FRUCTOSE-1,6-DIPHOSPHATASE OF ACINETOBACTER : INHIBITION BY ATP AND CITRATE

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Summary

Fructose-1,6-diphosphatase (FDPase) from a species of Acinetobacter was partially purified and some of its properties studied. The enzyme resembles other FDPases investigated in its dependence on a divalent metal like Mg^{++} or Mn^{++}, high affinity for the substrate, stimulation by EDTA and inhibition by high substrate concentrations. However, it differs from most other FDPases in its insensitivity to AMP and inhibition by ATP and citrate.

Introduction

Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) from a variety of sources is strongly inhibited by AMP and activated by ATP (Atkinson, 1965). However, Taketa and Pogell (1963) reported that ATP causes a reversible inactivation of the enzyme from rabbit liver. Rosen, et al. (1965) noted some inhibition of the enzyme in Candida utilis by high concentrations of ATP and ADP which they attributed to the traces of AMP present as a contaminant. In the slime mold Polysphondylium pallidum, fructose-1,6-diphosphatase is not appreciably inhibited by AMP or dAMP (Rosen, 1966). In this communication we report the presence of a fructose-1,6-diphosphatase in the gram negative bacterium Acinetobacter sp. (Mima polymorpha ATCC 9957, reclassified as Acinetobacter by Baumann, Doudoroff and Stanier, 1968) which is strongly inhibited in vitro by ATP

and to a lesser extent by citrate while being relatively unaffected by AMP.

Materials and Methods

The organism was grown on a simple mineral salts medium described earlier (Bell and Marus, 1966). The cells were harvested at the middle of the log phase and ruptured in the French Press. The enzyme was partially purified (40 fold) by lactic acid fractionation, subsequent ammonium sulfate precipitation and passage of the 80% ammonium sulfate fraction through a Sephadex G-25 column. The enzyme was assayed spectrophotometrically according to the method of Rosen, Rosen and Horecker (1966) by measuring the rate of reduction of nicotinamide adenine dinucleotide phosphate (NADP) at room temperature in a Gilford model 2000 multiple sample absorbance recorder at 340 mμ. The standard reaction mixture contained: 0.60 ml glycine buffer, 0.2 M, pH 9.5; 0.03 ml $MgCl_2$, 0.1 M; 0.03 ml NADP, 0.05 M; 0.003 ml each of phosphohexose isomerase (2.0 mg/ml) and glucose-6-phosphate dehydrogenase (5.0 mg/ml); 0.06 ml sodium fructose-1,6-diphosphate, 0.01 M; and 0.1 ml of a suitably diluted enzyme preparation. The final volume was adjusted to 3.0 ml with water. The reaction was initiated by adding the substrate (fructose-1,6-diphosphate) after equilibration and maximum rates were measured between 6 and 12 minutes. The specific activity of the enzyme is expressed as units per milligram protein. A unit of enzyme is the amount which converts 1 micromole of fructose-1,6-diphosphate to fructose-6-phosphate in 1 minute at room temperature. Protein was determined by the method of Lowry, et al. (1951). Fructose-1,6-diphosphate (98% pure), NADP, phosphohexose isomerase, glucose-6-phosphate dehydrogenase, AMP, ADP, ATP,

EDTA and citrate were purchased from Sigma Chemical Co., St. Louis. Before addition to the assay system, AMP, ADP, ATP, EDTA and citrate were neutralized.

Results and Discussion

Growth on various carbon sources - pyruvate, lactate, ethyl alcohol, acetate, succinate and fructose-1,6-diphosphate - does not affect the level of the enzyme, suggesting that it is constitutive for the organism. The specific activity of the enzyme in these different substrates is about 0.009-0.013. Like the FDPases from most other sources, the enzyme from our organism also requires a divalent cation (Mg^{++} or Mn^{++}), is stimulated by low concentrations of EDTA and is active at low (maximum rates of activities at 0.1 - 0.2 mM of FDP), but inhibited at high substrate concentrations. However, the response of the enzyme to allosteric modifiers like ATP, ADP, AMP, and citrate is quite different.

The curves marked A in Figs. 1 and 2 show that the addition of ATP or citrate to the assay mixtures results in a significant decrease in activity. While about 2.0 µmoles (in a 3.0 ml assay mixture) causes nearly 40-50% reduction in activity, it takes about 5-7 µmoles of citrate to give comparable results. ADP resembles ATP although it is less effective. Our enzyme is relatively insensitive to AMP which is a potent inhibitor in most other organisms (Atkinson, 1965). Since ATP and citrate have the capacity to bind Mg^{++} which is essential for the reaction, it was necessary to determine whether the observed inhibitions are due to the complexing of Mg^{++} or are due to a direct effect on the enzyme itself. It was observed that by increasing the concentration of Mg^{++} in the reaction mixture five fold, the activity of the enzyme could be restored

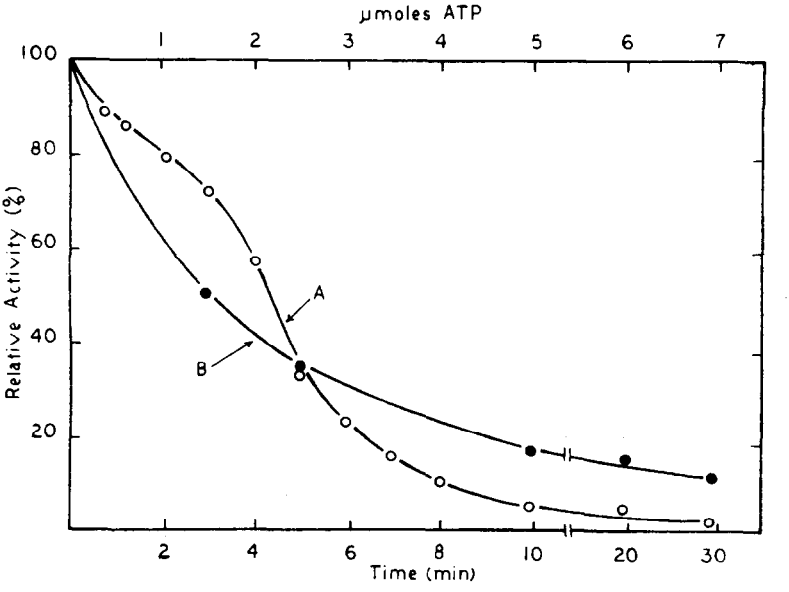

Figure 1. Inhibition of FDPase by ATP. The enzyme was assayed spectrophotometrically as described in the text. Curve A shows the effect of increasing concentrations of ATP while curve B shows the effect of preincubating the enzyme with ATP (2.0 µmoles/3.0 ml assay mixture) for different periods of time. Concentrations of ATP shown represent the amounts in 3.0 ml assay mixtures.

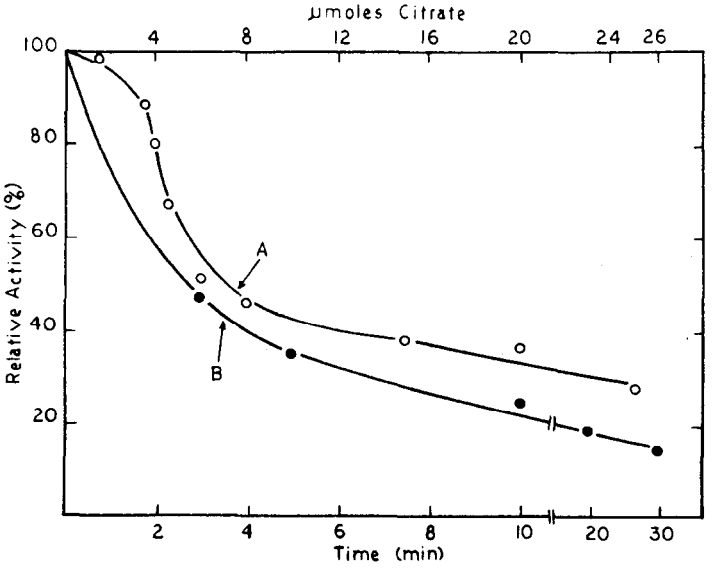

Figure 2. Inhibition of FDPase by citrate. The enzyme was assayed spectrophotometrically as described in the text. Curve A shows the effect of increasing concentrations of citrate while curve B shows the effect of preincubating the enzyme with citrate (5.0 µmoles/3.0 ml assay mixture) for different lengths of time. Concentrations of citrate shown represent the amounts in 3.0 ml assay mixtures.

to 90% of the control which suggested that the complexing of Mg^{++} may be the cause of the inhibitory effects.

Subsequent experiments (Table 1), however, showed that preincubation of the enzyme with ATP (2.0 µmoles/3.0 ml) or citrate (5.0 µmoles/3.0 ml) for 30 minutes results in significantly greater degrees of inhibition than those obtained when measurements are initiated immediately after adding ATP or citrate, although the amounts of magnesium in either case are the same. Table 1 also shows that the effect of either ATP or citrate addition to the enzyme preincubated with Mg^{++} for 30 minutes, is essentially the same as without prior incubation

Table 1

Effect of ATP and Citrate on FDPase activity

	Treatment	Relative activity (%)
2.0 µmoles ATP	Assayed immediately after addition	47.1
	Preincubated with $MgCl_2$ for 30 mins.	43.0
	Preincubated with ATP for 30 mins.	11.9
5.0 µmoles citrate	Assayed immediately after addition	50.0
	Preincubated with $MgCl_2$ for 30 mins.	48.4
	Preincubated with citrate for 30 mins.	14.1

Assays were run at alkaline pH as described in the text. The upper half of the Table shows the effect of 2.0 µmoles of ATP while the lower half shows the effect of 5.0 µmoles of citrate on the relative activity of the enzyme.

of the enzyme with the cation. It may be noted that in all assays the amounts of magnesium, ATP and citrate are constant in each case and the time of exposure of magnesium to ATP or citrate is the same. The very marked inhibition noted if the enzyme is exposed to ATP or citrate before the addition of Mg^{++} to the reaction mixture, clearly indicates that these compounds have a direct inhibitory effect on the enzyme which is quite distinct from Mg^{++} complexing. This was further confirmed by the results of experiments in which the enzyme incubated with ATP or citrate for 30 minutes was dialyzed overnight to remove free ATP and citrate and then assayed in the usual manner using an untreated but dialyzed sample of enzyme as a control. It was found that the ATP and citrate treated enzymes showed a residual activity of about 6.0 and 9.0% respectively of the control clearly demonstrating the inhibitory effects of these compounds on the enzyme protein itself.

The inhibition by ATP and citrate is dependent upon the time of incubation with the enzyme. The curves marked B in Figs. 1 and 2 show the effect of incubating the enzyme (in the absence of Mg^{++}) with ATP and citrate respectively for varying periods of time up to 30 minutes. Magnesium was added at the time of assay in each case. In these experiments we used an amount of the inhibitors which, in assays without prior incubation, would give 40-50% inhibition (2.0 µmoles of ATP/3.0 ml and 5.0 µmoles of citrate/3.0 ml). The time dependence of this phenomenon is clearly demonstrated. The lack of any structural relationships between the substrate (fructose-1,6-diphosphate) and the inhibitors (ATP and citrate) suggests that these interact with the enzyme

at an allosteric site or sites. Studies are being directed to determine the nature of these sites.

As in Escherichia coli (Fraenkel, et al., 1966) the fructose-1,6-diphosphatase in our organism is also constitutive. However, the insensitivity to AMP and susceptibility to ATP and citrate inhibition make the FDPase of Acinetobacter quite distinctive. The physiological significance is not clear. It is interesting to note that inhibition by ATP and citrate is characteristic of the catabolic enzyme phosphofructokinase (Atkinson, 1965) and not of the usually anabolic FDPase. It may be that the FDPase of Acinetobacter has both a catabolic and anabolic role.

Acknowledgment

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References

Atkinson, D.E., Science, 150, 851 (1965).

Baumann, P., Doudoroff, M. and Stanier, R.Y., J. Bacteriol., 95, 1520 (1968).

Bell, E.J. and Marus, A., J. Bacteriol., 91, 2223 (1966).

Fraenkel, D.G., Pontremoli, S. and Horecker, B.L., Arch. Biochem. Biophys., 114, 4 (1966).

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem., 193, 265 (1951).

Rosen, O.M., Rosen, S.M. and Horecker, B.L., Arch. Biochem. Biophys. 112, 411 (1965).

Rosen, O.M., Arch. Biochem. Biophys., 114, 31 (1966).

Rosen, O.M., Rosen, S.M. and Horecker, B.L., in Methods in Enzymology IX, W.A. Wood, ed., Academic Press, New York, 1966, p. 632.

Taketa, K. and Pogell, B., Biochem. Biophys. Res. Commun., 12, 229 (1963).