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INACTIVATION OF LIVER PHOSPHOFRUCTOKINASE BY PHOSPHOFRUCTOKINASE PHOSPHATASE OR BY LOW pH IS INHIBITED BY FRUCTOSE 2,6-BISPHOSPHATE

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1. Introduction

Recent work from various laboratories [1–7] has shown that glucagon can affect the kinetic behaviour of rat liver phosphofructokinase (PFK). Although it had been concluded that this might result from a phosphorylation of the enzyme [4,5], Van Schaftingen et al. [6] have presented evidence that the effect of glucagon is mainly based on the decrease in the concentration of a low molecular weight ligand, later identified as fructose 2,6-bisphosphate [8]. The nature of the compound has been confirmed by Pilkis et al. [9].

Rat liver phosphofructokinase can be inactivated by an enzyme which exhibits the characteristic features of a phosphofructokinase phosphatase (removal of ^{32}P-labelled inorganic phosphate from [^{32}P]phosphofructokinase, requirement of Mg^{2+} [10], inhibition by phosphate and inhibition by polyamines and polyarginine). Since we have found [11] that the PFK-phosphatase-catalyzed inactivation of purified rat liver phosphofructokinase could be inhibited by low concentrations of fructose 1,6-bisphosphate, we have examined in the present work the effect of fructose 2,6-bisphosphate on phosphofructokinase inactivation by PFK-phosphatase and on the spontaneous inactivation of phosphofructokinase at low pH. In addition we have studied the influence of fructose 2,6-bisphosphate on the inhibition by ATP and by citrate of purified rat liver phosphofructokinase, and compared the results with the effects of fructose 2,6-bisphosphate on purified rat liver L-type pyruvate kinase.

The results show that fructose 2,6-bisphosphate is about 50–1000 times more active in comparison to fructose 1,6-bisphosphate on all parameters tested with phosphofructokinase, but is much less active in comparison to fructose 1,6-bisphosphate with respect to L-type pyruvate kinase.

2. Materials and methods

Rat liver phosphofructokinase was purified as described earlier [12]. Rat liver phosphofructokinase phosphatase (PFK-phosphatase) was purified to apparent homogeneity (SDS-PAA electrophoresis) by a method which will be described elsewhere (Brand et al., manuscript in preparation). The activities of the PFK-phosphatase preparations used ranged from 3100 U mg^{-1} protein to 3600 U mg^{-1} protein corresponding to a purification factor over the initial high speed supernatant of about 120 000 (1 unit is defined as the activity which inactivates 1 unit of purified rat liver phosphofructokinase per min). L-type rat liver pyruvate kinase was purified according to Riou et al. [13]. The specific activity was 143 U mg^{-1}.

The conditions for testing phosphofructokinase and pyruvate kinase activities as well as for measuring phosphofructokinase inactivation are given in the legend to fig.1–5. Spontaneous inactivation of phosphofructokinase was measured as indicated in the legend to fig.2.

Biochemicals and auxilliary enzymes were purchased from the Böhringer Mannheim Corp., Mannheim, FRG; all other chemicals (analytical grade) were from E. Merck, A. G., Darmstadt, FRG. Fructose 2,6-bisphosphate was kindly donated by Professor G. Hers and Dr E. van Schaftingen, Laboratoire de Chimie Physiologique, Université Catholique de Louvain, Belgium.

3. Results

3.1. *Effects on phosphofructokinase inactivation by PFK-phosphatase*

After addition of PFK-phosphatase the V_{max} activity of purified rat liver phosphofructokinase decreased by 43% within 10 min under the chosen conditions. A control incubation under the same conditions but in the absence of PFK-phosphatase showed no change of phosphofructokinase activity. The presence of either fructose 1,6-bisphosphate or fructose 2,6-bisphosphate inhibited the enzyme-catalyzed inactivation of phosphofructokinase (fig.1) in a concentration-dependent manner. However, fructose 2,6-bisphosphate had a much higher efficiency than fructose 1,6-bisphosphate (app. $K_{i\ F\text{-}2,6\text{-}BP} \approx 3.5 \cdot 10^{-8}$ M; app. $K_{i\ F\text{-}1,6\text{-}BP} \approx 1 \cdot 10^{-6}$ M).

As fructose 1,6-bisphosphate and fructose 2,6-bisphosphate protect phosphofructokinase against inactivation by PFK-phosphatase, and since fructose 1,6-bisphosphatase is also able to inactivate phosphofructokinase [14], we have examined whether PFK-phosphatase inactivates phosphofructokinase by its ability

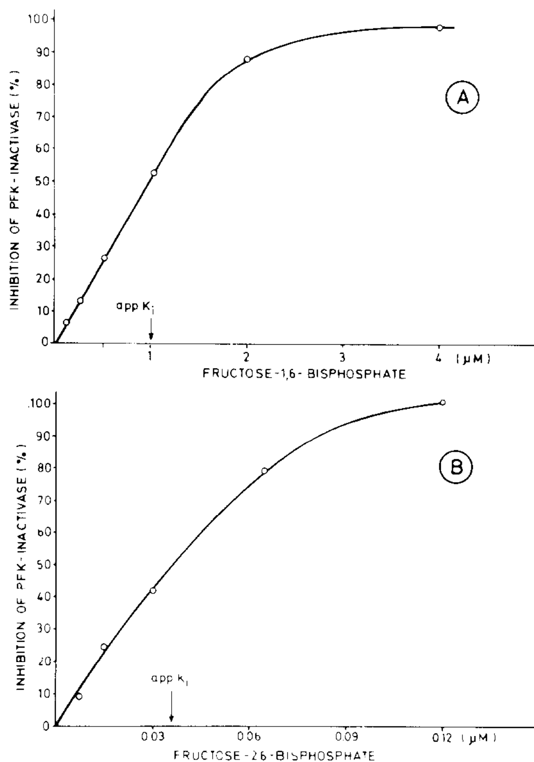

Fig.1. Concentration-dependent inhibition by fructose 1,6-bisphosphate (A) or fructose 2,6-bisphosphate (B) of PFK-phosphatase (= PFK-inactivase) catalyzed inactivation of purified rat liver phosphofructokinase. Phosphofructokinase was inactivated under the following conditions (final concentrations): K-phosphate 0.4 mM; Tris–HCl 30 mM; $MgCl_2$ 5 mM; 2-mercaptoethanol 1.5 mM; glycerol 5.1 M; phosphofructokinase 0.72 U ml^{-1}; PFK-phosphatase 0.072 U ml^{-1}. The inactivation reaction was followed over 10 min at 20°C. The final pH was 7.0. Thereafter, 10 μl were taken for the measurement of phosphofructokinase activity under optimum conditions (triethanolamine–Cl 50 mM, pH 8.0; $MgCl_2$ 2.8 mM; 2-mercaptoethanol 5 mM; $(NH_4)_2SO_4$ 40 mM; NADH 0.35 mM; 5'-AMP 1.9 mM; fructose 6-phosphate 2.1 mM; ATP 1.2 mM; aldolase 0.24 U ml^{-1}; 3-glycerophosphate dehydrogenase 2.1 U ml^{-1}; triosephosphate isomerase 6.1 U ml^{-1}).

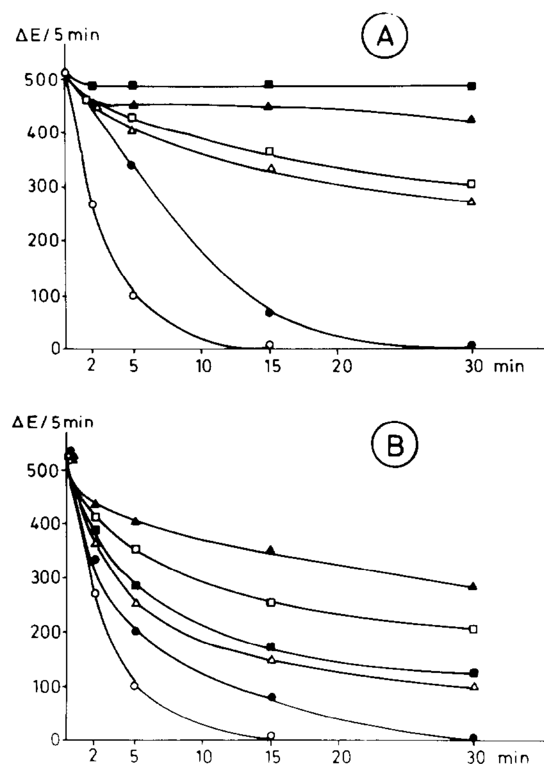

Fig.2. Inhibition of spontaneous inactivation of purified rat liver phosphofructokinase by fructose 1,6-bisphosphate (A) and fructose 2,6-bisphosphate (B). Phosphofructokinase (0.72 U ml^{-1}) was incubated for the times indicated in the figure under the following conditions (final concentrations): K-phosphate 0.4 mM; Tris–HCl 30 mM; $MgCl_2$ 5 mM; 2-mercaptoethanol 1.5 mM; glycerol 5.1 mM. The temperature was 37°C, the pH 6.5. (A) ○——○ control; ●——● 0.5 μM F-1,6-BP; △——△ 1 μM F-1,6-BP; □——□ 2 μM F-1,6-BP; ▲——▲ 3 μM F-1,6-BP; ■——■ 5 μM F-1,6-BP. (B) ○——○ control; ●——● 10 nM F-2,6-BP; △——△ 20 nM F-2,6-BP; ■——■ 30 nM F-2,6-BP; □——□ 50 nM F-2,6-BP; ▲——▲ 0.1 μM F-2,6-BP.

to act as a fructose 1,6-bisphosphatase and/or fructose 2,6-bisphosphatase. However, even in the presence of high activities of PFK-phosphatase and incubations up to 12 h, no degradation of either sugar bisphosphate could be measured (results not shown here).

3.2. *Effects on spontaneous inactivation of phosphofructokinase*

At pH 6.5 and 37°C, purified rat liver phosphofructokinase exhibits a rapid spontaneous inactivation (fig.2). This inactivation was inhibited or abolished by fructose 1,6-bisphosphate and fructose 2,6-bisphosphate in a concentration-dependent manner, fructose 2,6-bisphosphate again being much more effective than fructose 1,6-bisphosphate. Treatment of fructose 1,6-bisphosphate with 0.2 M HCl to destroy possibly contaminating fructose 2,6-bisphosphate did not diminish the effect of fructose 1,6-bisphosphate.

3.3. *Effects on inhibition of phosphofructokinase by ATP*

The inhibition of rat liver phosphofructokinase by ATP could be effectively inhibited or overcome by fructose 2,6-bisphosphate. Phosphofructokinase was almost completely inhibited by 5 mM ATP. This inhibition was already partially relieved by $4 \cdot 10^{-8}$ M fructose 2,6-bisphosphate (fig.3).

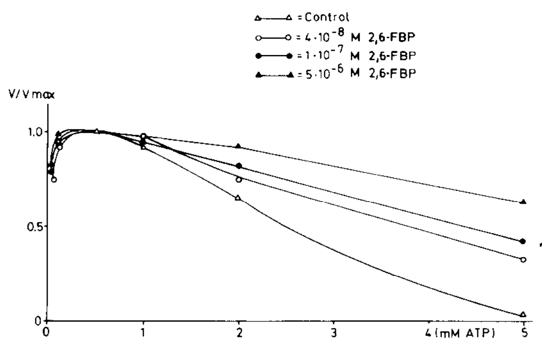

Fig.3. Effects of fructose 2,6-bisphosphate on inhibition of rat liver phosphofructokinase by ATP. The test conditions were as follows (final concentrations): Hepes 50 mM; EDTA 1 mM; $MgCl_2$ 5 mM; NH_4Cl 1 mM; NADH 0.33 mM; dithioerythritol 2.5 mM; fructose 6-phosphate 1 mM; aldolase 0.18 U ml^{-1}; 3-glycerophosphate dehydrogenase 0.72 U ml^{-1}; triosephosphate isomerase 0.8 U ml^{-1}. The final pH was 7.15, the reaction temperature 25°C. The auxilliary enzymes were desalted prior to use.

3.4. *Effects on inhibition of phosphofructokinase by citrate*

Under the conditions used (see legend to fig.4) citrate is a very strong inhibitor of rat liver phosphofructokinase. An effect of citrate via an effect on the activity of $[Mg^{2+}]$ was ruled out by the conditions selected.

In the aldolase-coupled test system for phosphofructokinase fructose 2,6-bisphosphate concentrations as low as $5 \cdot 10^{-9}$ M were able to reduce significantly the inhibitory effects of citrate. Fructose 2,6-bisphosphate at a concentration of 0.1 μM abolished almost completely the inhibitory effect of 3 mM citrate (fig.4A). Fig.4B also gives a comparison with the effect of fructose 1,6-bisphosphate in the pyruvate kinase-coupled test system. It is evident that fructose 2,6-bisphosphate is more than 1000 times more effective than fructose 1,6-bisphosphate.

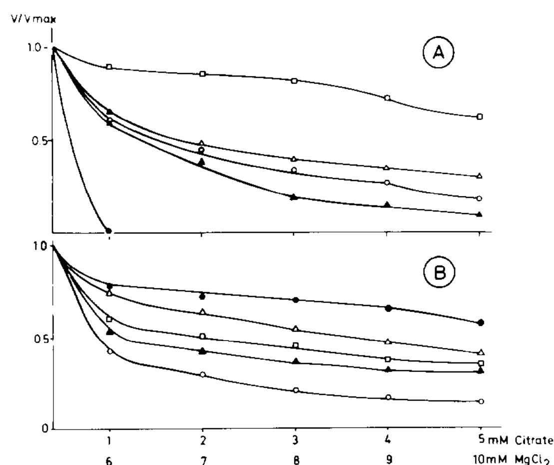

Fig.4. Effects of fructose 2,6-bisphosphate and fructose 1,6-bisphosphate on the inhibition of rat liver phosphofructokinase by citrate. The test conditions in (A) were the same as those given in the legend to fig.3, except that citrate was present and that the magnesium concentration was varied as indicated. The test conditions in (B) were as follows (final concentrations): Hepes 50mM; EDTA 1 mM; $MgCl_2$ 5 mM; NH_4Cl 1 mM; NADH 0.33 mM; dithioerythritol 2.5 mM; fructose 6-phosphate 0.56 mM; KCl 24 mM; ATP 0.47 mM; phosphoenolpyruvate 3.7 mM; phosphofructokinase 0.12 U ml^{-1}; pyruvate kinase 10 U ml^{-1}; lactate dehydrogenase 11 U ml^{-1}. The auxilliary enzymes had been desalted prior to use. Citrate and magnesium were added as given in the figure. The final pH was 7.16, the reaction temperature 25°C. Symbols in (A): ●——● control; ▲——▲ 5 nM F-2,6-BP; ○——○ 10 nM F-2,6-BP; △——△ 20 nM F-2,6-BP; □——□ 0.1 μM F-2,6-BP. Symbols in (B): ○——○ control; ▲——▲ 0.5 nM F-1,6-BP; □——□, 20 nM F-2,6-BP; △——△, 1 mM F-1,6-BP; ●——●, 0.4 μM F-2,6-BP.

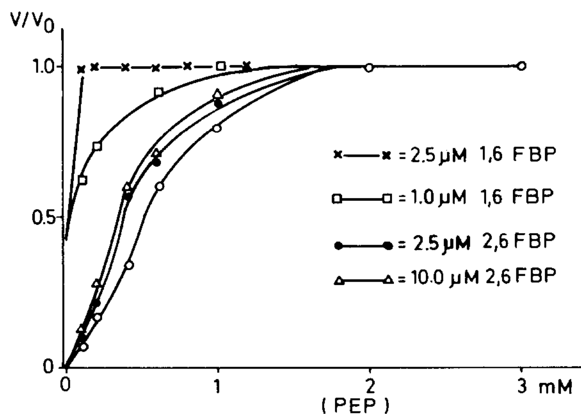

Fig.5. Comparison of the effects of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate on L-type rat liver pyruvate kinase. Test conditions (final concentrations): triethanolamine–Cl 50 mM; $MgCl_2$ 10 mM; KCl 100 mM; ADP 1 mM; NADH 0.33 mM; pyruvate kinase 0.16 U ml^{-1}; lactate dehydrogenase 2 U ml^{-1}. Phosphoenolpyruvate and the sugar bisphosphates were added as indicated in the figure. The final pH was 7.5, the reaction temperature 25°C. The reaction was started by the addition of phosphoenolpyruvate.

3.5. *Effects on L-type rat liver pyruvate kinase*

L-type pyruvate kinase exhibits a homotropic cooperativity with respect to its substrate phosphoenolpyruvate, as reflected by the sigmoidal shape of the velocity/substrate concentration curve (fig.5). The degree of cooperativity can be reduced by fructose 1,6-bisphosphate as first shown by Hess et al. [16]. This effect is depicted in fig.5. Comparing different concentrations of fructose 1,6-bisphosphate and fructose 2,6-bisphosphate it becomes evident, that fructose 2,6-bisphosphate is far less effective than fructose 1,6-bisphosphate (fig.5).

4. Discussion

The results presented are in support of a central regulatory importance of fructose 2,6-bisphosphate.

This ligand is not only a positive allosteric effector (especially against inhibition by citrate), but stabilizes liver phosphofructokinase also against inactivation by PFK-phosphatase or against spontaneous inactivation at low pH. It is not clear yet whether inactivation of phosphofructokinase by PFK-phosphatase results from the dephosphorylation itself or from a different catalytic activity of this enzyme. Experiments with in vivo ^{32}P-labelled phosphofructokinase are in progress to decide this question. There is indirect evidence that fructose 1,6-bisphosphate and fructose 2,6-bisphosphate interact with the binding of PFK-phosphatase to phosphofructokinase: immunoprecipitation of liver phosphofructokinase is inhibited in the presence of PFK-phosphatase. This inhibition is overcome completely by addition of low concentrations of fructose 1,6-bisphosphate or fructose 2,6-bisphosphate (experiments not shown here). The specificity of the metabolic actions of fructose 2,6-bisphosphate is underlined by the very low activity with respect to L-type pyruvate kinase.

In view of the recent finding of Van Schaftingen and Hers [15] that low concentrations of fructose 2,6-bisphosphate inhibit fructose-2,6-bisphosphatase it appears that this metabolite plays a specific role for the regulation of the fructose-2,6-bisphosphatase/phosphofructokinase step, most likely synergistically with 5′-AMP.

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