FEBS Letters, 2:209, 1969.

11. Greengard, P. and Kuo, J.F., In "Role of Cyclic AMP in Cell Function," (ed. P. Greengard and E. Costa) Raven Press, New York, p. 288, 1970.
12. Cheung, W. Y., Life Sciences, Part II, 9:861, 1970.
13. Gill, G.N. and Garren, L.D., Biochem. Biophys. Res. Commun., 39:335, 1970.
14. Solomon, Y. and Schramm, M., Biochem. Biophys. Res. Commun., 38:106, 1970.
15. Tao, M., Salas, M.L., and Lipmann, F., Proc. Nat. Acad. Sci., 67:408, 1970.
16. Pastan, I. and Perlman, R., Science, 169:339, 1970.
17. Zubay, G., Schwartz, D. and Beckwith, J., Proc. Nat. Acad. Sci., 66:104, 1970.
18. Walton, G.M. and Garren, L.D., Biochemistry, 9:4223, 1970.
19. Gilman, A., Proc. Nat. Acad. Sci., 67:305, 1970.