IDENTITY IN ESCHERICHIA COLI OF CARBAMYL PHOSPHOKINASE AND AN ACTIVITY WHICH CATALYZES AMINO GROUP TRANSFER FROM GLUTAMINE TO ORNITHINE IN CITRULLINE SYNTHESIS*

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Received December 31, 1964

Piérard and Wiame (1964) showed recently that extracts of wild type Escherichia coli were able to catalyze transfer of an amino group from glutamine to ornithine in ATP-mediated citrulline formation. Presumably this is the same type of activity discovered by Levenberg (1962) in Agaricus bisporus. Previously it had been considered that carbamyl phosphokinase was the sole enzyme activity responsible for carbamyl phosphate synthesis,

$$CO_2 + NH_3 + ATP \xrightarrow[Mg^{++}]{E} O{=}C\begin{matrix}NH_2\\ OPO_3H^-\end{matrix}$$

in the step leading to carbamyl aspartic acid formation and also citrulline biosynthesis in E. coli. The present report deals with purification of the glutamine-dependent activity (GDA) from E. coli B, and its apparent identity to carbamyl phosphokinase (CPK).

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*Aided by grant #E-345 from the American Cancer Society

Evidence for identity includes the following observations:

1. Purification. Wild type E. coli B was grown in minimal medium plus glucose (Davis and Mingioli, 1950) with addition of 3% casamino acids. Cells were harvested during the exponential phase of growth, sonicated, and then purified as indicated in Table 1. Details of the purification procedure will be included in a later publication. Overall purification of approximately 75-fold was achieved for the glutamine-dependent activity and about 36-fold for the carbamyl phosphokinase activity. Ratios of GDA to CPK activity

Table 1

Purification Procedure for Carbamyl Phosphokinase from E. coli

The assay mixture for glutamine dependent citrulline synthesis contained per ml 100 μmol Tris pH 8.5, 24 μmol $MgCl_2$, 12 μmol L-ornithine HCl, 24 μmol ATP, 6 μmol L-glutamine, 30 μmol $KHCO_3$, and excess ornithine transcarbamylase from S. faecalis. The assay mixture for ammonia-dependent citrulline synthesis contained per ml 200 μmol Tris pH 8.5, 20 μmol $MgCl_2$, 10 μmol L-ornithine HCl, 20 μmol ATP, 100 μmol $(NH_4)_2CO_3$, and excess ornithine transcarbamylase from S. faecalis. Incubations were carried out at 37° for 15 min; the reaction was stopped with an equal volume of 10% TCA. Citrulline was determined by a modification of the Archibald method (1944). One unit of either enzyme represents the ability to form 1 μmol of citrulline per hr.

	A	B	A/B
Step	S.A.	S.A.	
Cell extract	0.40	0.42	0.95
Protamine sulfate ppt'n (5 mg/ml)	1.13	.50	2.26
Ammonium sulfate ppt'n (35-60%)	4.60	2.18	2.11
Sephadex G-200	7.60	4.20	1.90
Protamine sulfate ppt'n (.01-.05 mg/ml)	30.00	14.40	2.08

A = GDA; B = CPK

changed once during purification; see protamine sulfate step (Table 1). This change appears to be due to the presence of a phosphokinase in the cell extract that is not adsorbed to any appreciable extent by protamine sulfate. The activity which remains in the supernatant at this step has both acetokinase and carbamyl phosphokinase activity (see Grisolia and Harmon, 1962) but no glutamine-dependent activity. The greater increase in specific activity of GDA relative to CPK at this stage of the purification accounts for the 2-fold discrepancy in the degree of purification for the two activities shown in Table 1. In all other steps the two activities showed a remarkably constant ratio. This was observed in many procedures not reported in the table, such as adsorption to alumina C-γ and calcium phosphate gel, and chromatography in various media (see below).

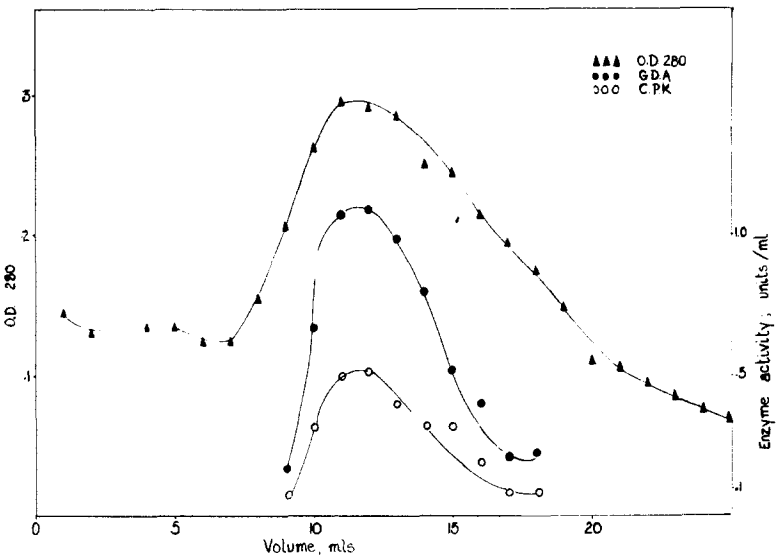

Figure 1. Chromatography on Sephadex G-200.

Five mg of purified material was layered on a 50 x 1 cm column made up with 0.02 M phosphate buffer, pH 6.0, containing 20% glycerol. Elution was carried out with the same solution. Ratio of GDA:CPK for the starting material was 2.3.

2. Chromatography. All attempts to resolve the two activities by chromatographic techniques failed. These included application to columns containing DEAE-cellulose in the presence or absence of guanidine hydrochloride (2M), Sephadex G-200, and hydroxylapatite. Figure 1 shows the pattern of elution from a G-200 column.

3. Co-sedimentation. When the purified material was applied to a sucrose density gradient the two activities traveled together as a single peak.

4. Electrophoresis. The purified material was applied to cellulose acetate strips in a Shandon electrophoresis apparatus. The two activites were compared after sufficient migration had occurred as determined by staining a longitudinal section of each strip. Both the carbamyl phosphokinase and the glutamine-dependent activity moved together as a single band. This observation was repeated at pH 4.5, pH 6, and also at pH 8.7. In the pH 8.7 strips GDA was lower than carbamyl phosphokinase activity; this corresponds to the observation that GDA is relatively unstable in alkaline solutions.

Electrophoresis in starch gel was carried out at pH 7 and 8.6. In each case a single band contained both enzyme activities. In addition, electrophoresis in starch gel was performed with a sample heated at 60° C until most of the GDA was destroyed. One band contained both residual activities. When a sample of the purified enzyme was subjected to starch gel electrophoresis in the presence of 2M formamide no separation of the enzyme activities was demonstrable. Results of starch gel electrophoresis are presented in Table 2.

Table 2

Starch Gel Electrophoresis

The gel column was that described by Smithies (1955), 20 mm wide x 250 mm long x 6 mm deep. The experiments were carried out for 3 to 4 hrs with a gradient of 400 volts. Protein bands were located on a thin slice of starch gel stained with amido black. Corresponding bands of the unstained starch were divided and incubated in the two assay mixtures described in Table 1. Note that this particular preparation had a higher GDA/CPK ratio than the one described in Table 1. At pH 7 the buffer system employed was .076 M Tris-citrate (starch) and .3 M borate (bridge solution) (Poulik, 1957). At pH 8.6 the buffer system was 0.01 M barbital (starch) and 0.1 M barbital for the bridge solution.

pH	Ratio, GDA:CPK Starting material	Radio, GDA:CPK Starch band
7	3	2.2
7 + 2M formamide	1.2	1.1
8.6	3	1.8

5. Reaction to inhibitors. Both activities were sensitive to the same set of inhibitors, although there were some differences in degree of depression. The GDA was especially sensitive to azaserine and potassium fluoride, both of which have been used to inhibit transfer of an amino group from glutamine in other enzyme studies (Meister, 1962). Both reactions were sensitive to the presence of α-keto-glutarate. These results are shown in Table 3. Both enzymes are sensitive to parachloromercuribenzoate.

Table 3

Inhibition of Enzyme Activities

Preincubation with each of the inhibitors, α-ketoglutarate, azaserine, and potassium fluoride was carried out at 37° for 10 min. Activities were assayed as described in Table 1.

Inhibitor	Conc.	*GDA	*CPK	% inhib GDA	% inhib CPK
Control		100	40		
α-ketoglutarate	6×10^{-3} M	14	0	84	100
azaserine	10^{-4} M	64	40	36	0
KF	10^{-3} M	60	40	40	0

*Activities are expressed as units per ml

6. Treatment with thiol reagents. Dimercaptoethanol at a concentration of .01 M did not produce resolution in any of the preparative steps. Incubation with dithioerythritol, 10^{-3} M (Cleland, 1964), and subsequent chromatography on Sephadex G-200 did not separate the two activities.

There were data that did not show completely parallel behavior for the two activities. The carbamyl phosphokinase activity was relatively insensitive to azaserine and potassium fluoride. In addition, it was found that the glutamine-dependent activity was unstable at room temperature, at 0° C, and at -20° C in the presence of .001 M ethylenediaminetetraacetic acid and .01 M mercaptoethanol. The addition of 20% glycerol to all solutions markedly improved stability. Even under these conditions GDA was unstable at alkaline pH's. Purification was carried out at pH 6. The carbamyl phosphokinase activity was relatively stable under ordinary conditions. Extreme lability of the glutamine-dependent activity

in the absence of glycerol may be responsible for difficulties in purification mentioned by Levenberg (1962) and for the time lag before it was discovered in bacteria. Heat denaturation studies did not yield parallel curves of inactivation. The procedure was complicated by the fact that the K_m for NH_3 (carbamyl phosphokinase) changed from about 1×10^{-3} M for a control sample to 6×10^{-2} M for a sample that had been 75% inactivated. The K_m for glutamine (GDA activity) did not change for a similar degree of inactivation.

This report presents evidence for the identity of carbamyl phosphokinase with an enzyme that catalyzes transfer of an amino group from glutamine in the synthesis of citrulline, an enzyme activity first reported in E. coli by Piérard and Wiame (1964). The evidence includes a parallel increase in specific activities during purification, co-chromatography in several media, co-sedimentation, identical behavior in electrophoresis, no resolution when treated with guanidine, formamide, or thiol reagents, and some degree of similarity in response to inhibitors.

Several enzymes which catalyze amino group transfer from glutamine have the capacity to use NH_3 also (Meister, 1962). An example is the xanthosine 5'-phosphate aminase isolated by Moyed and Magasanik (1957) from Aerobacter aerogenes. During the purification process this enzyme appeared to lose its ability to utilize glutamine although it retained ability to use NH_3 as a source of the amino group in guanosine 5'-phosphate. This experience is similar to our own earlier attempts at purification of GDA whereby only the CPK activity persisted in the absence of glycerol.

The data presented suggest that two enzyme activities

which catalyze the formation of carbamyl phosphate are properties of a single protein. Of the protein present in the purified material about 30-50% appeared to represent the active fraction (as estimated from electrophoretic patterns) which contained both activities. It is probable that a single enzyme may utilize either of two substrates, NH_3 and glutamine, for which it has different active sites, and the two sites differ somewhat in their sensitivity to chemical and physical treatments. It is possible that further attempts at separation will be successful. In any case, if there are two distinct proteins our data indicate that they must be tightly bound together or share very similar physical properties.

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