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Fixation of carbon dioxide by carbamyl phosphate synthetase of *Escherichia coli*. Evidence for a reversibly formed intermediate

Carbamyl phosphate synthetase (EC 2.7.2.5) of *Escherichia coli* has been isolated and purified in this laboratory. The reactions involving this enzyme are particularly interesting, since they involve the chemical activation of CO_2 and the subsequent synthesis of carbamyl phosphate. (For simplicity, we assume that CO_2 reacts as bicarbonate, the dominant form at the pH used in these studies.) We have demonstrated that Reaction 1 is catalyzed by this enzyme, and have described certain physical and kinetic properties of the enzyme[1,2].

$$\text{Glutamine} + 2\ \text{ATP} + HCO_3^- \underset{Mg^{2+}}{\overset{E}{\rightleftharpoons}} \text{glutamate} + \text{carbamyl phosphate} + 2\ \text{ADP} + P_i \quad (1)$$

This reaction has also been studied by ANDERSON AND MEISTER[3] who first determined its stoichiometry. On the basis of their experiments, they proposed the mechanism shown in Eqns. 2–4.

$$HCO_3^- + \text{ATP} + E \rightleftharpoons E\text{-}[HCO_3PO_3^{3-}] + \text{ADP} \quad (2)$$

$$E\text{-}[HCO_3PO_3^{2-}] + \text{glutamine} + H_2O \rightleftharpoons E\text{-}[NH_2CO_2^-] + \text{glutamate} + P_i \quad (3)$$

$$E\text{-}[NH_2CO_2^-] + \text{ATP} \rightleftharpoons E + NH_2COOPO_3^{2-} + \text{ADP} \quad (4)$$

The first step is thought to be discrete because of ATP hydrolysis in the absence of glutamine[4]. In this communication we present evidence that Reaction 2 is reversible. Thus, carbamyl phosphate synthetase in the presence of ATP fixes CO_2 reversibly to give an intermediate which is converted to carbamyl phosphate in the presence of glutamine and ATP.

Carbamyl phosphate synthetase was prepared from *E. coli* B (ref. 2). The material used had a specific activity of 250 (1 unit = 1 μmole of citrulline per h where carbamyl phosphate formed from ATP, glutamine and HCO_3^- is allowed to react with ornithine in the presence of ornithine transcarbamylase to form citrulline). Ornithine transcarbamylase was prepared as described by RAVEL *et al.*[5]. It was free of carbamate kinase and carbamyl phosphate synthetase activity. The specific activity was $1.5 \cdot 10^5$ (1 unit = 1 μmole of citrulline per h from carbamyl phosphate and ornithine).

ATP, ADP and DPNH were obtained from P–L Biochemicals. [^{3}H]ADP and ^{3}[H]ATP were obtained as the tri- and tetralithium salts, respectively, from Schwarz BioResearch. Carbamyl phosphate as the dilithium salt, phosphoenolpyruvate and lactate dehydrogenase containing pyruvate kinase were obtained from the Sigma Chemical Co.

The assay mixture for Reaction 2 contained 0.1 M Tris buffer (pH 8.5); 0.012 M $MgCl_2$, 0.012 M ATP, 0.03 M $KHCO_3$, and 90 μg/ml of carbamyl phosphate synthetase. Incubation was carried out at 37°; aliquots of 50 μl were taken at appropriate times. Immediately, each aliquot was stoppered and put in a boiling water bath for 2 min, then frozen in a mixture of dry ice and isopropanol and stored at − 20°.

For the determination of ADP formation by measurement of radioactivity, $6.5 \cdot 10^5$ counts/min (specific activity, $4.1 \cdot 10^3$ μC/μmole) per ml of [^{3}H]ATP were added to the assay mixture described above. Aliquots of 10 μl were applied to Whatman 3MM chromatography paper, and electrophoresis was carried out by the method of MARKHAM AND SMITH[6]. Spots that absorbed ultraviolet light were marked, and the appropriate sections of paper were cut into 3-mm-wide strips which were counted in 10 ml of 0.4% 2,5-bis-[2-(5-*tert.*-butylbenzoxazolyl)]-thiophene in toluene in a Tri-Carb liquid scintillation counter. Control experiments showed that the counting efficiency was the same for [^{3}H]ADP and [^{3}H]ATP. The enzymatic determination of ADP was carried out on aliquots of the frozen samples by the method described previously[2]. In studies on the formation of ATP from ADP, $2.14 \cdot 10^6$ counts/min (specific activity, $5.3 \cdot 10^3$ μC/μmole) per ml of [^{3}H]ADP were added to the assay and the experiment carried out as in the determination of [^{3}H]ADP.

The assay mixture[2] for Reaction 1 was the same as that for Reaction 2, except for the addition of 0.006 M glutamine. Determinations of [^{3}H]ADP were carried out as described above.

Carbamyl phosphate synthetase in the presence of bicarbonate but without glutamine shows ATPase activity. The conversion of ATP to ADP can be followed both by radioactive tracers and by enzymatic assay. Both give identical results. This conversion rate is 1/5 as fast as ATP breakdown in the presence of glutamine[2], independent of ADP concentration, and undetectable in the absence of bicarbonate.

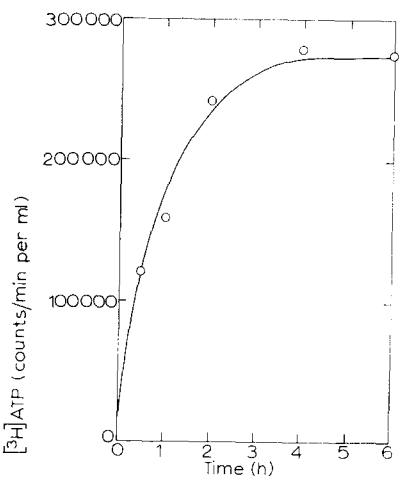

Fig. 1. Bicarbonate-dependent formation of ATP from ADP; Reaction 2, reverse.

Under identical reaction conditions, when radioactive ADP was present, radioactive ATP was produced (see Fig. 1). This reaction, too, proceeds only in the presence of bicarbonate. Under reaction conditions optimal for ATP cleavage, the rate of conversion of ADP to ATP is approx. 20% of the forward reaction (ATP $\rightarrow$ ADP).

In the presence of glutamine, conversion of ADP to ATP is drastically reduced (or, possibly, stopped). Thus, when glutamine was added to a portion of the reaction mixture containing [^{3}H]ADP, the [^{3}H]ATP present began to decrease. This is in

contrast to the portion without glutamine in which [^{3}H]ATP continued to increase.

All of these observations are consistent with the reversible formation of an enzyme-bound intermediate which reacts rapidly in the presence of glutamine. The appearance of phosphate in the absence of glutamine may be connected with dissociation of the intermediate. The intermediate, which could be a bicarbonate–phosphate anhydride, would be rapidly hydrolyzed in solution to inorganic phosphate and bicarbonate. Alternatively, hydrolysis may occur on the enzyme itself; the net result in either case is irreversible hydrolysis of ATP. The actual intermediate appears to be relatively stable, and the slower rate of ATP disappearance in the absence of glutamine suggests that the enzyme has a high affinity for the intermediate. Thus, it appears that decomposition of the intermediate is the rate-limiting step of ATP hydrolysis in the absence of glutamine: at this stage ADP cannot be enzyme-bound, since exchange occurs between [^{3}H]ADP in solution and ADP produced in Reaction 2. The observation that the rate of ATP formation from ADP is diminished in the presence of glutamine indicates that the intermediate reacts with glutamine to give products more rapidly than it reacts with ADP to return to bicarbonate and ATP. The overall stoichiometry shows that decomposition of the intermediate to give, ultimately, phosphate and bicarbonate is also slow compared with carbamyl phosphate formation in the presence of glutamine. Otherwise, more than 2 moles of ADP would be produced in the production of one mole of carbamyl phosphate. The rate of ATP hydrolysis in the absence of glutamine being independent of ADP concentration indicates that the enzyme is largely present as a complex which can react directly with ADP. These conclusions are also supported by the observation of ANDERSON AND MEISTER[3] that ADP is produced in an initial burst when glutamine is absent.

In summary, we have obtained evidence consistent with a mechanism in which carbamyl phosphate synthetase fixes bicarbonate reversibly producing ADP, and in which, in the absence of glutamine, the enzyme is present largely as a complex which can react directly with ADP and decomposes slowly to produce inorganic phosphate and bicarbonate.

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