pH Dependence of the Kinetic Properties of Allosteric Phosphofructokinase from *Escherichia coli*†

Dominique Deville-Bonne,* Florence Bourgain,‡ and Jean-Renaud Garel
Laboratoire d'Enzymologie, CNRS, F-91198 Gif-sur-Yvette, France
Received July 18, 1990; Revised Manuscript Received December 27, 1990

ABSTRACT: The pH dependence of the activity of the allosteric phosphofructokinase from *Escherichia coli* has been studied in the pH range from 6 to 9, in the absence or presence of allosteric effectors. The sigmoidal cooperative saturation of phosphofructokinase by fructose 6-phosphate has been analyzed according to the Hill equation, and the following results have been obtained: (i) the apparent affinity for Fru-6P, as measured by the half-saturating concentration, $[Fru\text{-}6P]_{0.5}$, does not change with pH; (ii) the cooperativity, as measured empirically by the Hill coefficient, n_H, increases markedly with pH and reaches a value of 5.5–6 at pH 9; (iii) the catalytic rate constant, k_{cat}, is controlled by the ionization of a critical group which has a pK of 7 in the absence of effector and must be deprotonated for phosphofructokinase to be active. The observation that pH affects both the cooperativity and the maximum velocity suggests that the catalytic efficiency of a given active site could be modified by the binding of fructose 6-phosphate to other remote sites. Finding values of the cooperativity coefficient larger than the number of substrate binding sites indicates that slow conformational changes may occur in phosphofructokinase. The cooperative saturation of phosphofructokinase by fructose 6-phosphate appears more complex than described by the classical concerted model at steady state and could involve two slowly interconverting states which differ in both their turnover rate constants and their affinities for fructose 6-phosphate. The presence of GDP shifts the pK of the critical group which controls k_{cat} from 7 to 6.6. This decrease in pK results in an activation of phosphofructokinase by GDP at saturation in fructose 6-phosphate below pH 8. The presence of GDP also increases k_{cat} by 10–20% independently of pH. This increase in k_{cat} as well as the pH-mediated activation by GDP occurs in addition to the increase in the affinity of phosphofructokinase for fructose 6-phosphate upon the classical allosteric transition.

Phosphofructokinase (PFK)[1] catalyzes the reaction:

ATP + fructose 6-phosphate → ADP + fructose 1,6-bisphosphate

In *Escherichia coli*, the enzyme present as the major species is a tetramer of four identical subunits of 34 817 daltons each (Blangy et al., 1968; Hellinga & Evans, 1985). The sequence of the 320 amino acids of the polypeptide chain has been deduced from the nucleotide sequence of the corresponding gene, *pfk A* (Hellinga & Evans, 1985), and the three-dimensional structure of *E. coli* PFK has been solved at a resolution of 0.24 nm (Shirakihara & Evans, 1988; Rypniewsky & Evans, 1989). When determined by steady-state activity measurements, the saturation of PFK by its substrate fructose 6-phosphate (Fru-6P) is highly cooperative, while the saturation by ATP is hyperbolic. In addition, the activity of PFK is regulated by different allosteric effectors: it is activated by the purine nucleoside diphosphates ADP or GDP and inhibited by phosphoenolpyruvate (PEP) (Blangy et al., 1968). Because of this highly regulated behavior, PFK is thought to be one of the key enzymes controlling the flux through the glycolytic pathway (Uyeda, 1979).

A thorough analysis of the steady-state kinetics of *E. coli* PFK at pH 8.2 has shown that its enzymatic properties were consistent with the concerted allosteric mechanism (Blangy et al., 1968). However, this detailed image of the enzymatic behavior of PFK results from investigations performed under a limited set of conditions. The present study focuses on the influence of pH (in the range from pH 6 to pH 9) on the steady-state kinetic properties of PFK, and several results suggest that the simple concerted allosteric model (Blangy et al., 1968; Blangy, 1971) may not be sufficient to describe the enzymatic properties of PFK.

Materials and Methods

Chemicals. All chemicals were of analytical grade. The auxiliary enzymes used in the PFK coupled assay (aldolase, triosephosphate isomerase, glycerol-phosphate dehydrogenase, and creatine kinase) as well as GDP, PEP, and phosphocreatine were from Boehringer Mannheim; ATP, DTT, Fru-6P, and NADH were from Sigma. Other reagents were from Merck.

Purification of PFK. PFK was prepared from HE_1 cells transformed with the plasmid pHL_1 as previously reported (Deville-Bonne et al., 1989) with the following modifications: after being loaded with the cell extract, the Blue Dextran–agarose column was washed with 0.5 M NaCl in phosphate buffer (50 mM phosphate buffer, pH 7.6, containing 1 mM magnesium acetate and 2 mM DTT) before elution of the enzyme by 2 mM ATP–Mg^{2+} in the same buffer. For enzymatic activity measurements, the $(NH_4)_2SO_4$ precipitate stored enzyme was extensively dialyzed against three changes of 1.5-L total volume of 50 mM imidazole hydrochloride buffer containing 1 mM magnesium acetate and 2 mM DTT, pH 7. Protein concentration was determined from the molar ab-

† This work was supported by grants from the Centre National de la Recherche Scientifique (LP 2401) and the Université Paris 6 (Division de Biochimie 92703).
* Address correspondence to this author.
‡ Present address: Laboratoire de Génétique Moléculaire, Ecole Normale Supérieure, 46, rue d'Ulm, 75005 Paris, France.

[1] Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); DTT, DL-dithiothreitol; Fru-6P, D-fructose 6-phosphate; PEP, phosphoenolpyruvate.

0006-2960/91/0430-5750$02.50/0

sorbance coefficient (Kotlarz & Buc, 1982).

Measurements of PFK Activity. The enzymatic activity of PFK was measured at 28 °C by using a coupled assay for the formation of fructose 1,6-bisphosphate, in which 0.2 mM NADH, 10 mM magnesium acetate, and the auxiliary enzymes aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase are present in the assay mixture (Kotlarz & Buc, 1982). The concentration of ATP was set constant to the saturating value of 1 mM by an ATP-regenerating system composed of creating phosphate and creatine kinase. Instead of the usual Tris buffer, a buffer composed of 0.1 M MES, 0.051 M *N*-ethylmorpholine, and 0.051 M diethanolamine (MND buffer) was used (Ellis & Morrison, 1982). This MND mixture of three components has the advantage of maintaining an almost constant ionic strength over a wide pH range. The pH was adjusted by either HCl or NaOH using a combined electrode coupled to a microprocessor-equipped Knick 763 pH meter.

It was observed that the saturation of PFK by Fru-6P measured at pH 8.2 in the MND tribuffer system is slightly more cooperative than that in Tris buffer at the same pH, with a Hill coefficient, n_H, around 4.5 instead of values of 3.8–4 reported previously (Blangy et al., 1968; Kotlarz & Buc, 1977; Le Bras & Garel, 1982). The half-saturating concentration of Fru-6P has the same value in the two buffers at pH 8.2, $[\text{Fru-6P}]_{0.5}$ = 0.4 mM. It was verified that the stability of PFK and the auxiliary enzymes was unaffected by the replacement of Tris buffer by the MND mixture and that the coupled assay is a valid measurement of activity in all of the studied pH range.

In some experiments, 2 mM GDP was also present in order to transform the sigmoidal saturation of PFK by Fru-6P into a hyperbolic one. 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 (Blangy et al., 1968). Beside GDP, 2 mM magnesium acetate was also added so that the concentration of free Mg^{2+} ions remained the same as in the absence of GDP.

Kinetic Analysis of the Data. The saturation curves of PFK by Fru-6P were analyzed by using the Hill equation. The three parameters related to a Hill sigmoid curve: (i) the turnover rate constant at saturating Fru-6P, k_{cat}; (ii) the half-saturating concentration, $[\text{Fru-6P}]_{0.5}$, and (iii) the cooperativity coefficient, n_H, were determined from the experimental data either manually by using a double-logarithmic plot or by nonlinear regression analysis with a computer [an IBM-AT compatible computer equipped with the program Enzfitter (Leatherbarrow, 1987) or an Apple IIe equipped with a locally written program (Tauc, 1987) were used]. The values obtained for k_{cat}, $[\text{Fru-6P}]_{0.5}$, and n_H from these three different procedures were not always identical, but the differences were within experimental error.

The variations of k_{cat} with pH were analyzed according to the titration curve of a single group with a limiting value of zero at acidic pH. The values for plateau at alkaline pH and the p*K* were also determined by computer fitting.

Results

The shape of the saturation curve of PFK by Fru-6P changes when the pH is varied between 6 and 9, as shown in Figure 1 for three saturation curves obtained at pH 6.2, 7.3, and 8.3 and normalized between pH 0 and 1. We have chosen not to use the concerted model (Monod et al., 1965) to describe the sigmoidal saturation curves of PFK for two reasons. First, this model contains very specific assumptions about the mechanism responsible for the cooperative binding (Blangy et al., 1968), which may not be valid for PFK under all conditions. Second, the representation of a cooperative saturation curve is rather complex and requires the adjustment of three parameters. The pH-dependent changes of the saturation curves were analyzed by using the Hill equation, which provides the simplest way to represent the sigmoidal curves observed for the cooperative binding of ligands by proteins. Only two parameters are needed to determine the fraction saturated: the midpoint or half-saturating concentration, $S_{0.5}$, and the maximum slope or Hill cooperativity coefficient, n_H (Hill, 1910). The Hill equation is only a mathematical description and contains no mechanistic hypothesis about the origin of cooperativity, and we have used it as a simple phenomenological description of the behavior of PFK. Two features of each sigmoidal saturation curve can be quantitated: $S_{0.5}$, which can be taken as an empirical measure of the apparent affinity for the ligand, and n_H, which indicates the cooperativity of its binding.

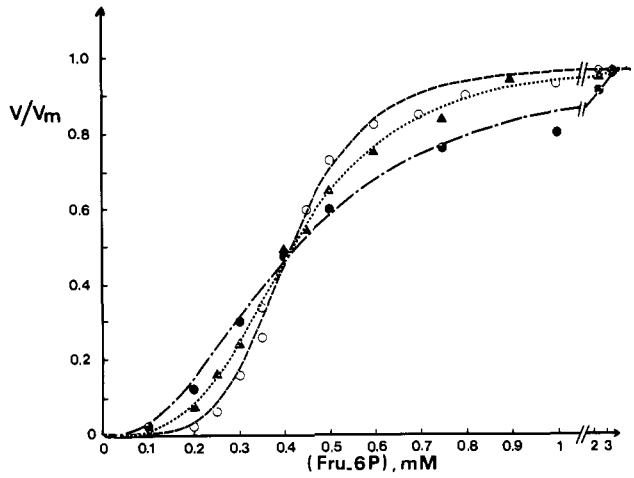

FIGURE 1: pH dependence of the cooperativity of the saturation of PFK by Fru-6P. The results obtained for the saturation of PFK by Fru-6P at pH 6.2 (●), 7.3 (▲), and 8.3 (○) are shown after normalization to the same overall amplitude. The curves are drawn according to the Hill equation using cooperativity coefficients n_H of 2.25, 3.1, and 4.7, respectively.

pH Dependence of the Half-Saturating Concentration $[\textit{Fru-6P}]_{0.5}$. The half-saturating concentration $[\text{Fru-6P}]_{0.5}$, which reveals the apparent affinity of PFK for Fru-6P, does not depend on pH between pH 6 and pH 9 (Figure 2), with an average value of $[\text{Fru-6P}]_{0.5}$ = 0.41 ± 0.05 mM. $[\text{Fru-6P}]_{0.5}$ is certainly related to the binding constant of Fru-6P to PFK. Fru-6P is a charged substrate which ionizes with a p*K* of 6.1, and which binds to an enzyme in which a catalytic residue has a p*K* of 7.0 (see below). It is therefore surprising that the binding of Fru-6P to PFK is independent of the changes in the ionization states of either interacting partners which occur between pH 6 and pH 9.

The concentration of Fru-6P needed for half-saturation of PFK is also independent of pH in the presence of 2 mM GDP. The average value of $[\text{Fru-6P}]_{0.5}$ is 60 ± 10 μM, except at pH 6 where a slightly higher value, $[\text{Fru-6P}]_{0.5}$ = 90 ± 10 μM, is obtained (Figure 2). Although the value of 60 μM for $[\text{Fru-6P}]_{0.5}$ at pH 8.2 is slightly higher than that of 12 μM reported by Blangy et al. (1968), these results agree with their conclusion that the presence of GDP increases the affinity of PFK for Fru-6P.

At pH 8.2, the saturation of PFK by the other substrate ATP is hyperbolic in both the absence and presence of GDP (Blangy et al., 1968; Le Bras & Garel, 1982). The same is

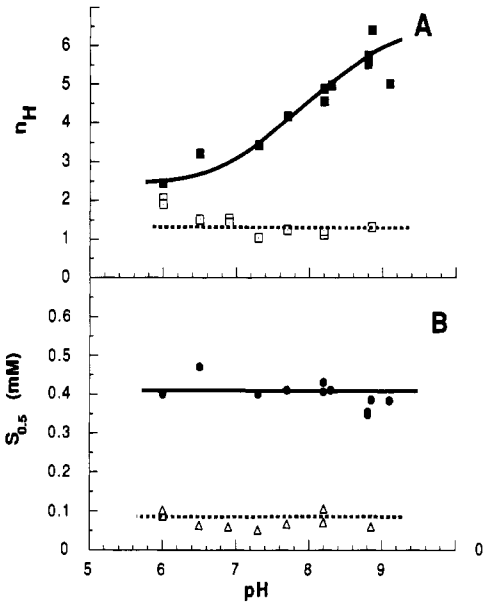

FIGURE 2: pH dependence of (A) the Hill cooperativity coefficient, n_H, and the apparent affinity, $[Fru\text{-}6P]_{0.5}$, of PFK. At each pH value, the activity of PFK in MND buffer was measured as a function of Fru-6P concentration using 1 mM ATP. The values of $[Fru\text{-}6P]_{0.5}$ and n_H were obtained by fitting each saturation curve to the Hill equation. Closed symbols, no effector added; open symbols, 2 mM GDP.

found between pH 6 and 9, although PFK seems to have a slightly higher affinity for ATP at pH 6 than at pH 9 (results not shown). The concentration of 1 mM ATP used in all the present experiments is largely sufficient to saturate PFK.

pH Dependence of the Cooperativity Coefficient n_H for the Saturation of PFK by Fru-6P. In the presence of the allosteric activator GDP, the saturation of PFK by Fru-6P at pH 8.2 is no longer cooperative but hyperbolic (Blangy et al., 1968). Such a hyperbolic saturation by Fru-6P was observed in almost all pH ranges studied, with an average value of the Hill coefficient $n_H = 1.1 \pm 0.2$ (Figure 2). Only at pH 6, a value of $n_H = 1.9 \pm 0.3$ indicates that some residual cooperativity could still exist.

In the absence of GDP, contrary to $[Fru\text{-}6P]_{0.5}$ which remains constant between pH 6 and pH 9, the cooperativity of the saturation of PFK by Fru-6P increases with pH (Figure 2). The value found for the Hill coefficient increases from $n_H = 2\text{–}2.5$ at pH 6 to $n_H = 5.5\text{–}6$ at pH 9 (Figure 2). There is too much scatter in the values of n_H calculated at different pH values to determine whether the pH dependence of n_H is governed by a linear increase or by a sigmoidal titration-like curve. It is unexpected that the Hill coefficient reaches values larger than the number of Fru-6P binding sites, $n_H = 5.5\text{–}6$ as compared to 4 sites.

pH Dependence of the Catalytic Rate Constant k_{cat} of PFK in the Absence and Presence of Allosteric Effector. The catalytic rate constant k_{cat} of PFK has been determined at different pH values by using the Hill equation for extrapolation to saturating concentrations of both substrates Fru-6P and ATP. Figure 3 shows that the turnover constant k_{cat} varies with pH and that its pH dependence follows the titration curve of one ionizable group with a p$K = 7.1 \pm 0.1$. Various attempts to fit the experimental data suggest that the catalytic efficiency of PFK is reduced at least 20-fold upon protonation of this group. The maximum slope of the k_{cat} vs pH curve shows that the number of protons involved is 1 ± 0.3. PFK is a tetramer, and any ionizable group is present on each of the four chains. The overall pH profile of a single group

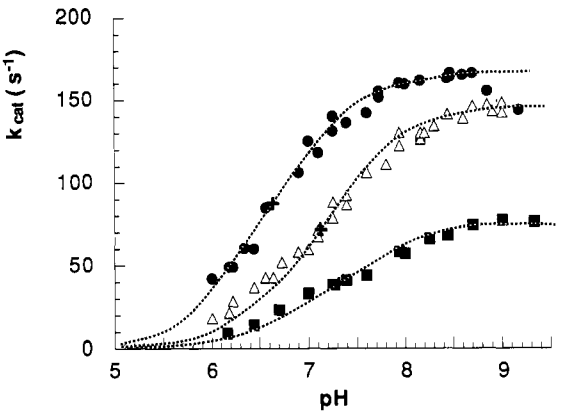

FIGURE 3: pH dependence of the catalytic rate constant k_{cat} of PFK. The values of the catalytic constant k_{cat} were determined by extrapolation to saturation in Fru-6P using the Hill equation in the presence of (△) no allosteric effector or (●) 2 mM GDP. Also shown is the turnover rate constant measured (■) at 1 mM Fru-6P in the presence of 3 mM PEP. The dashed curves correspond to the ionization of a single group with a pK of 7.1 in the absence of effector, of 6.6 in the presence of 2 mM GDP, and of 7.0 in the presence of 3 mM PEP.

suggests that the four critical groups with a pK of 7.1 ionize independently and that the deprotonated state of these groups is required for PFK to be catalytically active.

The catalytic rate constant of PFK in the presence of GDP is also pH dependent: k_{cat} increases with pH according to the titration curve of one ionizable group with a p$K = 6.6 \pm 0.1$ (Figure 3). As above, this result shows that one critical group, within each subunit, needs to be deprotonated for PFK to be catalytically active. These four groups ionize independently so that the pH profile of k_{cat} reveals the involvement of one proton. It is likely that this critical group is the same as that with the pK of 7.1 which controls the catalytic efficiency of PFK in the absence of GDP. Figure 3 also shows the pH dependence of the enzymatic activity of PFK measured at a single Fru-6P concentration of 1 mM in the presence of 3 mM PEP. The same inhibition of about 50% is observed at 3 mM PEP independently of pH. Under these conditions, the activity of PFK varies with pH according to the titration curve of a group with a pK of 7.0 ± 0.2.

The pK of the ionizable group which controls k_{cat} is shifted from 7.1 to 6.6 in the presence of GDP, suggesting that the binding of the allosteric activator induces a conformational change in the protein. Because of this pK shift of 0.5 pH unit, there is more PFK deprotonated and thus active in the presence of GDP than in its absence. The addition of GDP to PFK at constant pH therefore increases k_{cat}. This pH-mediated activation by GDP is not important above pH 8, where PFK is fully active in the absence of GDP. It becomes significant as the pH decreases: below pH 6, the addition of GDP raises the fraction of deprotonated PFK, and thus k_{cat}, by a factor 2.5–3. Figure 4 shows the pH dependence of the activation by GDP of the catalytic efficiency of PFK. This activation occurs by a different mechanism than that analyzed in detail at pH 8.2, where GDP activates PFK by increasing the apparent affinity for Fru-6P (Blangy et al., 1968). It has also been reported that the activity of PFK is increased by 15–20% by the presence of GDP even at a saturating concentration of Fru-6P [S. A. Berger, personal communication; quoted by Rypniewsky and Evans (1989)], and this is indeed the case up to pH 8.6 (Figures 3 and 4).

The pH dependence of the activity of PFK was measured by using different concentrations of Fru-6P as a substrate. An average value of 6.95 ± 0.10 is obtained for the pK of the

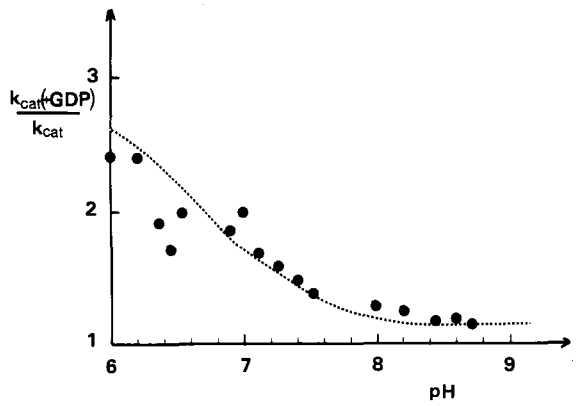

FIGURE 4: pH dependence of the activation by GDP of the catalytic constant of PFK. The activation by GDP is expressed as the ratio between the activities of PFK measured at saturation in Fru-6P either in the presence of 2 mM GDP or without effector. The curve corresponds to the difference between two titration curves, one with a pK of 6.6 and the other with a pK of 7.05 and an amplitude smaller by 15%.

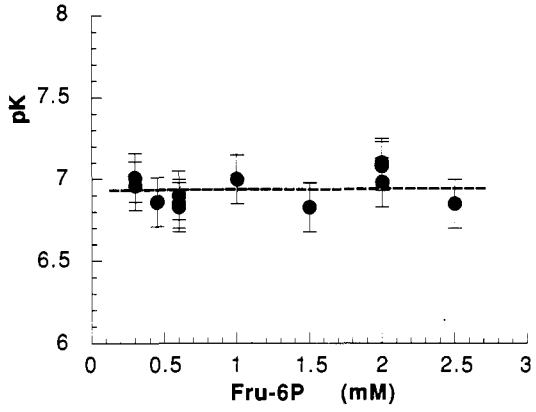

FIGURE 5: Dependence on Fru-6P concentration of the pK of the ionizable group which controls the catalytic efficiency of PFK. The pH dependence of the enzymatic activity was measured at various Fru-6P concentrations, and the pK value corresponding to each concentration of Fru-6P was determined by fitting this dependence to the titration curve of a single group.

catalytic residue of PFK for Fru-6P concentrations below or above $[Fru\text{-}6P]_{0.5}$ (Figure 5). This value is not significantly different from that of 7.1 determined from the pH dependence of k_{cat} (Figure 3). The affinity for the proton which binds to PFK and inhibits its activity is independent of the degree of saturation of PFK by Fru-6P. There is no mutual influence between the binding of Fru-6P to the active site and the binding of a proton to the crucial catalytic residue.

Discussion

The ionization of a group controls the pH dependence of the catalytic rate constant k_{cat} of PFK (Figure 3). This group has a pK of 7.1 in the absence of effector and of 6.6 in the presence of GDP. These pK values suggest that this ionizable group could be a carboxyl or an imidazole belonging to the protein. Because its ionization affects k_{cat} and not the apparent affinity for the substrate, this group could be directly involved in the chemical reaction of phosphate transfer from ATP to Fru-6P and could thus belong to the active site. The three-dimensional structure of *E. coli* PFK reveals several groups not far from the substrates: residues such as Asp-12, Asp-103, Asp-127, Asp-129, Glu-222, His-160, or His-249 to which this pK of 6.6 or 7.1 could be attributed (Shirakihara & Evans, 1988). Site-directed mutagenesis suggests that Asp-127 may be a plausible candidate. Suppression of the negative charge on Asp-127 by the mutation Asp → Ser-127 reduces the catalytic activity of PFK by a factor 18000 (Hellinga & Evans, 1987). The present results cannot ascertain whether the residual catalytic activity of PFK at low pH (when the critical group of pK 6.6 or 7.1 is protonated) is really zero or some low level like that of the Asp → Ser-127 mutant, around 5 × 10^{-5} that of wild type. Further work is obviously needed to identify this critical residue unambiguously.

The presence of PEP does not appreciably shift the pK of the critical ionizable group (Figure 3). This is in agreement with results of X-ray crystallography showing that the binding of a PEP analogue to the PFK from *Bacillus stearothermophilus* causes a conformational change which disrupts the Fru-6P binding site (Schirmer & Evans, 1990). This suggests that, at low or moderate ligand concentration, PFK can bind either Fru-6P or PEP, but not both. If so, the active PFK molecules are only those with Fru-6P bound, and thus without PEP, and the same pH dependence as in the absence of inhibitor is indeed observed.

Depending upon pH and/or Fru-6P concentration, GDP can activate the PFK from *E. coli* by three different mechanisms, which seem to be additive: (i) GDP increases the affinity for Fru-6P (Figure 2; Blangy et al., 1968); (ii) GDP increases the catalytic rate constant k_{cat} by 15–20% (Figure 3); and (iii) GDP shifts the pK of a critical residue from 7.1 to 6.6 (Figure 3). This indicates that the conformation of PFK saturated by GDP is different from that of PFK saturated by Fru-6P.

A concerted mechanism has been proposed to explain the regulatory properties of PFK at pH 8.2 (Blangy et al., 1968; Blangy, 1971; Perutz, 1989). The protein exists in equilibrium between two conformational states, R and T, which have the same affinity for ATP, but different affinities for Fru-6P: the binding constant K_R of the R state for Fru-6P is 2000 times smaller than that of the T state, K_T. In the absence of ligand, PFK is mainly in the T state with an equilibrium constant L_0 between the T and R states of 4 × 10^6 (Blangy et al., 1968). The question arises of whether this concerted mechanism can explain two of the results obtained here: first, that the cooperativity coefficient n_H changes with pH while the half-saturating concentration $[Fru\text{-}6P]_{0.5}$ remains constant (Figures 1 and 2); second, that n_H reaches values larger than the number of Fru-6P binding sites.

In the concerted mechanism, the shape of the saturation curve depends on the values taken by three parameters: L_0, K_R, and the ratio c between K_R and K_T (Monod et al., 1965; Blangy et al., 1968). The simplest interpretation would be that the changes with pH of only one of these three parameters explain the pH dependence of n_H without concomitant change in $[Fru\text{-}6P]_{0.5}$ (Figures 1 and 2). Simulations of the concerted model show that changes in K_R or in L_0 result in large variations of $[Fru\text{-}6P]_{0.5}$. Also, although small changes in c can modify the apparent cooperativity without large changes in the half-saturating concentration [see Figure 1b of Monod et al. (1965)], large changes in c do affect $[Fru\text{-}6P]_{0.5}$ (Blangy et al., 1968). Thus, the large increase in n_H observed upon raising the pH from 6 to 9 can be explained only in part by a decrease in c. Simulations in which two out of the three parameters L_0, K_R, and c vary independently with pH lead to a much greater degree of freedom and can reproduce large changes in n_H without concomitant change in $[Fru\text{-}6P]_{0.5}$, but they cannot account for the actual values reached by n_H (see below).

The finding that both k_{cat} and n_H vary with pH suggests that catalysis and cooperativity could be linked in PFK. In the original paper on the concerted mechanism for allosteric

regulation (Monod et al., 1965), it was proposed that cooperativity could be related either to catalysis (V systems), or to binding (K systems). Indeed, steady-state measurements of enzymatic activity cannot easily distinguish between these two cases. The actual rate of product formation, v, of an active site is $v = k_{cat}Y$ where k_{cat} is the catalytic rate constant of this site and Y is the fraction of this site saturated by the substrate. The sigmoidal dependence of v on substrate concentration indicates that k_{cat}, Y, or both are influenced by the presence of substrate molecules bound at other sites. In the concerted mechanism proposed for PFK, only Y, i.e., the saturation of one site by Fru-6P, depends on the binding of Fru-6P at other sites (Blangy et al., 1968). However, PFK could behave (at least partly) as a V system, and its positive cooperativity could be also due to the enhancement of the catalytic efficiency k_{cat} of one active site upon binding Fru-6P at remote sites. The decrease in the catalytic rate constant k_{cat} upon lowering the pH (Figure 3) could then be viewed as the allosteric inhibition of a V system, the effector being the proton binding to the crucial group of pK 7. Direct measurements of the binding of Fru-6P to PFK are now in progress in our laboratory to compare the cooperativity of Fru-6P binding to that of steady-state velocity and test the hypothesis that k_{cat} depends on the degree of saturation of other sites. Nevertheless, no V, K, or mixed K-V system at steady state can lead to n_H values larger than 2 for a dimer or 4 for a tetramer (Schramm & Morrison, 1971; Segel, 1975; Neet, 1980).

Such a situation where n_H values are larger than the number of substrate binding sites is not frequent. An example is that of the apparent cooperative behavior observed in some monomeric enzymes, where kinetic cooperativity arises from a slow transition between two states of the protein with different catalytic properties (Frieden, 1970; Neet & Ainslie, 1980; Ricard, 1989). Thus, superimposition of apparent kinetic cooperativity resulting from nonequilibrium situations and of genuine cooperativity due to site interactions can lead to large values of n_H, as stated by Neet and Ainslie (1980): "The complete rate equation will be nth order in substrate, when n is the number of oligomeric sites times the number of states due to slow transitions." Therefore, in order to account for values of n_H larger than the number of Fru-6P sites, the concerted mechanism must be extended beyond steady state, and one possibility is to consider slow transitions between different conformations which bring kinetic contributions to cooperativity (Neet & Ainslie, 1980; Ricard, 1989).

In conclusion, the present results suggest that the concerted mechanism used previously (Blangy et al., 1968) is too simple to describe the pH dependence of the enzymatic properties of PFK. In the absence of any ligand, the structure of PFK resembles that of the R state, i.e., that in the presence of both substrate and activator (Rypniewsky & Evans, 1989), which indicates that the cooperative saturation of PFK by Fru-6P is not related to a major conformational change. The concerted mechanism could be refined so as to consider (i) the behavior of PFK as a mixed K-V system, (ii) the existence of slow transitions within the protein, and (iii) the ionization of at least one group which controls k_{cat}. We feel, however, that it is premature to propose a defined specific mechanism using a limited set of steady-state data. Direct evidence of conformational changes upon ligand binding and/or determinations of the fraction of saturated sites in various conditions is needed to establish an unambiguous model of the enzymatic properties of PFK.

Acknowledgments

We are grateful to Dr. P. R. Evans for communication of his results prior to publication as well as for many stimulating discussions. We are indebted to Gisèle Le Bras for her continuous cooperation throughout this work. We thank Pierre Antoine and Sylvie Tournier for their friendly help.

References

Blangy, D. (1971) *Biochimie 53*, 135–149.
Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.
Deville-Bonne, D., Le Bras, G., Teschner, W., & Garel, J. R. (1989) *Biochemistry 28*, 1917–1922.
Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.
Frieden, C. (1970) *J. Biol. Chem. 245*, 5788–5799.
Hellinga, H. W., & Evans, P. R. (1985) *Eur. J. Biochem., 149*, 363–373.
Hellinga, H. W., & Evans, P. R. (1987) *Nature (London) 327*, 437–439.
Hill, A. V. (1910) *J. Physiol. (London) 40*, iv.
Kotlarz, D., & Buc, H. (1977) *Biochim. Biophys. Acta 484*, 35–48.
Kotlarz, D., & Buc, H. (1982) *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, The Netherlands.
Le Bras, G., & Garel, J. R. (1982) *Biochemistry 21*, 6656–6660.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 3*, 318–356.
Neet, K. E. (1980) *Methods Enzymol. 64*, 139–192.
Neet, K. E., & Ainslie, G. R. (1980) *Methods Enzymol. 64*, 192–226.
Perutz, M. (1989) *Q. Rev. Biophys. 22*, 139–236.
Ricard, J. (1989) in *Allosteric Enzymes* (Hervé, G., Ed.) pp 1–25, CRC Press, Boca Raton, FL.
Rypniewski, W. R., & Evans, P. R. (1989) *J. Mol. Biol. 207*, 805–821.
Schirmer, T., & Evans, P. R. (1990) *Nature (London) 343*, 140–145.
Schramm, V. E., & Morrison, J. F. (1971) *Biochemistry 10*, 2272–2277.
Segel, I. H. (1975) in *Enzyme Kinetics*, pp 346–464, Wiley, New York.
Shirakihara, Y., & Evans, P. R. (1988) *J. Mol. Biol. 204*, 973–994.
Tauc, P. (1987) Ph.D. Thesis, Université de Paris-Sud.
Uyeda, K. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 193–244.