31

PROTEIN BIOSYNTHESIS BY A CELL-FREE BACTERIAL SYSTEM

IV. EXCHANGE OF DIPHOSPHONUCLEOSIDES WITH HOMOLOGOUS TRIPHOSPHONUCLEOSIDES BY THE "AMINO ACID INCORPORATION ENZYME"

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SUMMARY

A highly purified preparation of the "amino acid incorporation enzyme" is capable of catalysing the transfer of phosphate from triphosphonucleosides to homologous diphosphonucleosides (ATP–ADP; UTP–UDP; CTP–CDP; GTP–GDP). Thermal inactivation of this enzyme preparation suggests the existence of four different and specific enzymes each capable of catalysing the Mg^{++} dependent exchange of one pair of nucleotides. The purified enzymic preparation is free of diphosphonucleoside kinase (ATP + UDP ⇌ UTP + ADP), monophosphonucleoside kinase (ATP + UMP ⇌ ADP + UDP) and myokinase (2 ADP ⇌ ATP + AMP).

INTRODUCTION

We have shown that a purified fraction obtained from *Alcaligenes faecalis* promotes the incorporation of amino acids into peptide bonds when added to a particulate fraction[1,2]. The purified fraction which we have called the "amino acid incorporation enzyme" also catalyses the exchange reaction of [^{14}C]ADP with ATP.

In this paper we present data with respect to the ability of this enzymic preparation to catalyse the exchange of all four di- with triphosphonucleosides and have attempted to determine whether these activities were due to a single or to several enzymes. A brief summary of these results has been presented in a preliminary note[3].

MATERIALS AND METHODS

Enzyme purification

The method of purification of the amino acid incorporation enzyme already described[1] has been slightly modified. In step 2, the ammonium sulfate concentration was increased to 0.70%. It was found that in step 5 the use of an ammonium sulfate

Abreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; PP, pyrophosphate; Tris, tris(hydroxymethyl)aminomethane.

TABLE I

EXCHANGE OF DI- WITH TRIPHOSPHONUCLEOSIDES BY THE "AMINO ACID INCORPORATION ENZYME"

Incubation mixture: 5 μM of $MgCl_2$; 50 μM of Tris, pH 7.3; 0.5 μM of each nucleotide; 3 μg of purified "amino acid incorporation enzyme". Final volume 0.5 ml. Incubation 10 min in air at 34°. Blank values representing from 3 to 5 % of exchange have been substracted. Calculations according to DE MOSS AND NOVELLI[9].

System	*Time of incubation (min)*	*Radioactivity, counts/min*								*Exchange per cent of final equilibrium*	*Rate of exchang µmoles/mg of protein/h*
		ADP–ATP		*GDP–GTP*		*UDP–UTP*		*CDP–CTP*			
Complete	0	2,506	0	15,900	0	16,080	0	11,820	0	0	0
Complete	10	1,670	770							62	486
Complete	10			10,810	4,808					61	483
Complete	10					10,700	5,280			65	530
Complete	10							7,800	3,800	58	430
No $MgCl_2$	10	2,460								0	0
No enzyme	10	2,470								0	0
No ATP	10	2,458								0	0
Heated enzyme	10	2,465								0	0

concentration of 0.40 % instead of 0.55 % resulted in a better yield of the "amino acid incorporation enzyme" while still allowing a clear cut separation of this enzyme from the "amino acid activating enzymes" fraction. The former enzyme was then precipitated from the supernatant solution by 0.70 % ammonium sulfate, dissolved in a minimum volume of phosphate buffer, 0.01 M, pH 7.4, and dialysed against 200 volumes of the same buffer at 3° for 1 h with one change of dialysing solution.

ATP–PP exchange. The method of assay of the exchange between ATP and ^{32}P labeled pyrophosphate has been described[1].

Diphosphonucleosides–triphosphonucleosides exchange

The conditions of measurement of the exchange of labeled diphosphonucleosides with unlabeled triphosphonucleosides are described in the legend to Table I. The reaction was stopped with 0.05 ml of 40% TCA. The nucleotides were separated by chromatography on Whatman No. 1 paper[4] and the u.v. absorbing spots detected. After elution the radioactivity of nucleotides was measured. [8-^{14}C]ADP was purchased from the Schwartz Lab. (Mount Vernon, New York). GDP, UDP and CDP were labeled in the terminal phosphate with ^{32}P using a polynucleotide phosphorylase preparation from *Alcaligenes faecalis* (S.A. 4) according to the method of GRUNBERG-MANAGO AND OCHOA[5]. The labeled diphosphonucleosides were twice chromatographed on Whatman 3 MM paper, eluted with water and lyophylised. All unlabeled di- and triphosphonucleosides were purchased from Sigma Company (St Louis Ill. U.S.A.).

Diphosphonucleoside kinase activity

The presence of diphosphonucleoside kinase in the purified "amino acid incorporation enzyme" was tested by incubation of [^{14}C]ADP with either CTP, GTP or UTP and subsequent analysis for the appearance of labeled ATP[6]. The incubation conditions are described in the legend to Table II. After stopping the reaction with 0.05 ml of 40 % TCA, the nucleotides were chromatographed and eluted as described for the exchange of diphosphonucleosides with their homologous triphosphonucleosides.

TABLE II

ASSAY OF NUCLEOSIDE DIPHOSPHOKINASE ACTIVITY[6] IN THE PURIFIED "AMINO ACID INCORPORATION ENZYME"

Conditions of incubation: 5 μM of $MgCl_2$; 50 μM of Tris buffer pH 7.3; 1 μM of each nucleotide ([^{14}C]ADP w'th 2,300 counts/min); 3 μg of purified "amino acid incorporation enzyme". Final volume 0.5 ml. Incubation 10 min in air at 34°. Nucleotides were separated as described in MATEPIALS AND METHODS.

Labeled substrate	*Addition*	*counts/min found in ATP*
[^{14}C]ADP	ATP	1,000
[^{14}C]ADP	GTP	0
[^{14}C]ADP	UTP	0
[^{14}C]ADP	CTP	0

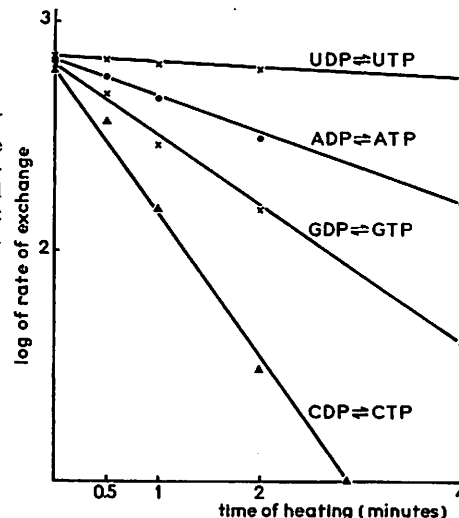


Fig. 1.

Myokinase activity. The myokinase activity was tested as previously described[3]. No activity was detected.

RESULTS AND DISCUSSION

In previous experiments it was observed that certain preparations of the "amino acid incorporation enzyme" which catalysed a very rapid exchange of [^{14}C]ADP with ATP, failed to stimulate the incorporation of amino acids into *Alcaligenes* particles. These preparations which were active only for ADP–ATP exchange did not show the exchange activity between pairs of the other three homologous di- and triphosphonucleosides. On the other hand purified preparations of "amino acid incorporation enzyme" which were able not only to catalyse ADP–ATP exchange, but also to stimulate the incorporation of ^{14}C amino acids into *Alcaligenes* particles, were able to exchange orthophosphate between each pair of homologous nucleotides (Table I).

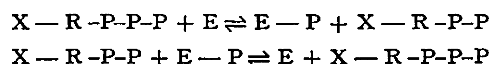
The exchange reactions are absolutely dependent on the presence of Mg^{++} ions. Mn^{++} only partially replaces Mg^{++} while Ca^{++} has no effect. In the pH range from 5 to 8 the rate of the exchange is essentially constant.

The rate of the exchange reaction is not influenced by a mixture of L-amino acids, by 2,4-dinitrophenol (up to $2 \cdot 10^{-4}$ M) chloramphenicol (up to 0.1 mg/ml) or streptomycin and penicillin at various concentrations. Orthophosphate, purines or pyrimidines added in excess to the incubation mixture had no effect. The failure of monophosphonucleosides at a 20 fold excess each to influence the exchange of diphosphonucleosides with homologous triphosphonucleosides suggests the absence of monophosphonucleoside kinase[7,8] in the purified "amino acid incorporation enzyme".

Diphosphonucleoside kinase, the enzyme described by BERG AND JOKLIK[6], is able to transfer the terminal phosphate from ATP to heterologous diphosphonucleosides (for example: ATP + UDP ⇌ UTP + ADP) but acts equally well in the presence of Mg^{++}, Mn^{++} or Ca^{++} ions. Since the "amino acid incorporation enzyme" is completely

active only in the presence of Mg^{++}, it would appear probable that the two enzymes are different. This was found to be the case. Table II shows that incubation of the purified "amino acid incorporation enzyme" with [^{14}C]ADP, Mg^{++} ions and either CTP, GTP or UTP does not result in appearance of labeled ATP, thus indicating the absence of diphosphonucleoside kinase in the purified enzyme preparation.

In view of the fact that the concentration of the two homologous nucleotides is constant during the exchange reaction, the orthophosphate is probably exchanged according to the following scheme:



$$X - R\text{-}P\text{-}P\text{-}P + E \rightleftharpoons E - P + X - R\text{-}P\text{-}P$$
$$X - R\text{-}P\text{-}P + E - P \rightleftharpoons E + X - R\text{-}P\text{-}P\text{-}P$$

X = purine or pyrimidine

The inability of the "amino acid incorporation enzyme" to exchange the heterologous nucleotides suggests that the enzyme preparation contains four different and specific enzymes, each capable of exchanging the orthophosphate of one pair of homologous di- and tri-phosphonucleosides. In order to differentiate these four reactions a highly purified enzyme preparation was heated at 55° for various periods of time (100 µg of enzyme protein in 1 ml of Tris buffer, 0.05 *M*, pH 7.3). The exchange for each pair of nucleotides was then determined. The results are presented in the figure (data from Table I).

The thermal inactivation of the "amino acid incorporation enzyme" follows a simple monomolecular law. The rate of inactivation is very different for each exchange reaction, suggesting that four different enzymes are present.

We propose to call these enzymes: adenosine triphosphate-ADP kinase, guanosine triphosphate-GDP kinase, uridine triphosphate-UDP kinase and cytidine triphosphate-CDP kinase.

The formation of a true peptide bond has been demonstrated by SNOKE AND BLOCH[10] with a purified preparation of glutathione synthetase. This enzyme, which catalyses glutathione formation from γ-glutamyl-cysteine and glycine in the presence of ATP and Mg^{++} is also able to promote a rapid exchange of ADP with ATP. These properties of glutathione synthetase suggest that the promotion of amino acid incorporation by the *Alcaligenes* preparation may be due to a similar type of reaction.

It was of interest to determine if glutathione synthetase contained the activity for exchange of other pairs of homologous nucleotides. A preparation of glutathione

TABLE III

EXCHANGE OF LABELED DI- WITH UNLABELED TRIPHOSPHONUCLEOSIDES BY GLUTATHIONE SYNTHETASE

Conditions of incubation as in Table I with 100 µg of glutathione synthetase. Incubation 20 min in air at 34°.

Nucleotides	*Exchange per cent of final equilibrium*	*Rate of exchange µM/mg of protein/h*
[^{14}C]ADP–ATP	90	33
[^{32}P]GDP–GTP	0	0
[^{32}P]UDP–UTP	0	0
[^{32}P]CDP–CTP	0	0
[^{14}C]ADP–ATP (heated enzyme)	1	0

synthetase, kindly supplied by Dr. K. BLOCH, was tested for exchange of all four nucleotides and for amino acid incorporation activity in *Alcaligenes* particles. No effect on incorporation was observed. As shown in Table III, glutathione synthetase did not exchange other diphosphonucleosides with triphosphonucleosides.

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