Protein Science (1992), *1*, 254–258. Cambridge University Press. Printed in the USA.

pH dependence of the reverse reaction catalyzed by phosphofructokinase I from *Escherichia coli*: Implications for the role of Asp 127

ISABELLE AUZAT AND JEAN-RENAUD GAREL
Laboratoire d'Enzymologie du CNRS, 91198 Gif-sur-Yvette, France
(RECEIVED July 24, 1991; ACCEPTED September 23, 1991)

Abstract

The kinetics of the reverse reaction catalyzed by *Escherichia coli* phosphofructokinase, i.e., the synthesis of ATP and fructose-6-phosphate from ADP and fructose-1,6-bisphosphate, have been studied at different pH values, from pH 6 to pH 9.2. Hyperbolic saturations of the enzyme are observed for both substrates. The affinity for fructose-1,6-bisphosphate decreases with pH following the ionization of a group with a pK of 6.6, whereas the catalytic rate constant and perhaps the affinity for ADP are controlled by the ionization of a group with a pK of 6. Several arguments show that the pK of 6.6 is probably that of the carboxyl group of Asp 127, whereas the pK of 6 is tentatively attributed to the carboxyl group of Asp 103.

The pK of 6.6 is assigned to the carboxyl group of Asp 127 in the free enzyme, and a simple model suggests that the same group would have an abnormally high pK, above 9.6, in the complex between phosphofructokinase and fructose-1,6-bisphosphate. It is proposed that the large pK shift of more than 3 pH units upon binding of fructose-1,6-bisphosphate is due to an electrostatic repulsion that could exist between the 1-phosphate group and the carboxyl group of Asp 127, which are close to each other in the crystal structure of phosphofructokinase (Shirakihara, Y. & Evans, P.R., 1988, *J. Mol. Biol. 204*, 973–994). The same interpretation would also explain the much higher affinity of the enzyme for fructose-1,6-bisphosphate when Asp 127 is protonated. Because of its pK above 9.6, Asp 127 would be protonated in the complex between phosphofructokinase and fructose-1,6-bisphosphate, and could act as a proton donor, in agreement with its catalytic role as a proton acceptor in the forward reaction (Hellinga, H.W. & Evans, P.R., 1987, *Nature 327*, 437–439).

Keywords: active site; enzymatic catalysis; pH dependence; phosphate transfer; phosphofructokinase

Phosphofructokinase (PFK) catalyzes the transfer of the γ-phosphate from ATP to the 1-OH group of fructose-6-phosphate (Fru-6-P):

$$\text{Fru-6-P} + \text{ATP} \rightarrow \text{Fru-1,6-P}_2 + \text{ADP}.$$

The inversion of the configuration of the transferred phosphate group (Jarvest et al., 1981) and the structure of the complex between the enzyme, Fru-6-P, and ADP (Kinemages 1, 2; Shirakihara & Evans, 1988) suggest that this transfer is direct and occurs by an in-line nucleophilic attack of the 1-OH group on the γ-phosphate of ATP. It has been proposed that the carboxylate group of Asp 127 enhances the reaction rate by acting as a base that removes the proton from the 1-OH group of Fru-6-P and thus makes the oxygen a stronger nucleophile (Hellinga & Evans, 1987). Replacement of Asp 127 by a serine decreases the catalytic efficiency of PFK by four orders of magnitude, without markedly changing the affinity for the substrates (Hellinga & Evans, 1987). The pH dependence of the catalytic rate constant of PFK shows that an ionizable group must be deprotonated for PFK to be active: this group has a pK of 7.1 in the absence of allosteric effector and of 6.6 in the presence of the activator GDP (Deville-Bonne et al., 1991). This crucial group with a pK of 6.6–7.1 could be the carboxyl group of Asp 127, in agreement with its role as a base in catalysis.

If indeed the side chain of Asp 127 acts as a base in the forward reaction, microscopic reversibility dictates that this residue acts as an acid in the reverse reaction:

$$\text{Fru-1,6-P}_2 + \text{ADP} \rightarrow \text{Fru-6-P} + \text{ATP}.$$

Reprint requests to: Jean-Renaud Garel, Laboratoire d'Enzymologie du CNRS, 91198 Gif-sur-Yvette, France.

That Asp 127 is also crucial for the reverse reaction is shown by the reduction by three to four orders of magnitude of the catalytic rate constant in this direction of the Asp → Ser 127 mutant of PFK (Hellinga & Evans, 1987). The participation of Asp 127 as an acid catalyst in the reverse reaction requires its protonated form for PFK to be active. In this case, raising the pH should deprotonate Asp 127 and thus inactivate PFK. In order to check this possibility and assess the actual role of Asp 127 in the catalytic mechanism of PFK, we have measured the pH dependence of the kinetics of the reverse reaction.

Results and discussion

At all pH values, the saturation of PFK by either substrate, ADP or Fru-1,6-P_2, was hyperbolic and followed Michaelis–Menten kinetics. This kinetic behavior is probably due to the presence of ADP, which is needed as a substrate but is also an allosteric activator of the direct reaction of PFK (Blangy et al., 1968). In the presence of 10 mM Mg^{2+} ions, the ionization of Fru-1,6-P_2 occurs with a pK around 5 (unpubl.) and is outside the studied pH range of 6–9.2. In the case of a single-substrate enzyme obeying the Michaelis–Menten mechanism, the pH dependences of $1/K_m$ or k_{cat}/K_m reflect the ionization(s) occurring within the free enzyme, whereas the pH dependences of k_{cat} or K_m are related to the ionization(s) occurring within the enzyme–substrate complex (Fersht, 1985). This simple analysis can be used here assuming that the substrates bind to PFK rapidly and independently and that the only monomolecular step is the slow rate-limiting phosphate transfer step. Under these restrictive hypotheses, the Michaelis constant K_m for one substrate will be equal to the equilibrium constant of its dissociation from the enzyme, and the kinetically determined pKs will directly reflect the ionization of specific groups in particular species. This hypothesis of a sequential random mechanism operating at quasi-equilibrium may not be strictly valid for PFK (Hellinga & Evans, 1987; Shirakihara & Evans, 1988; Deville-Bonne et al., 1991), but it is the simplest that can be done.

The Michaelis constant for Fru-1,6,-P_2, K_m(Fru-1,6-P_2), determined at saturating ADP, increases with pH. Figure 1 shows that $1/K_m$(Fru-1,6-P_2) decreases with pH

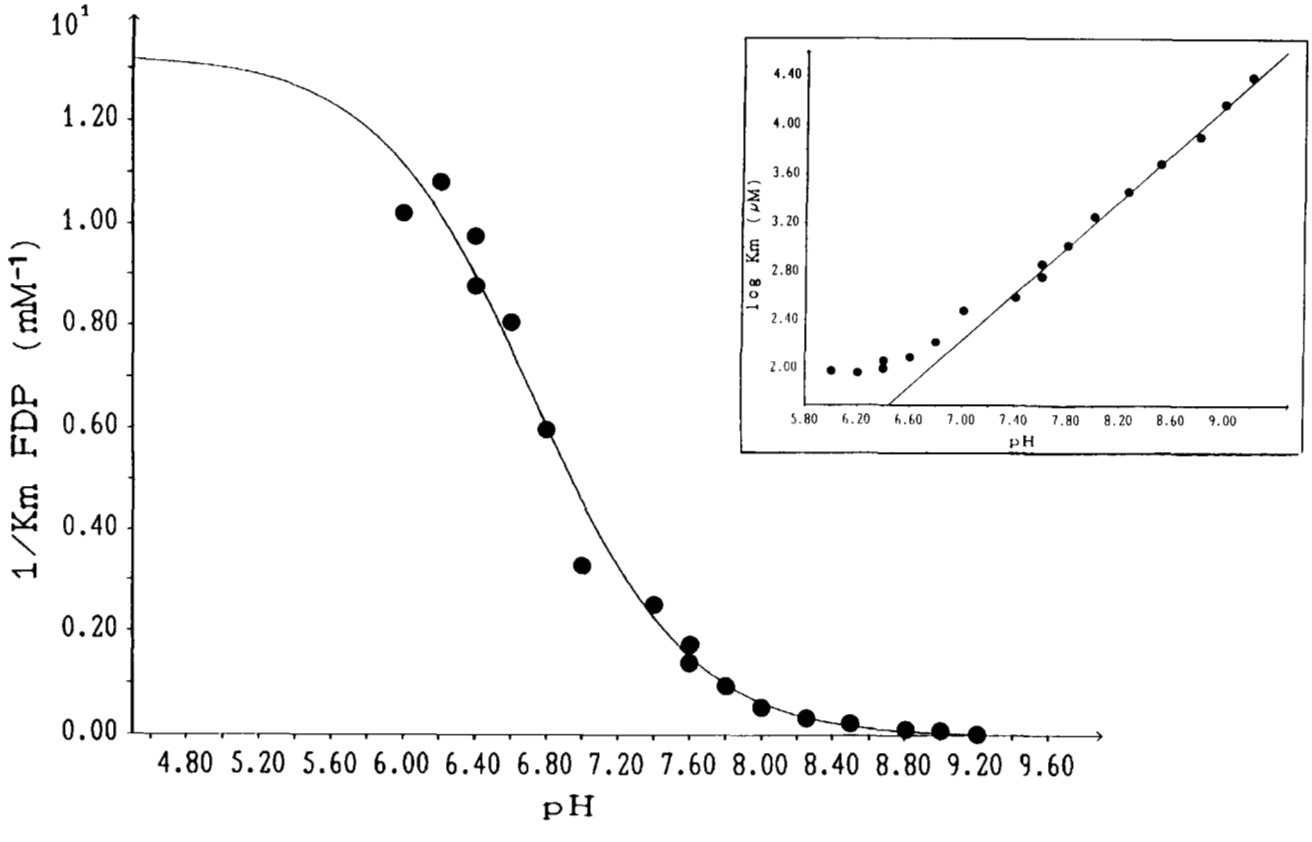

Fig. 1. The variation of $1/K_m$(Fru-1,6-P_2) (FDP) with pH at a fixed concentration of ADP of 2 mM. The solid line corresponds to the titration curve of a single ionizable group with a pK of 6.6 and limiting values at low pH and high pH of 1.32×10^4 M^{-1} and 0 for $1/K_m$(Fru-1,6-P_2), respectively, i.e., of 75 μM at low pH and ∞ at high pH for K_m(Fru-1,6-P_2). **Inset**: The variation of log K_m(Fru-1,6-P_2) with pH. The straight line at high pH has a slope of 1, showing that K_m(Fru-1,6-P_2) increases by a factor of 10 for each pH unit, and thus that the decrease of $1/K_m$(Fru-1,6-P_2) is controlled by the unique titration curve corresponding to the pK of 6.6.

following the ionization of a single group with a pK of 6.6 ± 0.2, indicating that an ionizable group of PFK has a pK of 6.6 in the free enzyme and must be protonated to allow binding of Fru-1,6-P_2. A limiting value of 75 ± 10 μM for K_m(Fru-1,6-P_2) at low pH is obtained by extrapolation using the assumption that the pH dependence of $1/K_m$(Fru-1,6-P_2) obeys a simple titration curve. Above pH 7.5 and up to pH 9.2, K_m(Fru-1,6-P_2) increases by a factor of 10 for each pH unit (as seen from the plot of log K_m(Fru-1,6-P_2) vs. pH given in the inset of Fig. 1), confirming that Fru-1,6-P_2 is bound only by the small fraction of protonated form of the group with a pK of 6.6 and not by its deprotonated form, even at a Fru-1,6-P_2 concentration of 60 mM. Therefore, the deprotonation of the group with a pK of 6.6 decreases the affinity of PFK for Fru-1,6-P_2 by three orders of magnitude or more.

Such a pH-dependent affinity of PFK for the sugar substrate is not observed for Fru-6-P in the direct reaction (Deville-Bonne et al., 1991), which suggests that the ionization of the group of pK 6.6 does not have the same influence on the binding of the substrate Fru-6-P and of the product Fru-1,6-P_2. It is possible that the group with the pK of 6.6 acquires a negative charge upon deprotonation, which repels the negative phosphate group at position 1 in Fru-1,6-P_2 but not the neutral OH group of Fru-6-P. The three-dimensional structure of PFK indicates that the group of pK 6.6 could be the carboxyl group of Asp 127 (Kinemage 2; Shirakihara & Evans, 1988). Indeed, electrostatic repulsion between deprotonated Asp 127 and Fru-1,6-P_2 has been proposed as an aid in the release of products (Hellinga & Evans, 1987). Therefore PFK activity in the reverse reaction would require the protonated state of the carboxyl group of Asp 127 for both binding Fru-1,6-P_2 and acid-catalyzing the phosphate transfer to ADP.

Contrary to expectation, no pH-dependent decrease of the catalytic rate constant k_{cat} corresponding to a possible deprotonation of Asp 127 is observed for the reverse reaction of PFK. Instead, k_{cat} increases with pH following the ionization of a single group with a pK of 6 ± 0.2 and shows no decrease up to pH 9.2 (Fig. 2). There is an ionizable group in PFK with a pK of 6, which must be deprotonated for the enzyme to be active (Fig. 2), and it is unlikely that this group of pK 6 belongs to Asp 127 because a carboxyl group cannot act as an acid catalyst when deprotonated.

The pH-dependent decrease in k_{cat} expected upon deprotonation of Asp 127 is not observed (Fig. 2). A first explanation is that this expectation was unjustified because the role of Asp 127 in the reverse reaction is not that of an acid catalyst, and thus the protonated form of its carboxyl group is not required for PFK activity. Another possibility could be that Asp 127 acts indeed as an acid catalyst but that its carboxyl group does not ionize within the studied pH range, between pH 6 and pH 9.2. Under the above assumption of rapid substrate binding

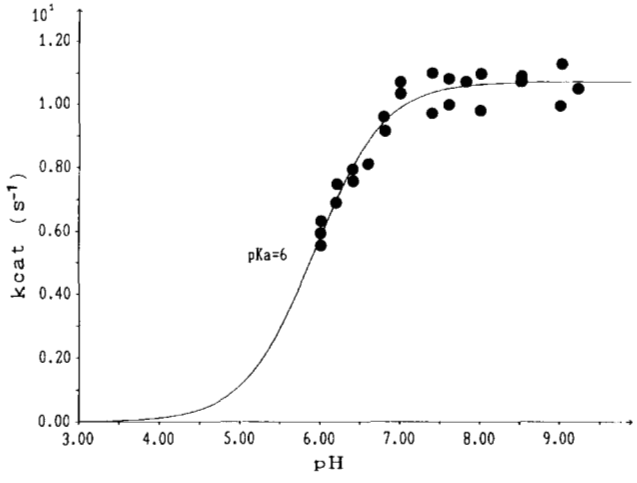

Fig. 2. pH dependence of the catalytic rate constant k_{cat}. Between pH 6 and pH 8.5, the values of k_{cat} are determined at saturating concentrations of both substrates Fru-1,6-P_2 and ADP. Because of the low affinity of PFK for Fru-1,6-P_2 above pH 8.5, extrapolation to saturating Fru-1,6-P_2 is needed to determine the values of k_{cat} at high pH. The solid line corresponds to the titration curve of a single ionizable group with a pK of 6.0 and limiting values of k_{cat} at low and high pH of 0 and 10.8 s^{-1}.

followed by a slow chemical step, one simple model representing the interaction between PFK, Fru-1,6-P_2, and one proton on Asp 127 could be as follows:

$$\begin{array}{ccc} H^+ + PFK + \text{Fru-1,6-}P_2 & \overset{K'_m}{\rightleftharpoons} & H^+ + \text{PFK-Fru-1,6-}P_2 \\ \Big\uparrow\Big\downarrow K_e & & \Big\uparrow\Big\downarrow K_{es} \\ \text{H-PFK} + \text{Fru-1,6-}P_2 & \underset{K_m}{\rightleftharpoons} & \text{H-PFK-Fru-1,6-}P_2 \\ & & \Big\downarrow k_{cat} \\ & & \text{products} \end{array}$$

In this scheme (1) all forms of PFK are saturated by ATP, (2) K_m and K'_m, the Michaelis constants for Fru-1,6-P_2 of protonated and deprotonated PFK, respectively (with K_m = 75 μM), can be treated as thermodynamic dissociation constants, (3) K_e and K_{es} are the dissociation constants of the proton from Asp 127 in free PFK and in the PFK–Fru-1,6-P_2 complex, respectively (with $K_e = 4 \times 10^{-7}$ M and pK_e = 6.6), and (4) only protonated PFK is catalytically active. The apparent affinity of PFK for Fru-1,6-P_2 increases by at least 3 orders of magnitude upon deprotonation of the group of pK 6.6 (see above), so the ratio K'_m/K_m is larger than 10^3. Because $K_m \cdot K_e = K'_m \cdot K_{es}$, the observation that $K'_m/K_m > 10^3$ implies that $K_e/K_{es} > 10^3$, that is $(pK_{es} - pK_e) > 3$ pH units, and thus that $pK_{es} > 9.6$. In the complex between PFK and Fru-1,6-P_2, the pK of the proton of Asp 127 would have a pK above 9.6, an unusually high pK value for a carboxyl group, and would therefore not dissociate in the pH range from 6 to 9.2 studied here.

In the crystal structure of PFK, the carboxyl group of

Asp 127 is close to the 1-phosphate group of Fru-1,6-P_2 (Shirakihara & Evans, 1988). A reasonable interpretation is that binding Fru-1,6-P_2 by the deprotonated form of Asp 127 would be difficult because an electrostatic repulsion could exist between these negative groups. If so, the same electrostatic repulsion would make the dissociation of the proton from Asp 127 more difficult and shift its pK by more than 3 pH units, from 6.6 to above 9.6.

Between pH 7.5 and pH 8.5, Asp 127 would be deprotonated in free PFK ($pK_e = 6.6$) and inactive as a proton donor. One possibility for PFK to protonate and become catalytically active for the reverse reaction would then be that a large increase in the pK of Asp 127 ($pK_{es} > 9.6$) occurs upon Fru-1,6-P_2 binding. The binding of Fru-6-P to PFK is not accompanied by such a large pK shift of Asp 127, which has a pK of 6.6–7.1 in the complex between PFK and Fru-6-P (Deville-Bonne et al., 1991). Above pH 7.5, its carboxyl group is deprotonated and can act as a base (Hellinga & Evans, 1987), so that PFK is catalytically active for the forward reaction.

The second group that has a pK of 6 and controls the pH dependence of k_{cat} (Fig. 2) is unknown. The value of 6 for its pK indicates that it could be a carboxyl group, and the three-dimensional structure of PFK suggests that the deprotonated forms of the carboxyl groups of Asp 103, Asp 129, and Glu 222 are required for PFK to be active (Shirakihara & Evans, 1988). A possible candidate could be the carboxyl group of Asp 103, which binds the Mg^{2+} ion (Kinemage 3; Shirakihara & Evans, 1988). In the reaction between ADP and Fru-1,6-P_2, the Mg^{2+} ion participates first in the binding of ADP, and then bridges the β-phosphate of ADP and the 1-phosphate of Fru-1,6-P_2 in order to facilitate phosphate transfer (Shirakihara & Evans, 1988). Therefore if the group of pK 6 is Asp 103, its ionization should not only affect k_{cat} but also K_m(ADP), as both depend upon the proper interaction of the negative charge with the Mg^{2+} ion. The Michaelis constant for ADP, K_m(ADP), determined at saturating Fru-1,6-P_2, does not depend on pH above 6.8, with a value around 50 μM, and increases at acidic pH, being 3–4 times higher at pH 6 than at neutral pH (Fig. 3). Although the experimental accuracy is limited, the changes of K_m(ADP) around pH 6 are compatible with the ionization of a group with a pK of 6.1 (Fig. 3).

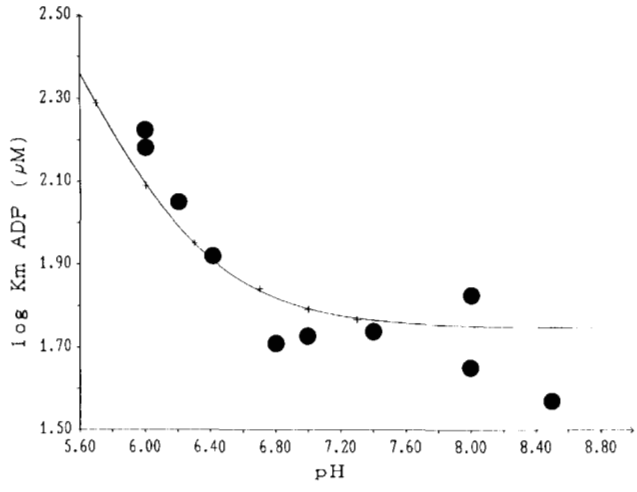

Fig. 3. The variation of log K_m(ADP) with pH. The concentration of Fru-1,6-P_2 ranges from 1 mM at pH 6 to 40 mM at pH 8.5, so that it is saturating. The solid line corresponds to the titration of a single ionization group with a pK of 6.1 and limiting values for K_m(ADP) of ∞ and 55 μM at low and high pH.

Conclusions

The present results show that two ionizable groups are important for the activity of PFK. The first group is probably the carboxyl group of Asp 127, which has a pK of 6.6 in free PFK and of 6.6–7.1 in the complex between PFK and Fru-6-P (Deville-Bonne et al., 1991). A simple model suggests that the pK of Asp 127 in the Fru-1,6-P_2 complex could be higher than 9.6. A value above 9.6 would be one of the highest reported for the pK of a carboxyl group in proteins and would be consistent with an electrostatic repulsion between two negatively charged groups, deprotonated Asp 127 and 1-phosphate of Fru-1,6-P_2 within the active site of PFK (Shirakihara & Evans, 1988). The same electrostatic interpretation would also explain the higher affinity for Fru-1,6-P_2 of the neutral form of Asp 127 (Fig. 1). At pH values between 7.5 and 8.5, the pK shift of more than 3 pH units that might occur upon Fru-1,6-P_2 binding would induce proton uptake by Asp 127, so that its protonated carboxyl group could acid catalyze the phosphate transfer from Fru-1,6-P_2 to ADP. The same pK shift does not take place upon binding of Fru-6-P (Deville-Bonne et al., 1991), so that Asp 127 remains deprotonated and can base catalyze the phosphate transfer from ATP to Fru-6-P (Hellinga & Evans, 1987). It would be because the pKs of Asp 127 are different in the complexes of PFK with Fru-1,6-P_2 and Fru-6-P that the same carboxyl group could be both a proton acceptor in the forward reaction and a proton donor in the reverse reaction, and that the enzyme could satisfy the requirement of microscopic reversibility.

The second ionizable group has a pK of 6 in the PFK–substrates complex and has been tentatively attributed to the carboxyl group of Asp 103. This group must be deprotonated for an efficient catalysis of phosphate transfer (Fig. 2) and a higher affinity for ADP (Fig. 3). It is likely that the forward reaction between ATP and Fru-6-P also requires that Asp 103 be ionized (Shirakihara & Evans, 1988). If the pK of Asp 103 is around 6 and lower than that of 6.6–7.1 of Asp 127 (Deville-Bonne et al., 1991), this group will not appear in the pH dependence of the forward reaction as it requires both residues to be deprotonated. This role for Asp 103 is still hypothetical but could be tested by a combination of site-directed mu-

tagenesis and studies of the pH dependences of the mutant PFKs.

Materials and methods

Materials

PFK was prepared as described previously (Deville-Bonne et al., 1989), and its activity in the synthesis of Fru-1,6-P_2 was measured using the coupled assay of Kotlarz and Buc (1982). The concentration of PFK was determined according to Bradford (1976) using immunoglobulins as a standard. The auxiliary enzymes and substrates used in this assay and in that of the reverse reaction were obtained from Boehringer.

Methods

The synthesis of Fru-6-P in the reverse reaction of PFK was coupled to the formation of NADPH using the auxiliary enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The ATP produced was regenerated into ADP using glycerol kinase and glycerol. The activity was determined from the increase in absorbance at 340 nm upon formation of NADPH in the following conditions: 0.5–3 μg/mL PFK, 18 μg/mL phosphoglucose isomerase, 18 μg/mL glucose-6-phosphate dehydrogenase, 30 μg/mL glycerol kinase, 0.8 mM $NADP^+$, 3 mM glycerol, 10 mM $MgCl_2$, 100 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), 51 mM *N*-ethylmorpholine, 51 mM diethanolamine. The tribuffer composed of *N*-ethylmorpholine, MES, and diethanolamine has the advantage of maintaining an almost constant ionic strength over a wide range of pH values (Ellis & Morrison, 1982). Under these assay conditions, the absorbance at 340 nm corresponds to the initial velocity of the PFK-catalyzed reaction as judged from (1) the linear increase with time of $A_{340\ nm}$ during several minutes at any concentration of substrates Fru-1,6-P_2 and ADP, (2) the strict proportionality between the rate of increase in $A_{340\ nm}$ and the concentration of PFK, and (3) the fact that this rate is independent of a twofold change in the amount of any auxiliary enzyme. The coupled assay is valid in the whole pH range studied, from 6 to 9.2. Below pH 6, the low activity of the auxiliary enzymes results in a marked lag phase before a steady state is reached. Above pH 9, the coupled assay becomes dubious because of the instability of one or several auxiliary reagents. Analysis of the kinetic data using the Enzfitter program (Leatherbarrow, 1987) showed that the saturation of PFK by either Fru-1,6-P_2 or ADP was hyperbolic, and that the pH dependences of the catalytic rate constant k_{cat} and of the Michaelis constants for ADP and Fru-1,6-P_2 could be fitted to the titration curve of a single ionizable group.

Acknowledgments

The authors are grateful to D. Deville-Bonne, G. Le Bras, and R. Lainé for many interesting discussions and for their help in some experiments, and to P.R. Evans for the open communication of his results about PFK. This work has been supported by grants from the CNRS (UPR 2401) and Université Paris 6 (927-0300).

References

Blangy, D., Buc, H., & Monod, J. (1968). Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. *J. Mol. Biol. 31*, 13–35.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.

Deville-Bonne, D., Bourgain, F., & Garel, J.-R. (1991). pH dependence of the kinetic properties of allosteric phosphofructokinase from *Escherichia coli*. *Biochemistry 30*, 5750–5754.

Deville-Bonne, D., Le Bras, G., Teschner, W., & Garel, J.-R. (1989). Ordered disruption of subunit interfaces during the stepwise reversible dissociation of *Escherichia coli* phosphofructokinase with KSCN. *Biochemistry* 28, 1917–1922.

Ellis, K.J. & Morrison, J.F. (1982). Buffers of constant ionic strength for studying pH-dependent processes. *Methods Enzymol. 87*, 405–426.

Fersht, A.R. (1985). *Enzyme Structure and Mechanism*, 2nd Ed., pp. 155–175. W.H. Freeman, New York.

Hellinga, H.W. & Evans, P.R. (1987). Mutations in the active site of *Escherichia coli* phosphofructokinase. *Nature 327*, 437–439.

Jarvest, R.L., Lowe, G., & Potter, B.V.L. (1981). The stereochemical course of phosphoryl transfer catalysed by *Bacillus stearothermophilus* and rabbit skeletal-muscle phosphofructokinases with a chiral (^{16}O, ^{17}O, ^{18}O)phosphate ester. *Biochem. J. 199*, 427–432.

Kotlarz, D. & Buc, H. (1982). Phosphofructokinases from *Escherichia coli*. *Methods Enzymol. 90*, 60–70.

Leatherbarrow, R.J. (1987). *Enzfitter: A Non-linear Regression Data Analysis Program for the IBM PC*. Elsevier Science Publishers BV, Amsterdam.

Shirakihara, Y. & Evans, P.R. (1988). Crystal structure of the complex of phosphofructokinase from *Escherichia coli* with its reaction products. *J. Mol. Biol. 204*, 973–994.