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Allosteric Activation and Competitive Inhibition of Yeast Phosphofructokinase by D-Fructose

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Abstract. Purified phosphofructokinase from bakers yeast is activated by D-fructose in low concentrations (up to 1 mM) and inhibited by high concentrations. The stimulatory effect of D-fructose is similar, but smaller than that of AMP. In the presence of AMP (0.4 mM or higher) D-fructose does no longer stimulate, but its inhibitory effect persists (K_I = 8 mM). Its dualistic action on phosphofructokinase activity indicates that D-fructose might induce low frequency in glycolytic oscillations by direct interaction with the enzyme.

Key words: Biochemical Oscillations — Frequency Control — D-Fructose Metabolism — Glycolysis — Phosphofructokinase — Yeast Metabolism.

Phosphofructokinase (E.C.2.7.1.11) is an important control site in glycolysis (Passoneau and Lowry, 1962; Mansour, 1963; Atkinson, 1966) and the enzyme step responsible for oscillating metabolite levels in this pathway (Ghosh and Chance, 1964). The observation of different frequencies in NADH and other metabolite oscillations with D-fructose as the substrate instead of glucose (Becker and Betz, 1972; Pye, 1973), pointed to a direct influence of D-fructose on the activity of PFK. This would fit to the fact that fructose is taken up to 5–10 times higher concentrations than glucose in the yeast cell (Becker and Betz, 1972). This paper deals with the influence of D-fructose on the kinetics of purified yeast PFK.

Methods

The enzyme was isolated from baker's yeast, using a modified combination of the methods recommended by Jauch *et al.* (1970) and Nissler *et al.* (1972). The purification procedure is summarized in Table 1.

Cristalline preparations contained: 0.1% glucose-6-phosphate isomerase, 0.1% aldolase, 0.05% triosephosphatisomerase, 0.01% ATPase and less than 0.01% of the following enzyme activities: Glyceraldehyde-3-phosphate dehydrogenase, adenylate kinase, pyruvate kinase, fructose-1.6-phosphatase and lactic dehydrogenase. Hexokinase, which was still present in the cristalline preparation had to be removed by chromatography on Cibacron blau F 3 G-A (from Ciba Corp., Basel) bound to Sephadex G 200 and subsequent chromatography on Sepharose 6B and DEAE SS, following the instructions given by Diezel *et al.* (1973).

Table 1. Purification of PFK from bakers yeast

	Vol. (ml)	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification
Grinding with sand	1000	58000	18000	0.3	1
French press	3000	58000	24000	0.41	1.3
Protamin sulfate precipitation (120 ml 5%, pH 7.2)	2900	25300	22500	0.88	2.9
$(NH_4)_2SO_4$ precipitation (50% sat., pH 7.2)	450	9500	23300	2.45	8
Aceton precipitation (40% w/v)	290	1700	10200	6.0	20
$(NH_4)_2SO_4$ precipitation (65% sat., pH 7.2)	97	910	10900	11.9	39.6
Heat inactivation (10 min 47° C)	75	520	8500	16.3	54.3
$(NH_4)_2SO_4$ precipitation (40%)	20	210	7300	34.7	115.8
Dialysis and crystallization in $(NH_4)_2SO_4$ (37%)	7	41	2470	60.2	200.6

Non Standard Abbreviations. ALD = fructosediphosphate aldolase (4.1.2.13); ATPase = ATP phosphohydrolase (3.6.1.3); DAP = dihydroxyacetonephosphate; F-6-P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate; GAP = glyceraldehydephosphate; GDH = glycerol-3-phosphate dehydrogenase (1.1.1.8); PEP = phosphoenolpyruvate; PFK = phosphofructokinase (2.7.1.11); PK = pyruvate kinase (2.7.1.40); TIM = triosephosphate isomerase (5.3.1.1.); Tris = trishydroxymethylaminomethan.

In this way contaminating hexokinase could be reduced to less than 0.1% of PFK activity.

Both products of the PFK reaction were used for measuring the activity of the enzyme. During the preparation and in most of the kinetics the reaction was followed by monitoring the oxidation of NADH coupled with FDP aldolase, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase. In those kinetic studies, where FDP had to be added to the reaction mixture, the formation of ADP was estimated in a NADH consuming reaction with pyruvate kinase and lactate dehydrogenase as auxiliary enzymes. In both cases the reaction mixture (3 ml) contained Tris-HCl buffer (80 mM) pH = 7.2, mercaptoethanol (5 mM), $MgCl_2$ (2 mM), NADH (0.3 mM) and the substrates F-6-P and ATP as indicated. The auxiliary enzymes (ALD, TIM, GDH in the first case and PK and LDH in the latter) were added in excess, to make sure that only PFK was limiting.

During enzyme preparation and in the tests on contaminating enzymes the results are expressed in international units (micromoles FDP produced/min, at 25° C). In kinetics the velocity (v) was expressed as change in fluorescence per min, extrapolated to zero time and using the same NADH concentration throughout. Care was taken that fluorescence of a standard sample was constant for the time of the experiment. It had further to be considered that different ratios of NADH were oxidised if the test was based on ADP instead of FDP.

The graphs are presented in the manner recommended by Dixon and Webb (1964) for estimating inhibitory constants (K_I). All enzymes and biochemicals were products of Boehringer, Mannheim, the other reagents of p.a. or highest purity grade were purchased from Merck, Darmstadt.

D(-) fructose recommended "für biochemische Zwecke" (Merck 5322) was found pure in thin layer chromatography.

Results and Discussion

The dependence of PFK activity on fructose concentration is demonstrated in Fig. 1 for three different concentrations of F-6-P with 0.15 mM ATP. The influence of fructose was rather complex in every case.

If F-6-P was very low (0.15 mM), fructose enhanced the reaction with concentrations up to 2 mM, whereas higher sugar concentrations became slightly inhibitory, in so far that the reaction approached its rate observable in the absence of fructose. Inhibition by higher and activation with medium sugar concentrations could be detected with higher concentrations of F-6-P too (0.24 and 0.3 mM F-6-P in Fig. 1). But with these higher substrate concentrations a slightly inhibitory effect of very low sugar concentrations (<1 mM) could be observed. This limited inhibition by low sugar concentrations was observed repeatedly but it might anywhere be an artefact and it cannot be explained without further experiments.

The stimulating effect of fructose seemed to be independent of the actual concentration of F-6-P, however, the actual reaction rate was elevated with higher F-6-P concentrations. This is in accord with the dualistic role of F-6-P, which is not only a substrate in the reaction but an allosteric effector of PFK too (Betz, 1973).

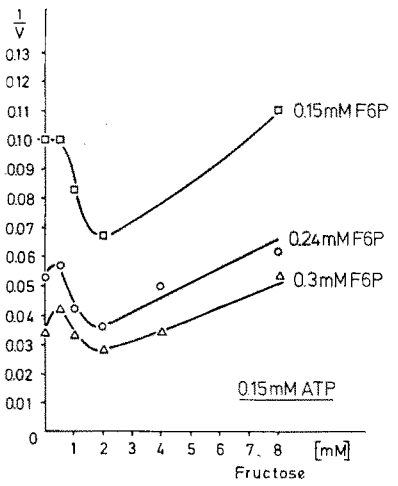

Fig. 1. The influence of D-fructose on the activity of yeast phosphofructokinase in the presence of 0.15 mM ATP (experimental details are described under "Methods")

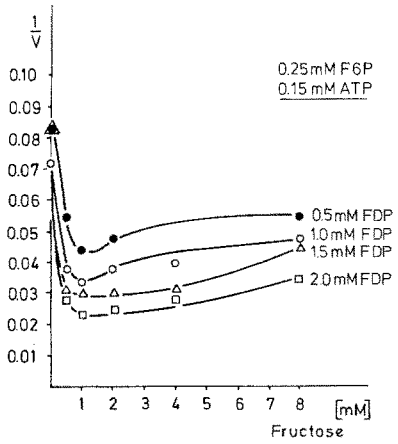

Fig. 2. Activity of yeast phosphofructokinase in the presence of D-fructose and fructose-1.6-diphosphate

In the presence of FDP which is known to activate this enzyme under certain conditions, D-fructose again stimulated with low concentrations (up to 1 mM) and became slightly inhibitory with higher concentrations (Fig. 2). The activating effect of fructose was similar to that observed with FDP alone but the inhibition with higher fructose concentrations was almost negligible in the presence of FDP. With respect to ATP even more complicated kinetics were observed (Fig. 3). The enzyme was also activated with low concentrations (up to 1 mM) of the hexose and slightly inhibited with higher sugar concentrations. The strength of the effect, however, depended mainly on the actual ATP-concentration.

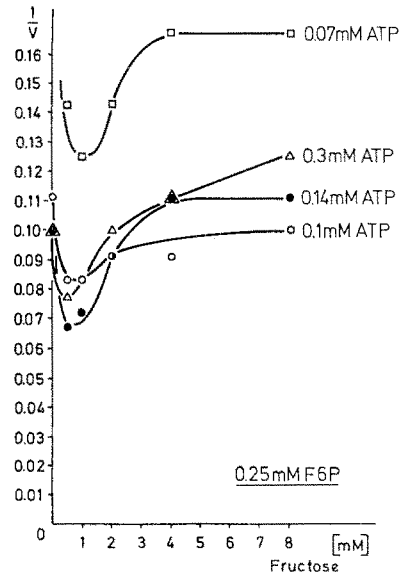

Fig. 3. The influence of D-fructose on yeast phosphofructokinase in the presence of ATP in different concentrations

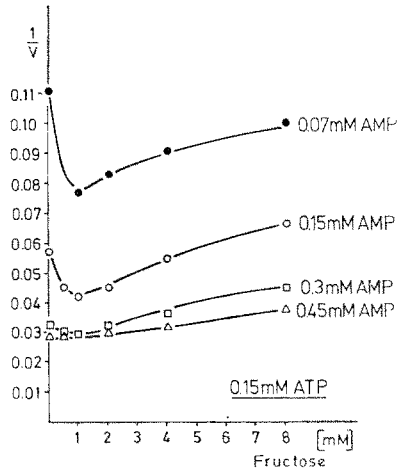

Fig. 4. The influence of D-fructose on yeast phosphofructokinase in the presence of AMP

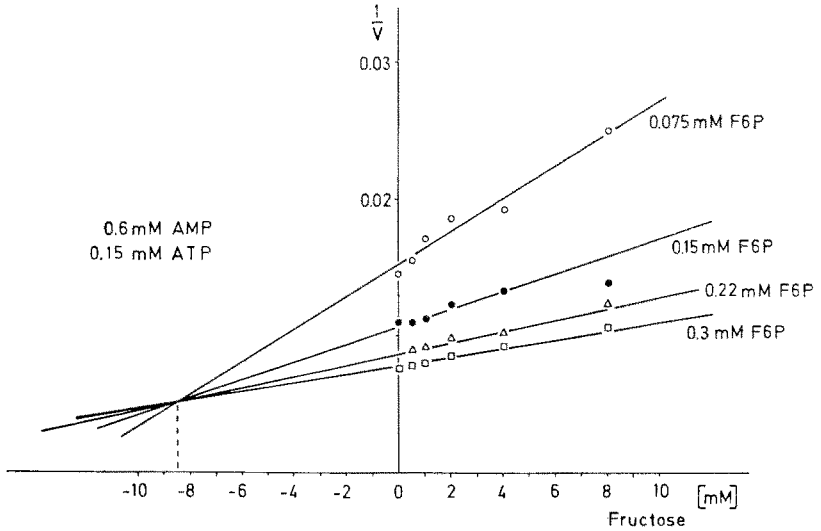

Fig. 5. In the presence of AMP in a concentration sufficient for complete activation, D-fructose is an inhibitor for phosphofructokinase, competitive with fructose-6-phosphate

The enhancing effect of fructose was rather small in the presence of low ATP concentrations (0.07 mM) since ATP is the limiting substrate. With high ATP concentrations the effect of fructose was limited too; it was obviously unable to overcome completely the inhibition by ATP. Fructose stimulated the PFK reaction most markedly at medium ATP concentrations (0.1; 0.14 mM). But with low concentrations (up to 1.5 mM) fructose was stimulatory at all concentrations of ATP. Its inhibitory effect was almost negligible.

ATP is like F-6-P not only a substrate for this enzyme, but an allosteric effector, as well. The inhibitory action of fructose should become clearer therefore if it could be followed under conditions in which allosteric interactions of ATP are excluded. For yeast PFK, AMP is not a substrate but a very efficient allosteric activator, antagonistic to ATP (Freyer *et al.*, 1970).

As seen in Fig. 4 with rising concentrations of AMP the enzyme became activated and the enhancing effect of fructose disappeared. However, the inhibitory effect with higher concentrations of the hexose persisted.

With PFK fully activated by AMP ($\geqq$0.4 mM) K_I could be determined. The result is given in Fig. 5,

from which it is evident that fructose is a competitive inhibitor of PFK, competing with F-6-P. The K_I = 8 mM fructose. The height of its K_I makes us understand that with low concentrations (beyond 2 mM) the inhibitory effect became negligible and only activation could be observed.

Conclusions

Fructose competes with F-6-P at the substrate binding site of yeast PFK. K_I is rather high (K_I = 8 mM) and the inhibitory effect is moderate only. But since in yeast cells fructose is taken up to concentrations as high as 5 mM, the effect can be significant in the control of fructose metabolism. With low concentrations ($\sim$ 1 mM) fructose stimulates PFK, which effect is similar to that of FDP but distinctly smaller than AMP. It is obvious that fructose does not only compete with F-6-P at the substrate binding site, but it is able to stimulate the enzyme similar and additive to AMP (see Fig. 4), which latter is known as its most potent allosteric effector (Betz, 1973). Depending on the concentration, fructose stimulates PFK or inhibits it. This dualistic role suggests that fructose controls the period length of glycolytic oscillations by direct interaction with PFK. The details of frequency control, however, still need further elucidation.

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