FRUCTOSE-6-PHOSPHATE AND AMP; EFFECTORS OF PROLINE BIOSYNTHESIS IN ESCHERICHIA COLI

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Summary: Fructose-6-phosphate and AMP have been found to inhibit the first step in proline bioshythesis in Escherichia coli. Inhibition is not affected by the sensitivity of the enzyme to end-product inhibition by proline. Both fructose-6-phosphate and AMP display uncompetitive inhibition with respect to glutamic acid, and apparent competitive inhibition with ATP in the proline-sensitive glutamokinase reaction. Sigmoidal kinetics are observed with both inhibitors, and separate binding sites for substrates, proline, and the regulatory compounds are proposed.

Proline is synthesized from glutamic acid in E. coli by a series of three reactions (1,2,3). The synthesis is largely controlled by end-product inhibition by proline of the first enzyme in the pathway (4). This enzyme catalyzes the phosphorylation of glutamic acid by ATP (5) to produce an intermediate which, by analogy with glutamine synthetase (6), is believed to be enzyme bound in vivo.

$$\text{Glutamic acid} + \text{ATP} \xrightarrow{MgCl_2} \gamma\text{-glutamyl phosphate} + \text{ADP}$$

In the course of investigating the effects of various carbon sources on the synthesis of the proline-sensitive glutamokinase, it was found that glucose-6-phosphate and fructose-6-phosphate exerted a direct inhibitory effect on the partially purified enzyme, and that fructose-6-phosphate was the more effective of the two. This suggested that the activity of glutamokinase might be regulated by available energy in the system, as well as by end-products, so AMP and ADP were also tested. Both of these adenylates inhibited the reaction.

Materials and Methods

The methods used in this paper were described previously (5), except that all of the colorimetric analyses for phosphate and protein and were

carried out using the Klett-Summerson colorimeter with the 66 filter. Fructose was determined by the Roe (7) method using the 54 filter. The enzyme preparation used was the streptomycin precipitate previously described (5). All of the reagents used were Sigma products.

Table 1

The effect of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase on the activity of fructose-6-phosphate in the enzyme reaction

Sample	fructose-6-phosphate, M/L	% Inhibition
treated	0.03	0
untreated	0.32	50
f-6p	0.50	64

The treated sample contained imidazole 0.05 M, 0.85 ml, fructose-6-phosphate 0.01 M, 0.05 ml TPN, 0.01 M, 0.05 ml, $MgCl_2$, 1 M, 0.01 ml. The reaction was initiated by the addition of glucose-6-phosphate dehydrogenase 5 µg/ml, 0.01 ml. After 15 minutes at 25°C, the sample was heated at 100° for 5 minutes. The samples of fructose-6-phosphate were tested in the usual incubation mixture, and adjusted so that the final concentration in the mixture was for the untreated samples, 1.6×10^{-4} M. For the untreated sample, the enzyme was added while the sample was being heated. The concentration of fructose-6-phosphate was verified by the Roe (7) test.

Results and Discussion

Both glucose-6-phosphate and fructose-6-phosphate inhibit the glutamokinase reaction. At 0.01M, the inhibition observed is about the same for both sugar-phosphates,but at a concentration of 0.001M, fructose-6-phosphate exerts a much greater effect. In crude extracts, glucose-6-phosphate and fructose-6-phosphate are in equilibrium because of the action of phosphoglucose isomerase (8). Since fructose-6-phosphate is an unexpected inhibitor of proline biosynthesis, and since contamination of chemicals is occasionally encountered, the activity of fructose-6-phosphate was verified by using a specific enzyme system to remove the sugar, and then testing to see if the preparation still had inhibitory activity. As is shown in Table 1, after treatment with glucose-6-phosphate dehydrogrenase (contaminated with

phosphoglucose isomerase) and TPN, the fructose-6-phosphate preparation no longer showed any inhibitory activity. The decrease of fructose-6-phosphate in the treated preparation was verified by the Roe test (7).

AMP and ADP also inhibit the reaction, and at a concentration of 0.001M these compounds show quantitative effects comparable to fructose-6-phosphate. Table 2 lists sugars and sugar-phosphate derivatives tested for activity in this reaction. Only fructose-6-phosphate, and to a smaller extent, glucose-6-phosphate are inhibitors.

Table 2

Metabolic inhibitors of the proline-sensitive glutamokinase reaction.

Metabolite	% Inhibition .001 M	0.01 M
fructose-6-phosphate	32	70
glucose-6-phosphate	0	45
ribose-5-phosphate		0
1-phosphoribosyl-5-pyrophosphate		0
fructose 1,6 diphosphate		0
glucose-1-phosphate		0
glucosamine-6-phosphate		0
ribose		0
deoxyribose		0
glucose		0
arabinose		0
fructose		0
3'-AMP		0
3'-5' cyclic AMP		0
5'-AMP	56	78
ADP	52	100

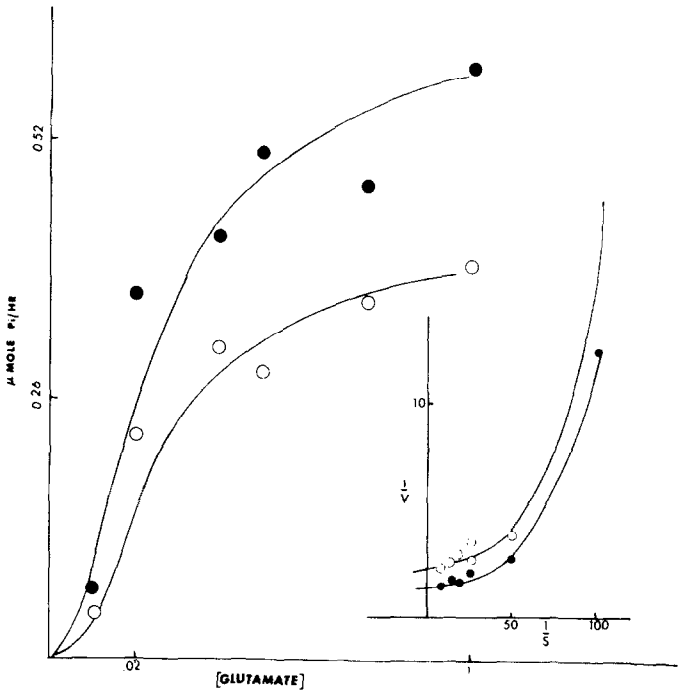

Figure 1: The effect of glutamic acid concentration on the velocity of the reaction in the presence (O) and absence (●) of 0.001 M fructose-6-phosphate.

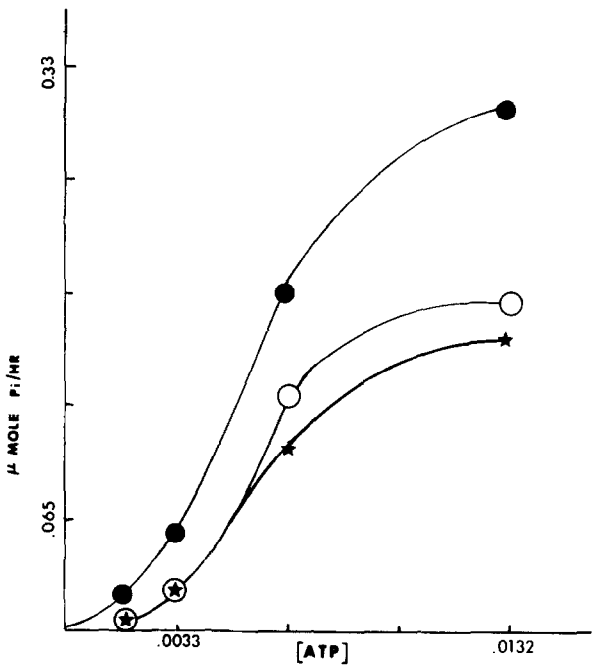

Figure 2: The effect of ATP on the velocity of the reaction (●) in the presence of 0.001 M fructose-6-phosphate (O) and in the presence of 0.001 M AMP (★). The concentration of $MgCl_2$ was varied so that the ATP/$MgCl_2$ ratio remained constant at 5.0.

Inhibition of glutamokinase by fructose-6-phosphate and by AMP is uncompetitive with glutamic acid. Glutamic acid shows cooperative stimulation of the reaction at lower concentrations, but the shape of the curve is not altered in the presence of fructose-6-phosphate or AMP (Fig. 1).

The Km for ATP/$MgCl_2$ is estimated to be 3×10^{-3} M. In the presence of 0.001M fructose-6-phosphate or in the presence of 0.001M AMP, the kinetics become sigmoidal (Fig. 2) and a Lineweaver-Burk analysis indicates competitive inhibition with ATP (Fig. 3). Raising of the concentration of fructose-6-phosphate increases the sigmoidicity of the reaction.

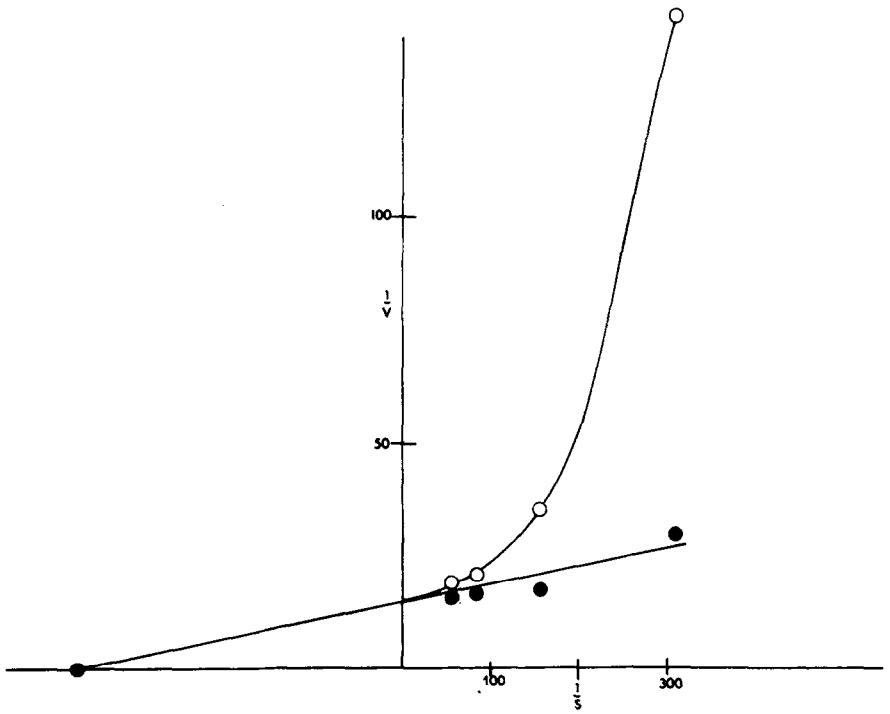

Figure 3: A Lineweaver-Burk analysis of the effect of ATP on the velocity of the reaction in the absence (●) and presence (O) of 0.001 M fructose-6-phosphate.

At concentrations of ATP greater than 0.02M, glutamokinase is inhibited an effect which has been observed previously in phosphofructokinase (9). At higher concentrations of ATP, fructose-6-phosphate diminishes the inhibitory effects of ATP (Fig. 4).

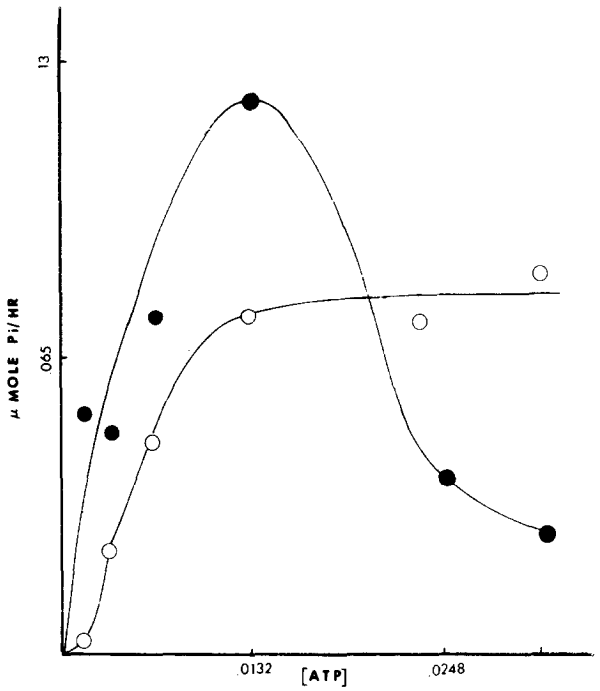

Figure 4: The effect of the ATP/$MgCl_2$ concentration on the velocity of the reaction in the absence (●) and presence (O) of 0.001 M fructose-6-phosphate.

A proline-insensitive glutamokinase obtained from a control-deficient strain of bacteria (4) retains its sensitivity to regulatory metabolites. This observation suggests that the binding site for proline is separate from the binding site for fructose-6-phosphate and AMP.

Conclusions

Inhibition of glutamokinase by ADP and AMP could be explained on the basis of competitive inhibition at the ATP site, however, inhibition by fructose-6-phosphate is not so easily interpreted. If competitive inhibition by the sugar-phosphate occurred at the ATP substrate binding site, it would be anticipated that ribose-5-phosphate or 1-phosphoribosyl-5 pyrophosphate, which are more similar in structure to ATP than fructose-6-phosphate, would also act as inhibitors. The observation that only fructose-6-phosphate and to a lesser extent glucose-6-phosphate, inhibits, suggests a specific control site for hexose-phosphates on the enzyme protein. The location of the binding

site for AMP is unclear, since it could be binding at the same site, competing with ATP, or it could bind at a separate site.

The proline-sensitive glutamokinase thus has at least two control sites apart from the active site which binds glutamic acid and ATP; the proline binding site for end-product inhibition, and the fructose-6-phosphate binding site, for metabolic regulation.

There are two regulatory systems involved in control of phosphofructokinase, whose activity is regulated by the level of ATP (9) at the adenylate level and by citrate (10) at the substrate level. In an analogous fashion, the activity of the proline-sensitive glutamokinase if regulated both by the energy availability in the cell, as indicated by its inhibition by ADP and AMP, and by a substrate level effector, fructose-6-phosphate.

This finding can be interpreted as providing a direct, substrate-level link between amino acid biosynthesis, and therefore, protein synthesis, and the availability of energy in the organism.

Acknowledgments

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