P-FRUCTOKINASE AND THE CONTROL OF THE CITRIC ACID CYCLE*

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It was reported earlier (Passonneau and Lowry, 1962a, 1962b) tha the strange kinetic properties of P-fructokinase (PFK) provide a reasonable explanation for the control of glycolysis beyond fructose-6-P (F6P). PFK is strongly inhibited by high levels of one substrate ATP (Lardy and Parks (1956), Bueding and Mansour (1957), Mansour and Mansour (1962)), and this inhibition is counteracted by the other substrate and both products as well as by AMP, P_i, and cyclic 3',5'-AMP.[1] Increases in activity of 10 or 20-fold are produced by small increases in these compounds, and combinations of some of them are particularly effective. These results have been confirmed for heart PFK (Mansour, 1963); and yeast PFK has been shown to exhibit a number of the same kinetic phenomena (Viñuéla et al., 1963).

It has now been found simultaneously in this and two other laboratories that citrate is a potent inhibitor of PFK from skeletal muscle (Parmeggiani and Bowman, 1963), heart (Garland et al., 1963),

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[1]In a more thorough study of the kinetics of PFK to be reported elsewhere it has been found that ADP, AMP and 3'5'-cyclic AMP act chiefly by enhancing activity at all ATP levels, whereas P_i, F6P, and fructose diphosphate overcome ATP inhibition. It has also been found, in confirmation of Muntz (1953), that NH_4^+ and K^+ enhance PFK activity but they are not effective in antagonizing ATP inhibition.

brain and liver (this report). Therefore PFK may control not only the rate of glycolysis but also the levels of substrates in the citric acid cycle.

EXPERIMENTAL

Most of the experiments to be described were performed with a PFK preparation purified 200-fold from sheep brain with activity of 1500 moles per kg protein per hour at 28° and pH 8. This is 15 or 20 % of that of muscle PFK prepared by Ling et al. (1955). Enzyme activity was followed directly in the fluorometer by measuring the rate of fructose diphosphate formation with the aid of aldolase, triose-P isomerase, glycero-P dehydrogenase and DPNH. The DPNH was treated with phosphatase to remove AMP, a major contaminant of commercial preparations. Amounts of enzyme were chosen to give velocities in the range of 10^{-6} to 10^{-7} moles L^{-1} min^{-1}. By thus restricting the rates, and by using high levels of muscle aldolase (30 μg per ml), very low steady state levels of fructose diphospnate were maintained. This is essential since fructose diphosphate is an exceedingly potent antagonist of both ATP and citrate inhibition. When the effects of fructose diphosphate were to be studied, the rate of formation of ADP was followed, using pyruvate kinase, lactate dehydrogenase, P-pyruvate and DPNH as auxiliary materials. In most cases the substrate was provided by a mixture of F6P and glucose-6-P together with P-glucoisomerase to maintain equilibrium.

RESULTS

Brain PFK is markedly inhibited by citrate at physiological concentrations and the degree of inhibition is increased by ATP and decreased by fructose-6-P, (Fig. 1), as Parmeggiani and Bowman found for the muscle enzyme. Under any given set of conditions the reciprocal of the velocity is a linear function of citrate

concentration (Fig. 2) from which an apparent inhibition constant, K_i, can be calculated. By variation of ATP and F6P concentrations K_i can be made to vary 100-fold (Table I) or more. Within limits K_i (ATP)/(F6P) is relatively independent of substrate concentrations, but is much greater at pH 8 than at pH 7 (Table III).

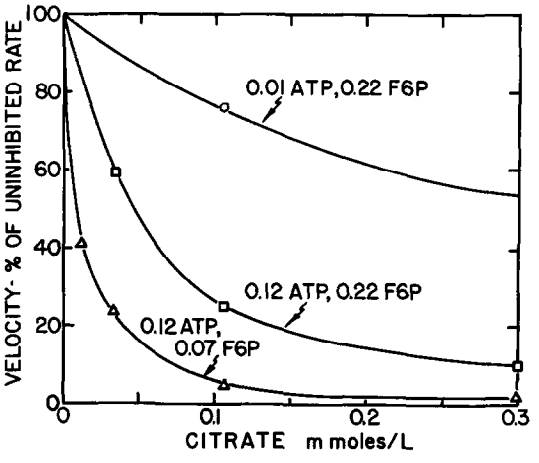

Figure 1. Velocity of brain PFK as a function of citrate concentration at several ATP and F6P levels. The assay conditions and the actual values for the uninhibited rates are given in Table I.

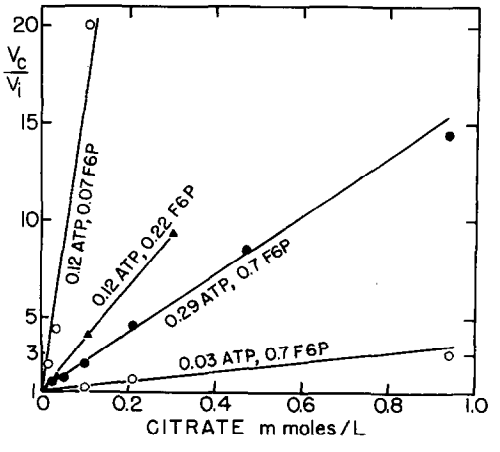

Figure 2. Ratios of control velocity, v_c, to velocity in presence of citrate, v_i, as a function of citrate concentration at several ATP and F6P levels. The assay conditions are given in Table I.

TABLE I

Effect of F6P and ATP on Citrate Inhibition of PFK

F6P	ATP	PFK velocity*			K_i		K_i (Obs.) x $\frac{ATP}{F6P}$
		Control	Citrate 0.1 mM	Citrate 1 mM	Obs.	Calc.+	
mmoles/L		moles L^{-1} $Hour^{-1}$			mmoles/L		
0.07	0.01	5.3	1.8	0.3	0.090	0.130	0.013
"	0.12	2.8	0.14		0.010	0.020	0.017
"	0.29	1.0	0.08		0.010	0.014	0.041
0.22	0.01	7.9	6.0	1.9	0.34	0.29	0.015
"	0.12	17.9	4.5	1.0	0.037	0.032	0.020
0.70	0.01	11.0	10.0**	5.4	0.95	0.89	0.014
	0.03	15.0	12.8	4.7	0.54	0.26	0.023
	0.29	26.2	10.0	1.7	0.065	0.036	0.027
	1.00	17.1	3.6	0.5**	0.030	0.018	0.039

*Activities calculated back to concentration of stock enzyme solution.
**Calculated from inhibition constants.
+Calculated assuming that
$E + F6P \longleftrightarrow E \cdot F6P$
$E + ATP \longleftrightarrow E \cdot ATP + citrate \longleftrightarrow E \cdot ATP \cdot citrate$
in which E·F6P is the active form of the enzyme. (Combination of ATP with its substrate site is not shown.) The Michaelis constants which were used for this calculation are $K_{F6P} = K_{ATP} = 0.05$ mM, and $K_{citrate} = 0.01$ mM.

The study of the mechanism of the citrate inhibition and its deinhibition is complicated by the great complexity of PFK kinetics in general. In addition to the two active substrate sites, it seems necessary to postulate two ATP inhibitor sites, and two enhancing sites (one for NH_4^+ and one for AMP).[1] It now seems necessary to postulate a separate citrate inhibitor site. In spite of the direct competition between citrate and F6P, the citrate site is probably not the F6P substrate site since P_i, AMP, and fructose disphosphate can also overcome citrate inhibition. Nor can the citrate site correspond to one of the ATP inhibitor sites since if this were the case the apparent citrate inhibition constant would be increased by raising ATP.

Inhibition by other polycarboxylic acids. Among the citric acid cycle intermediates Garland et al. (1963) found only citrate inhibitory to rat heart PFK under their assay conditions, whereas,

in the case of brain PFK, all members of the cycle tested, except fumarate, have inhibitory capacity but the K_i's are 3 to 100 times greater than for citrate (Table II). The tricarboxylic acids are the most active. Elimination of the hydroxyl group from citrate (tricarballylate) decreases activity 10-fold and maleate is at least 15-fold more active than fumarate. The only polycarboxylic acid compound found to be as active as citrate is 1,2,3,4-butanetetracarboxylate. The presence of four carboxyl groups does not in itself confer inhibitory capacity, thus 1,1,5,5-pentanetetracarboxylate is slightly stimulatory, whereas marked stimulation, particularly at pH 8, is shown by ethylenediamine tetraacetate, by N-hydroxyethyl-ethylenediamine triacetate, and by nitrilotriacetic acid. Glutamate is almost without effect.

TABLE II

Inhibitor Constants for Various Polycarboxylic Acids

PFK activity was measured at pH 8.0 in 0.02 M Tris with 3 mM ATP, 1 mM glucose-6-P, and 5 mM $MgCl_2$. The apparent inhibitor constants have been calculated assuming a linear relationship between inhibitor concentration and 1/v. Values for K_i are recorded as mmoles per liter.

Substance	K_i		Substance	K_i
Citrate	0.03		1,2,3,4-Butanetetracarboxylate	0.02
Cisaconitate	0.1		Tricarballylate	0.3
d,l-Isocitrate	0.2		Maleate	0.6
α-Ketoglutarate	2.5		Pyromellitate	0.2
Succinate	1.5		Trimesate	0.5
Fumarate	>10.		Carbonyldibenzoate	0.5
Malate	0.6		Sulfoisophthalate	0.8
			Itaconate	4.

Citrate antagonists. As shown by Parmeggiani and Bowman, P_i and AMP are capable of relieving citrate inhibition. However, the most potent citrate antagonist discovered is fructose diphosphate (Table III). Concentrations as low as 2×10^{-6} M can raise the K_i for citrate 5-fold, and higher levels can abolish citrate inhibition. AMP

is also effective at low levels, but is not capable of completely antagonizing citrate inhibition. Under most conditions P_i (at higher concentrations) is more affective than AMP, and the two substances can be shown to be synergistic (Table III). NH_4^+ shows

TABLE III

Effect of Fructose Diphosphate (FDP), AMP, P_i and NH_4^+ on Citrate Inhibition of PFK

The first experiment listed was conducted with the analytical system for measuring ADP formation, whereas in the other three experiments the system for measuring FDP formation was utilized. K_i refers to the apparent citrate inhibitor constant.

Expt.	pH	ATP	F6P	Mg	Other Addition	Control[c] velocity	K_i
		mmoles/L				mM	
1	8.0	0.09	0.11	5	None[a]	3.2	20
	"	"	"	"	Aldolase	3.0	1.9
	"	"	"	"	" , FDP,0.002[b]	3.7	10.
	"	"	"	"	FDP,0.4	8.5	30.
2	7.3	0.15	0.28	0.17	None	4.0	0.09
	"	"	"	"	AMP,0.3	4.4	0.36
	"	"	"	"	P_i,10	6.4	2.
3	8.0	0.09	0.07	5	None	4.3	1.4
	"	"	"	"	NH_4^+,10	15.7	2.6
	"	"	"	"	AMP,0.25	8.9	6.3
	"	"	"	"	P_i,10	17.	12.0
	"	"	"	"	NH_4,10; AMP, 0.25	5.7	24.5
4	7.0	0.01	0.23	5	None	5.8	0.40
	"	"	"	"	AMP,0.003	8.0	0.85
	"	"	"	"	",0.01	9.6	1.07
	"	"	"	"	",0.09	9.8	1.79
	"	"	"	"	",0.9	8.6	1.71
	"	"	"	"	P_i	8.6	0.84
	"	"	"	"	",10	17.3	1.07
	"	"	"	"	",2.0; AMP,0.09	10.5	3.8

[a]During the assay, in the absence of aldolase, a few µmoles per liter of FDP accumulated. In all samples for this experiment 10 mM NH_4Cl was present.
[b]Calculated equilibrium concentration; the initial fructose disphosphate concentration was 0.012 mM.
[c]Calculated back to concentration of stock enzyme solution, moles L^{-1} $Hour^{-1}$.

little or no specific antagonism toward citrate although it markedly increases the absolute enzyme velocity (Expt. 3, Table III). Garland et al. (1963) found that high levels of sulfate (as $(NH_4)_2SO_4$) can overcome citrate inhibition.

Liver PFK. PFK activity is very low in liver and the preparation used was only purified 5-fold, (from rat liver) to an activity of 2 moles per kg protein per hour but it was nevertheless free of interfering enzymes. It showed similar properties to brain PFK in regard to citrate inhibition, viz. 1) linearity between citrate concentration and the reciprocal of velocity, 2) increase in citrate inhibition with increase in ATP and 3) decrease in citrate inhibition with increase in F6P. Under a given set of conditions there was not much difference in the citrate inhibition constant for brain and liver PFK.

DISCUSSION

The fact that citrate can inhibit PFK provides a feedback mechanism for control of levels of metabolites in the citric acid cycle. This would require that these metabolite levels be influenced by the supply of pyruvate and acetyl-CoA. Garland et al. (1963) have found marked increases in citrate levels in hearts perfused with pyruvate, fatty acids, or keto acids, as well as in diabetes or after starvation. Parmeggiani and Bowman (1963) observed similar increases in diabetic hearts and after perfusion with fatty acids. Conversely Newsholme et al. (1962) found that perfusion with pyruvate, keto acids or fatty acids inhibits cardiac PFK in situ as evidenced by increased F6P and decreased fructose diphosphate concentration.

PFK emerges as a key enzyme subject to a multiplicity of coarse and fine adjustments. Under normal conditions its activity is kept low by ATP. When high activity is demanded (by anoxia for example) this can be brought about by a decrease in ATP and increases in P_i, AMP, possibly NH_4^+, and secondarily fructose diphosphate.

When fine control of the low normal rate is required, this can be provided by a delicate balance between citrate and F6P levels without necessary changes in ATP, P_i, or AMP. PFK is thus responsive on the one hand to the balance between $\sim$ P expenditure and $\sim$ P formation, and on the other to the metabolic mixture offered to the cell.

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