Volume 290, number 1,2, 173–176 FEBS 10191 September 1991

ADONIS 0014579391009025

Substrate antagonism in the kinetic mechanism of *E. coli* phosphofructokinase-1

Dominique Deville-Bonne, Romuald Laine and Jean-Renaud Garel

Laboratoire d'Enzymologie du CNRS, 91198 Gif-sur-Yvette, France

Received 18 June 1991; revised version received 26 July 1991

In the presence of its allosteric activator GDP, the major phosphofructokinase-1 from *Escherichia coli* K12 follows Michaelis–Menten kinetics. The kinetic behavior observed at steady-state using different concentrations of the substrates ATP and fructose-6-phosphate and the pattern of inhibition by the substrate analogs adenylyl-(β,γ-methylene)-diphosphonate and D-arabinose-5-phosphate are consistent with a random sequential mechanism in rapid equilibrium, rather than with an ordered binding as was suggested earlier. However, ATP and fructose-6-phosphate do not bind independently to the same active site, since the apparent affinity for one substrate is decreased about 20-fold when the other substrate is already bound. The antagonism between ATP and fructose-6-phosphate shows that a negative interaction occurs during the reaction with *E. coli* phosphofructokinase-1 which must be considered in addition to its allosteric properties.

Escherichia coli; Phosphofructokinase; Enzyme mechanism; Binding interaction

1. INTRODUCTION

Phosphofructokinase (PFK) catalyzes the transfer of phosphate from ATP to the 1-OH group of fructose-6-phosphate (Fru-6P) to yield ADP and fructose-1,6-bisphosphate (Fru-1,6P_2):

$$ATP + Fru\text{-}6P \rightarrow ADP + Fru\text{-}1,6P_2$$

Kinetic evidence shows that rabbit muscle PKF follows a random sequential mechanism [1], whereas the main enzyme from *Escherichia coli* K12 would respect an order in the binding of its substrates, with ATP binding before Fru-6P. The main argument in favor of this ordered mechanism is that no binding of Fru-6P to PFK could be detected by equilibrium dialysis, while binding of ATP could be measured [2]. A recent reinvestigation has shown, however, that the PFK from *E. coli* can indeed bind Fru-6P which raises the question of the actual mechanism of this enzyme.

In the present work, steady-state measurements of *E. coli* PFK activity are performed with various concentrations of both substrates to determine the order of their binding to the enzyme. To avoid interference from any cooperative process, PFK is studied in the presence of the allosteric activator GDP, where the enzyme follows Michaelis–Menten kinetics with hyperbolic saturations for both ATP and Fru-6P [3]. The presence of GDP suppresses the conformational change involved in the interactions between distant sites, so that the only interactions between the two substrates occur within one active site.

Abbreviations: Fru-6P, D-fructose-6-phosphate; Ara-5P, D-arabinose-5-phosphate; Fru-1,6P_2, D-fructose-1,6-bisphosphate; AMPPCP, adenylyl-(β,γ-methylene)-diphosphonate; PKF, phosphofructokinase (ATP-D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11)

Correspondence address: D. Deville-Bonne, Enzymologie-CNRS, 91198 Gif-sur-Yvette, France. Fax: (33) (1) 6982 3129.

2. MATERIALS AND METHODS

The activity of *E. coli* PFK, purified as described previously [4], was measured at 28°C using a buffer composed of 100 mM Tris-HCl, 10 mM magnesium acetate, 0.2 mM NADH, pH 8.2, and the auxiliary enzymes aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase [5]. Values determined using this coupled assay become less accurate for substrate concentration below 20–30 μM, since little steady-state can then be observed between the initial lag phase due to the coupling system and the final exhaustion of substrate. The concentration of ATP was set constant to a given value by an ATP regenerating system composed of creatine-phosphate and creatine kinase. In all experiments, 2 mM GDP was present to eliminate cooperative processes. GDP was used as the allosteric activator rather than ADP, because GDP binds only to the effector site, while ADP binds also to the active site and behaves as a competitive inhibitor of ATP [3]. Beside GDP, 2 mM magnesium acetate was added so that the concentration of free Mg^{2+} ions remained the same as in the absence of GDP. All the primary and secondary data related to the saturation of PFK by Fru-6P or ATP were analyzed using the program Enzfitter [6].

3. RESULTS AND DISCUSSION

In both the presence or absence of GDP, the saturation curves of PFK by ATP are hyperbolic at all Fru-6P concentrations. In the presence of 2 mM GDP, the saturation curves of PFK by Fru-6P are no longer sigmoidal

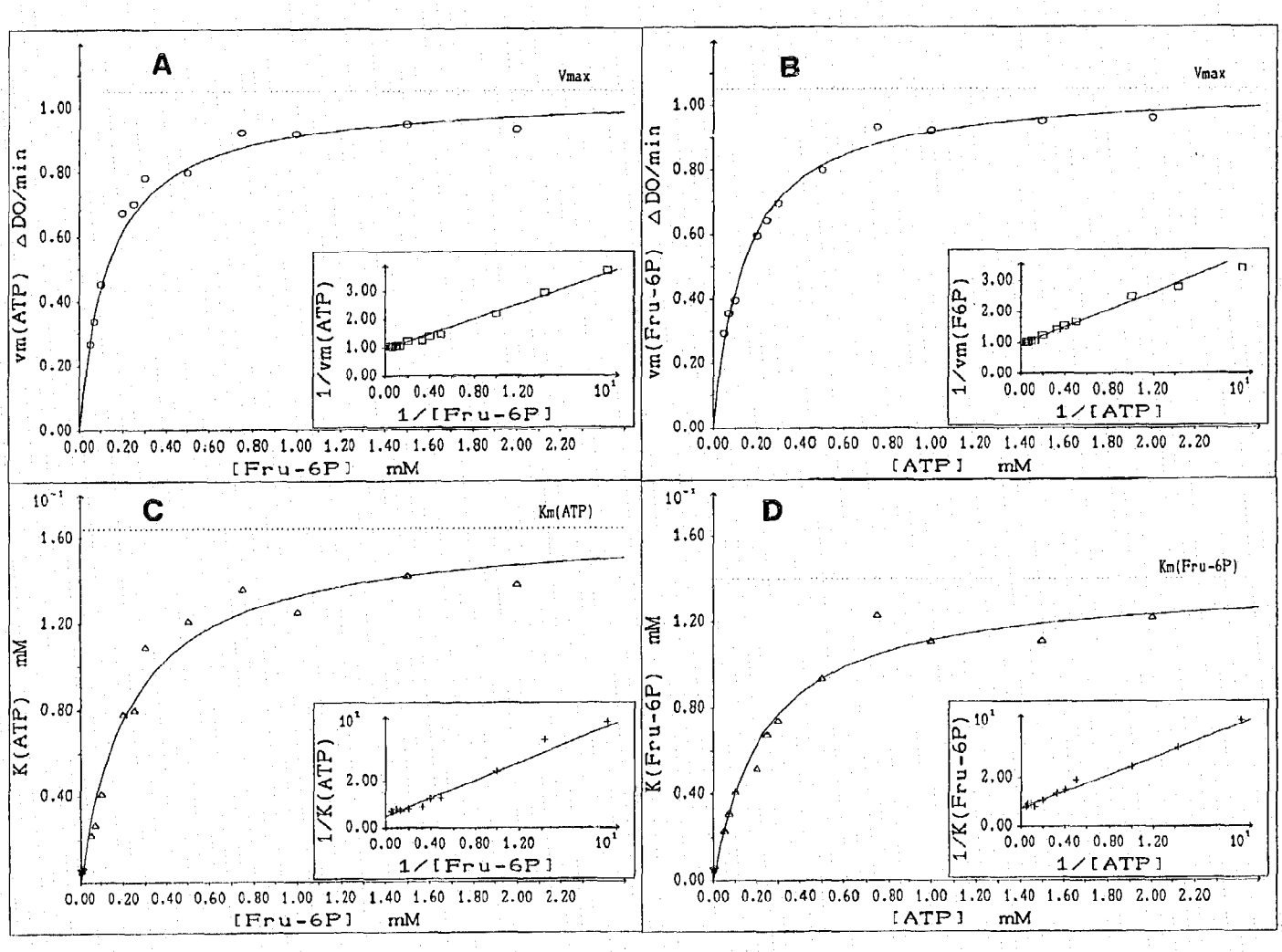

Fig. 1. Determination of the kinetic parameters of the PFK-catalyzed reaction in the presence of GDP. Initial velocities are measured with 0.3 μg/ml PFK in 100 mM Tris-HCl buffer, 10 mM magnesium acetate, at pH 8.2 and 27°C, using the coupled assay of Kotlarz and Buc [5], in which a decrease of 0.081 mM in Fru-6P concentration corresponds to a change of 1 unit in the absorbance at 340 nm. Each of the primary saturation curves for one substrate is fitted using the Michaelis–Menten equation to yield a value of v and for K. The secondary plots are: (A) v_m^{ATP} as a function of Fru-6P, (B) $v_m^{Fru\text{-}6P}$ as a function of ATP, (C) K^{ATP} as a function of Fru-6P, (D) $K^{Fru\text{-}6P}$ as a function of ATP. In each case, the inset is a double-reciprocal representation of the same data.

but hyperbolic, showing that cooperativity is abolished by the allosteric activator [3]. Attempts to fit these saturation curves with the Hill equation lead to values of the cooperativity coefficient n_H lower than 1.2 in any case, and thus all the data were analyzed according to the Michaelis equation.

The hyperbolic saturation curve of PFK by ATP, when measured at a fixed concentration of the other substrate Fru-6P, yields a value for the plateau, v_m^{ATP} and for the half-saturating concentration K^{ATP}, which are functions of Fru-6P. The values of v_m^{ATP} obtained at different Fru-6P concentrations are shown in Fig. 1A. v_m^{ATP} shows a hyperbolic dependence on Fru-6P concentration, with a half-saturating Fru-6P concentration $K_m^{Fru\text{-}6P} = 0.14 \pm 0.04$ mM. Fig. 1C shows that the half-saturating ATP concentration, K^{ATP}, increases with Fru-6P from about 0.01 mM or less at very low Fru-6P to 0.16 ± 0.05 mM at high Fru-6P. K^{ATP} is half of its maximum value for the Fru-6P concentration $K_m^{Fru\text{-}6P} = 0.24 \pm 0.07$ mM.

Similarly, the hyperbolic saturation curve of PFK by Fru-6P, at a fixed concentration of the other substrate ATP yields $v_m^{Fru\text{-}6P}$ for the plateau value and $K^{Fru\text{-}6P}$ for the half saturating concentration. Both $v_m^{Fru\text{-}6P}$ and $K^{Fru\text{-}6P}$ are functions of ATP. Fig. 1B shows the hyperbolic variation of $v_m^{Fru\text{-}6P}$ as a function of ATP, with a half-saturating ATP concentration $K_m^{ATP} = 0.15 + 0.04$ mM. $K^{Fru\text{-}6P}$ increases with ATP from about 0.01 mM or less at low ATP to 0.14 ± 0.05 mM at high ATP (Fig. 1D). $K^{Fru\text{-}6P}$ is half of its maximum value for a ATP concentration $K_m^{ATP} = 0.24 \pm 0.06$ mM. Table I shows that the values of K_m for a given substrate are similar,

Table I

Values of Michaelis constants K_m for ATP and Fru-6P obtained from the changes of v_m (midpoints of the curves in Fig. 1A and B), and from the changes of K (midpoints and plateaus of the curves in Fig. 1C and D)

	v_m	$K^{\text{other substrate}}$	
		Plateau	Midpoint
K_M^{ATP} (mM)	0.15 ± 0.04	0.16 ± 0.05	0.24 ± 0.06
$K_M^{Fru\text{-}6P}$ (mM)	0.14 ± 0.04	0.14 ± 0.05	0.24 ± 0.07

although that derived from the changes in v_m is slightly lower than from the changes in the K for the other substrate.

In the case where PFK behaves as a bireactant system in rapid equilibrium, the limit for K^{ATP} at zero concentration of Fru-6P should be equal to the equilibrium dissociation constant K_d^{ATP} of the binary complex between PFK and ATP [7,8]. Similarly, the limit for $K^{Fru\text{-}6P}$ for zero ATP concentration should be the equilibrium dissociation constant $K_d^{Fru\text{-}6P}$ of the complex PFK-Fru-6P. Although experimental accuracy does not allow a precise extrapolation of K^{ATP} to zero Fru-6P concentration, the limit of K^{ATP} at Fru-6P = 0 is consistent with the value of 0.006 mM determined by equilibrium dialysis for the dissociation constant K_d^{ATP} of the PFK-ATP complex [2] (Fig. 1C). The limiting value of $K^{Fru\text{-}6P}$ at ATP = 0 cannot be much higher than 0.01 mM (Fig. 1D), in agreement with the value of 0.125 mM found for PFK in the presence of GDP [3]. With such a low value of $K_d^{Fru\text{-}6P}$, it is surprising that direct measurements of Fru-6P binding by equilibrium dialysis have been unsuccessful [2]. Such measurements are now repeated in our laboratory, and preliminary results suggest that $K_d^{Fru\text{-}6P}$ is indeed of this magnitude. These results are consistent with a sequential mechanism in which ATP and Fru-6P bind to PFK at random, but with a negative mutual influence: the presence of the first substrate decreases the affinity for the other substrate of the same active site [7,8].

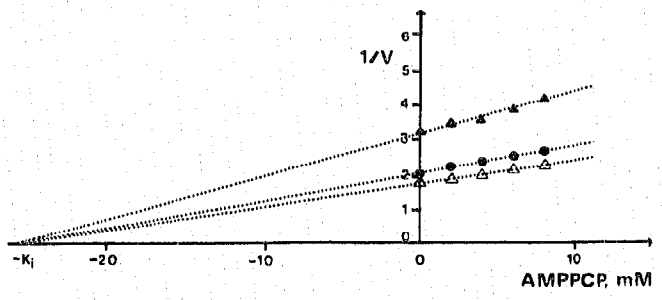

Fig. 2. Non-competitive inhibition of PFK by AMPPCP with respect to Fru-6P. Dixon reciprocal plot of the changes in the initial velocity with AMPPCP concentration measured in the presence of 0.1 mM (▲), 0.2 mM (●) or 1 mM Fru-6P (△). ATP is 1 mM, PFK concentration is 0.2 μg/ml and other conditions are as described in the legend of Fig. 1.

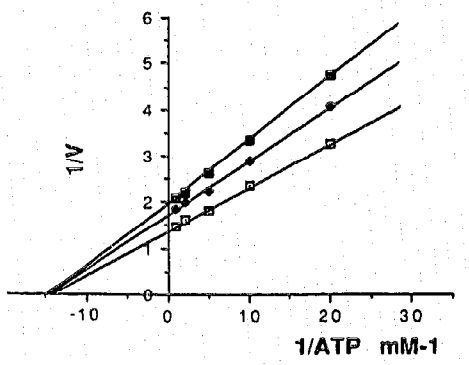

Fig. 3. Non-competitive inhibition of PFK by Ara-5P with respect to ATP. Double reciprocal plot of the changes in the initial velocity with ATP concentration in the absence (□) and presence of 10 mM (♦) or 20 mM Ara-5P (■). Fru-6P is 1 mM, PFK concentration is 0.2 μg/ml and other conditions are as described in the legend of Fig. 1.

Inhibition of PFK by substrate analogs is also consistent with such a mechanism. PFK activity is inhibited by adenylyl-(β,γ-methylene)-diphosphonate (AMPPCP), a non-hydrolyzable analog of ATP and by D-arabinose-5-phosphate (Ara-5P), a 5-carbon analog of Fru-6P. Inhibition by AMPPCP is competitive with respect to ATP at saturating Fru-6P, and non-competitive with respect to Fru-6P at saturating ATP (Fig. 2). Similarly, inhibition by Ara-5P is competitive with respect to Fru-6P at saturating ATP, and non-competitive with respect to ATP at saturating Fru-6P (Fig. 3). The values of the inhibition constants K_i are given in Table II. This inhibition pattern, which has been observed also for Ara-5P and AMPPCP with the PFK from rabbit muscle [1], shows that both inhibitors can bind to either the free enzyme or a binary complex and thus that the enzyme does not obey a ordered mechanism [7,8].

4. CONCLUSION

In the presence of GDP, the only interaction between the two substrates of PFK is that within an active site no cooperativity is observed. The kinetic behavior of *E. coli* PFK then resembles that of rabbit muscle PFK [1], and corresponds to a sequential mechanism for a random bireactant system in rapid equilibrium [7,8].

Table II

Type of inhibition and values of K_i determined by AMPPCP and Ara-5P

Inhibition by:	With respect to:	
	ATP	Fru-6P
AMPPCP K_i =	1.8 mM (C)	25 mM (NC)
Ara-5P K_i =	50 mM (NC)	6 mM (C)

(C), competitive; (NC), non-competitive.

The binding of ATP and Fru-6P to PFK takes place at random, but not independently of each other. There is a negative interaction within the same active site between the two substrates, which decreases about 20 times the apparent affinity for one substrate when the other is already bound. Such antagonism between ATP and Fru-6P could be due to steric hindrance and/or electrostatic repulsion, or to a conformational change of the protein. X-ray crystallography has shown that two conformations can be taken by the active site, an 'open' and a 'closed' one, which correspond to intermediates along the reaction pathway [9]. PFK is different from yeast hexokinase, in which a positive synergistic interaction exists between the two substrates [10]. In the absence of GDP, the saturation of PFK by ATP remains the same, but that by Fru-6P is markedly modified and becomes cooperative with a much higher half-saturating concentration, $[Fru\text{-}6P]_{0.5}$ = 0.35 mM [3]. A negative influence between Fru-6P and ATP has also been observed recently in the absence of GDP, where PFK behaves as an allosteric enzyme [11]. Therefore the apparent affinity for Fru-6P of a given active site in PFK is sensitive to: (i) the binding of ATP at the same site, through the antagonistic substrate interaction, (ii) the binding of Fru-6P at the other remote active sites, through the cooperative homotropic interactions [3], and (iii) the binding of effectors at the distant regulatory sites, through the allosteric heterotropic interactions [3,12]. Further studies are now in progress to determine the actual contribution of the three types of ligand interactions which superimpose in the complex regulatory properties of *E. coli* PFK.

Acknowledgements: The authors are grateful to Sybille Bachelier and Gisèle Le Bras for their help in several experiments, and to Dr Philip R. Evans for making available the crystallographic coordinates of PKF and many stimulating discussions. This work has been supported by Grants UPR 2401 from CNRS and 927-03 from Université Paris 6.

REFERENCES

[1] Hanson, R.L., Rudolph, F.B. and Lardy, H.A. (1973) J. Biol. Chem. 248, 7852–7859.
[2] Blangy, D. (1971) Biochimie 53, 135–149.
[3] Blangy, D., Buc, H. and Monod, J. (1968) J. Mol. Biol. 31, 13–35.
[4] Deville-Bonne, D., Le Bras, G., Teschner, W. and Garel, J.R. (1989) Biochemistry 28, 1917–1922.
[5] Kotlarz, D. and Buc, H. (1982) Methods Enzymol. 90, 60–70.
[6] Leatherbarrow, R.J. (1987) Enzfitter: A Non-linear Regression Data Analysis Program for the IBM PC. Elsevier, Amsterdam.
[7] Segel, I.H. (1975) in: Enzyme Kinetics. Wiley, pp. 273–345.
[8] Cleland, W.W. (1990) in: The Enzymes, 3rd Edn. (D.S. Sigman and P.D. Boyer, eds.) Vol. 19, pp. 99–158.
[9] Shirakihara, Y. and Evans, P.R. (1988) J. Mol. Biol. 204, 973–994.
[10] Viola, R.E., Raushel, F.M., Rendina, A.R. and Cleland, W.W. (1982) Biochemistry 21, 1295–1302.
[11] Kundrot, C.E. and Evans, P.R. (1991) Biochemistry 30, 1478–1484.
[12] Schirmer, T. and Evans, P.R. (1990) Nature 343, 140–145.