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Intermediates on the Reassociation Pathway of Phosphofructokinase I from *Escherichia coli*†

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ABSTRACT: The folding and association pathway of the allosteric phosphofructokinase from *Escherichia coli* has been investigated after complete denaturation of the protein in guanidine hydrochloride by spectroscopical methods, fluorescence and circular dichroism. Three successive processes can be observed during the renaturation of this protein. First, a fast reaction, detected by fluorescence, results in the formation of a (partially) structured monomer. Second, two monomers associate into a dimeric species. This step involves the shielding of the unique tryptophan residue, Trp 311, from the aqueous solvent, and it corresponds to the formation of the interface containing the effector binding site. The presence of ATP during renaturation increases the rate of formation of this dimeric species. The other ligands of the enzyme have no effect on this reaction as well as on the whole reactivation. Finally, the enzymatic activity is regained during the third slowest step. This last reaction is due to the association of two dimers into the native tetrameric structure. The presence of fructose 6-phosphate does not increase the rate of reactivation, even though this ligand strongly stabilizes the native enzyme against denaturation by bridging the interface corresponding to the active site. The self-assembly of phosphofructokinase from *E. coli* from its unfolded and separated chains follows a specific order in the formation of the interactions between subunits and involves a dimeric intermediate with a defined geometry.

Phosphofructokinase (PFK)[1] catalyzes the phosphorylation of fructose 6-phosphate (F6P) by adenosine triphosphate (ATP) to form fructose 1,6-bisphosphate (F-1,6-DP) in a reaction which controls the glycolysis in the cell:

$$\text{F6P} + \text{ATP} \xrightarrow{Mg^{2+}} \text{F-1,6-DP} + \text{ADP} \qquad (1)$$

In *Escherichia coli* the activity of PFK is regulated by different allosteric effectors; it is activated by the purine nucleotide diphosphates, ADP and GDP, and inhibited by phosphoenolpyruvate. Most of the enzymatic properties of *E. coli* PFK are consistent with a concerted model (Monod et al., 1965): the enzyme exists in two different conformational states in equilibrium, with the same catalytic efficiency and affinity for ATP and with largely different affinities for F6P and/or the effectors (Blangy et al., 1968).

The enzyme from *E. coli* is a tetramer of identical polypeptide chains of molecular weight 34817. The complete amino acid sequence of 320 residues, deduced from the nucleotide sequence of the *pfK* A gene, is known (Hellinga & Evans, 1985). X-ray diffraction studies (Shirakihara & Evans, 1988) show that each subunit consists of two domains, with a cleft between them which forms the active site. Each subunit is in close contact with only two of the other subunits in the tetramer. The F6P binding site belongs to one of these contact areas, whereas the effector binding site belongs to the other. In the case of an oligomeric enzyme such as PFK, the reconstitution of the native quaternary structure after complete unfolding in guanidine hydrochloride must be the consecutive folding and association of the polypeptide chains (Jaenicke, 1982). The simplest scheme which describes the self-assembly of PFK involves the folding of the monomers followed by two successive association steps:

$$4\text{U} \rightarrow 4\text{M} \rightarrow 2\text{D} \rightarrow \text{T} \qquad (2)$$

In this model U and T stand for the enzyme in its unfolded and native state, M stands for the monomeric protein, and D stands for the dimeric protein. It was recently shown that PFK from *E. coli* can be reassembled into a fully functional species after separating and unfolding its constituent polypeptide chains. The enzyme activity was quantitatively recovered as well as the allosteric behavior (Martel & Garel, 1984). The kinetics of reappearance of the native PFK can be described by a biphasic mechanism composed of a first-order reaction, which is generally attributed to the monomolecular fold-

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[1] Abbreviations: PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); ANS, 8-anilino-1-naphthalenesulfonic acid; F6P, D-fructose 6-phosphate; DTT, DL-dithiothreitol; Gdn-HCl, guanidine hydrochloride; CD, circular dichroism.

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ing/isomerization step, followed by a second-order reaction, which corresponds to the bimolecular association (Jaenicke & Rudolph, 1980). However, a kinetic analysis of the reactivation is not sufficient to determine which of the two association steps is actually rate limiting and responsible for the bimolecular character of the overall reaction.

In the present work the physical properties, fluorescence, and circular dichroism (CD) of PFK are compared to the enzymatic activity in order to get a more refined picture of the self-assembly and stability of the enzyme. The transition from the folded to the unfolded state induced by Gdn-HCl can be measured by CD spectroscopy, which is quite sensitive to the secondary structure of globular proteins (Adler et al., 1973), whereas fluorescence monitors the local environment of the aromatic side chains, mostly tryptophan residues (Brand & Witholt, 1967).

PFK from *E. coli* has a unique tryptophan residue at position 311, not far from the C-terminal end of the protein. Limited proteolysis has recently shown that the C-terminal segment is important for the stability of the tetrameric structure as well as for its sensitivity to allosteric effectors (Le Bras & Garel, 1982, 1985). Indeed, the tryptophan is located at an interface between two subunits corresponding to the effector binding site (Shirakihara & Evans, 1988). The protein fluorescence which is sensitive to the changes in the environment of Trp 311 should thus be a good probe to follow the formation of this interface.

ATP markedly increases the rate at which PFK regains its enzymatic activity after Gdn-HCl denaturation (Martel & Garel, 1984). The intrinsic protein fluorescence cannot be measured in the presence of ATP because of the strong interference of the nucleotide. An effect of ATP on the renaturation of PFK could however be observed by use of 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescent probe to follow the accessible hydrophobic surface (Winkler, 1962; Gally & Edelman, 1965).

Three successive reactions can be observed during the folding and assembly of PFK: (i) a fast folding step is detected by a small change of the intrinsic fluorescence of the protein, (ii) a subsequent bimolecular step leads to both a marked decrease in the accessible hydrophobic area and the regain of native fluorescence, and finally (iii) a second slower association step yields native enzyme. These results, as well as the sensitivity of these reactions to the presence of ligands, suggest that the renaturation of PFK proceeds via an inactive dimer, where the interface corresponding to the effector site is already built up.

Materials and Methods

Materials. All chemical reagents used for buffers, activity measurements, and phosphofructokinase (PFK) preparation were of analytical grade and either from Sigma Chemical Co. (Saint Louis, MO) or from Merck (Darmstadt, West Germany) or Boehringer (Mannheim, West Germany). Ultrapure guanidine hydrochloride was obtained from Schwarz/Mann (Orangeburg, NY). 8-Anilino-1-naphthalenesulfonic acid (hemimagnesium salt) was purchased from Sigma Chemical Co. (Saint Louis, MO).

PFK was prepared from *E. coli* K12 strain C600, transformed with a pGE 7 plasmid carrying the *pfK* A gene (a gift from A. Kimura and M. Shimosaka, Kyoto, Japan) according to the procedure of Kotlarz and Buc (1977), with the modifications described in the following paper in this issue (Deville-Bonne et al., 1989). The enzyme is stored as an ammonium sulfate precipitate in the presence of 1 mM F6P and is stable for several weeks. Before use, the protein is repeatedly dialyzed against 0.1 M sodium phosphate, pH 7.6, containing 1 mM magnesium chloride and 2 mM DTT. In the case of fluorescence and CD measurements, the buffer solutions are always prepared with quartz bidistilled water.

Methods. The enzymatic activity of PFK was measured with a coupled assay following the changes in absorbance at 340 nm (Kotlarz & Buc, 1977). Standard assays were performed at pH 8.2 in 100 mM Tris-HCl buffer containing 10 mM magnesium chloride, 0.2 mM NADH, 1 mM F6P, 1 mM ATP, and the auxiliary enzymes aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase.

The protein concentration of PFK was measured by a Bradford protein assay (Bradford, 1976) with immunoglobulin as a standard.

For measuring the transition curves, PFK was incubated for 24 h in a buffer containing 0.1 M sodium phosphate, pH 7.6, 1 mM magnesium chloride, 2 mM DTT, and a given concentration of Gdn-HCl. The amount of activity was measured by transferring the protein into the standard assay mixture, i.e., after the Gdn-HCl was diluted out. The residual Gdn-HCl concentration was always less than 50 mM and was checked not to interfere with the activity assay. No significant reactivation occurred during the measurement. For renaturation kinetics, PFK was first unfolded at least 60 min in 2 M Gdn-HCl and then diluted 20 times in 0.1 M sodium phosphate, 1 mM sodium phosphate, 1 mM $MgCl_2$, and 2 mM DTT, pH 7.6, before measuring activity and fluorescence.

Protein fluorescence was followed with a SPEX Fluorolog 2 fluorometer equipped with a SPEX DM1B spectroscopy laboratory coordinator. Equilibrium and kinetic fluorescence measurements were carried out in a buffer composed of 0.1 M sodium phosphate, pH 7.6, containing 2 mM DTT and 1 mM magnesium chloride. Changes in the emission spectrum of the protein when excited at 280 nm were monitored with the intensity emitted at a single wavelength as well as the ratio between the intensities emitted at two wavelengths, 330 and 310 nm. Such a ratio is more sensitive to small changes in a broad spectrum and has been useful in analyzing the complex transition between the monomer and various oligomers of ferritin (Gerl & Jaenicke, 1987).

For measuring the fluorescence kinetics with ANS, 0.1 mM ANS was present in the refolding assay. In this case the excitation wavelength was 390 nm, whereas the emission wavelength was 490 nm.

CD measurements made use of a Jobin-Yvon Mark V circular dichroism spectropolarimeter under the same conditions as those used for fluorescence measurements. The spectra were accumulated on an on-line computer (Apple II+).

Results

Fluorescence Spectra of PFK. Like its absorption spectrum, the fluorescence excitation spectrum of native PFK has a maximum at 280 nm. The fluorescence spectrum emitted by native PFK upon excitation at 280 nm is characterized by a maximum emission wavelength of 330 nm and a ratio between the fluorescence intensities emitted at 330 and 310 nm of 1.40 (Figure 1, inset). Upon dissociation and denaturation by Gdn-HCl, the fluorescence at 330 nm is reduced to about 30% of the native intensity while that at 310 nm does not change markedly (Figure 1, inset). This change is indicative for the exposure of the tryptophan residue from the interior of the enzyme into the aqueous solvent (Teipel & Koshland, 1971). At the same time the maximum emission wavelength is shifted to 307 nm, showing that the contribution of the tyrosine

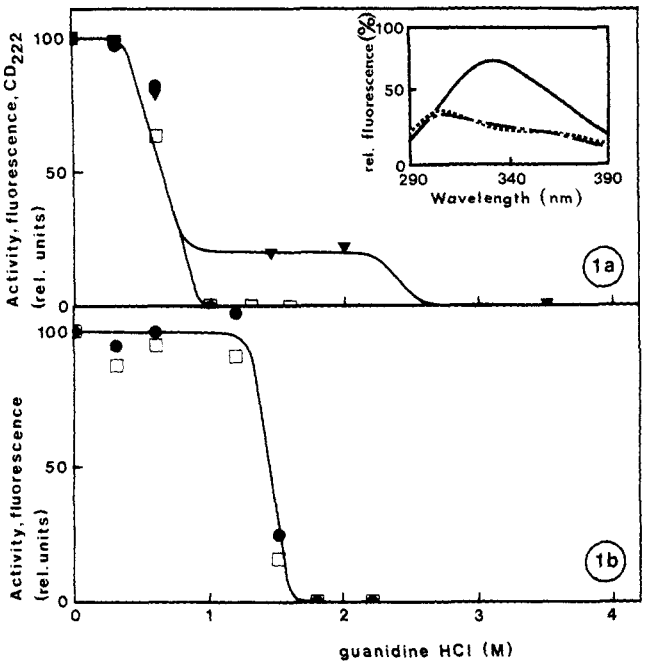

FIGURE 1: Gdn-HCl-dependent deactivation and denaturation at 20 °C of PFK in the absence of F6P (a) and the presence of 2 mM F6P (b): (●) enzymatic activity; (□) relative fluorescence (excitation wavelength = 280 nm) with the fluorescence of the denatured enzyme taken as zero; (▼) relative ellipticity at 222 nm with the signal of the denatured enzyme taken as zero. Enzyme subunit concentration: 8 μM (activity and CD) or 270 nM (fluorescence). (Inset) Fluorescence spectra of native PFK (—) and denatured enzyme in 6 M Gdn-HCl (- - -) compared to the spectrum of a mixture of free tyrosine and tryptophan in the molar ratio of 11:1 (– – –). The excitation wavelength was 280 nm. Enzyme subunit concentration: 270 nM. The contribution from the water Raman scattering was subtracted by the fluorometer.

residues is now dominant in the fluorescence emitted by PFK. The fluorescence ratio 330/310 nm is then reduced to 0.75, a value which is close to 0.68 measured for a mixture of free tyrosine and tryptophan in the same molar ratio of 11:1 as given by the amino acid composition of the enzyme (Figure 1, inset).

Unfolding Transitions of PFK. The unfolding transition curve of PFK was determined after 24-h incubation in Gdn-HCl at 20 °C, starting from either the native state or from the state completely unfolded by 6 M Gdn-HCl. The same transition is obtained for both fluorescence and activity, with a midpoint at 0.6 M Gdn-HCl (Figure 1). However, when determined by circular dichroism, the unfolding of PFK is characterized by a two-step transition. The first step is parallel to the inactivation and involves 80% of the change in ellipticity; the second step has a midpoint between 2 and 3 M Gdn-HCl and corresponds to a smaller signal change. Above 3.5 M Gdn-HCl the enzyme is completely unfolded as seen from its CD spectrum.

The presence of F6P markedly stabilizes the native state of PFK against Gdn-HCl-induced denaturation: the transition midpoint is shifted to 1.45 M Gdn-HCl for both fluorescence and activity (Figure 1).

One of the major complications encountered in studies of the unfolding/refolding of oligomeric and/or large monomeric proteins is irreversible aggregation. In the case of PFK no decrease in solubility is detected by light scattering, up to a protein concentration of 1 μM in chains. Aggregation can be observed in the range of 1–2 M Gdn-HCl at a protein concentration above 5 μM in chains; in this range, the protein has 20% of the ellipticity of the native state, suggesting that the

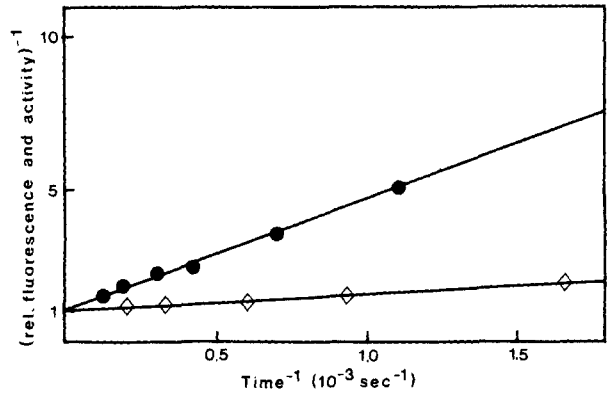

FIGURE 2: Second-order double-reciprocal plot of the kinetics of renaturation of PFK as measured by activity (●) and fluorescence (◇) at 20 °C (excitation wavelength = 280 nm). The final subunit concentration of PFK is 270 nM.

chains which undergo aggregation have a partially structured backbone. This observation and the similar ones reported for lactate dehydrogenase (Zettlmeissl et al., 1979) and octopine dehydrogenase (Zettlmeissl et al., 1984) are in agreement with the conclusion that aggregation results from wrong interdomain interactions between partially folded intermediates (Kim & Baldwin, 1982; Jaenicke, 1982).

Refolding Kinetics of PFK As Measured by the Changes in the Fluorescence of the Protein. In 2 M Gdn-HCl, the denaturation of PFK is rapid and takes less than 20 s, as judged from the loss of activity and change in fluorescence. The kinetics of reactivation of PFK after denaturation for at least 60 min in 2 M Gdn-HCl are biphasic, with an early lag phase of a few minutes followed by a bimolecular reaction (Figure 2) (Martel & Garel, 1984).

Two kinetic phases can also be monitored by fluorescence during the renaturation of PFK. A first rapid phase is complete within the time required for manual mixing. This fast fluorescence change, which has a small relative amplitude, could simply be due to the change in solvent upon dilution of Gdn-HCl. It could also reflect the folding of individual PFK chains, as suggested by results on other proteins (Teschner et al., 1987; Jaenicke, 1987).

The major part of fluorescence change of PFK can be observed during a second slower phase which corresponds to a bimolecular reaction; indeed, a double-reciprocal plot of regain of native fluorescence versus time yields a straight line (Figure 2). From experiments performed at different PFK concentrations, a value of 4×10^4 M^{-1} s^{-1} can be determined for the second-order rate constant of this slower reaction. The extrapolation to infinite time of these bimolecular kinetics shows that most of the original fluorescence can be recovered. The kinetics of fluorescence change are independent of the initial denaturation conditions within the range of 2–6 M Gdn-HCl. In the same final conditions, the reactivation of PFK is also controlled by a bimolecular reaction, but with a rate constant of the order of 10^3 M^{-1} s^{-1}. The reappearance of the native fluorescence is thus quite faster than the regain of activity, although they both follow second-order kinetics (Figure 2). This suggests that a first association reaction renders Trp 311 inaccessible to the aqueous solvent and that the active site becomes functional during a second slower association reaction, i.e., that an inactive dimeric intermediate exists.

The influence of various ligands of PFK on the kinetics of these fluorescence changes can be examined only for those which do not absorb light in the near-UV. The allosteric effector phosphoenolpyruvate and the cooperative substrate F6P have no effect on the rate at which the protein reaches

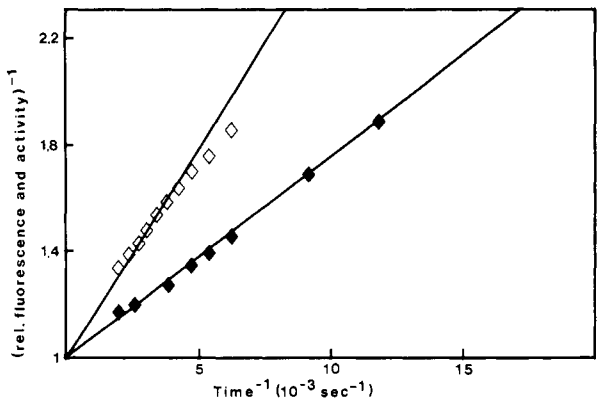

FIGURE 3: Second-order double-reciprocal plot of the kinetics of renaturation of PFK at 20 °C as measured by the regain of ANS fluorescence in the absence (◇) and in the presence (◆) of 1 mM ATP (excitation wavelength = 390 nm; emission wavelength = 490 nm). Enzyme subunit concentration: 270 nM.

its native fluorescence, even at concentrations well above those required for complete saturation of native PFK. It was also previously found that F6P and the allosteric effector GDP have no effect on the kinetics of reactivation (Martel & Garel, 1984). It seems as if F6P and GDP bind only to the final product of reactivation, i.e., the native tetrmer, and not to any intermediate species.

Refolding Kinetics of PFK Measured by the Changes in the Fluorescence of ANS. It is known that the noncooperative substrate ATP markedly increases the rate at which PFK regains its enzymatic activity (Martel & Garel, 1984). Because ATP strongly absorbs light in the near-UV, the intrinsic fluorescence of PFK cannot be followed in the presence of this ligand. However, ANS can be used as an external probe for the renaturation of PFK. Since ANS becomes highly fluorescent upon binding to accessible hydrophobic surface in proteins (Winkler, 1962; Gally & Edelman, 1965), the changes in ANS fluorescence are related to the decrease in this surface during renaturation of PFK. In the absence of ATP and under comparable conditions, the same bimolecular kinetics of renaturation are obtained for PFK by using the changes of either the intrinsic fluorescence of the protein or the fluorescence of ANS which shows that the binding of ANS to unfolded PFK does not interfere with its renaturation. This reaction is characterized by a second-order rate constant of $(3 \pm 1) \times 10^4$ M^{-1} s^{-1}. The presence of 1 mM ATP does not change the bimolecular character of the reaction, as shown by the two straight lines in Figure 3, but significantly increases the rate of fluorescence changes of ANS, i.e., the rate at which PFK decreases its accessible surface when renaturing. In the presence of 1 mM ATP, a value of $(1.5 \pm 0.5) \times 10^5$ M^{-1} s^{-1} is obtained for the second-order rate constant, about three to eight times larger than that without ATP.

Discussion

Phosphofructokinase from *E. coli* is an example of an oligomeric protein which can be reassembled into a fully native species after separating and unfolding its constituent polypeptide chains. Three different processes have been observed during the renaturation of PFK from the unfolded state U, obtained after complete denaturation by Gdn-HCl: (i) a fast reaction, complete within the time required for manual mixing, modifies the fluorescence of the protein; (ii) a bimolecular reaction with a second-order rate constant of $(3–4) \times 10^4$ M^{-1} s^{-1} decreases the binding of ANS and largely changes the fluorescence of the protein to its native value; (iii) a second slower bimolecular reaction with a second-order rate constant of about 10^3 M^{-1} s^{-1} governs the regain of enzymatic activity. These results, together with those obtained previously for the reactivation of PFK (Martel & Garel, 1984), lead to the following picture of the mechanism of PFK renaturation.

Renaturation begins with the rapid formation of a monomeric folded species M, in which Trp 311 is still quite exposed as judged from fluorescence measurements. This step has a half-life of at most a few seconds and yields a monomer M well folded enough so as to undergo further association (Martel & Garel, 1984). Indeed, the bimolecular kinetics of fluorescence change show no detectable lag phase. The formation of this folded monomer M could be rate limited by either the cis–trans isomerization of X–Pro peptide bonds (Lin & Brandts, 1983; Kuwajima & Schmid, 1984) or the pairing of already folded domains (Teschner et al., 1987; Vaucheret et al., 1987). The almost complete recovery of the activity suggests that no side reaction, leading to incorrectly folded inactive species, competes with the formation of the monomeric species M. The following step corresponds to the association of two folded monomers M into a dimeric species D. This reaction is accompanied by a decrease in the hydrophobic area accessible to ANS and by the shielding of Trp 311 from the aqueous solvent. Although its intrinsic fluorescence is that of native PFK, this intermediate D is inactive.

The last step corresponds to the reappearance of the enzymatic activity. During the reactivation of PFK, the activity present at any time is sensitive to allosteric effectors (Martel & Garel, 1984). The three-dimensional structure of PFK is such that only a tetrameric species can have both active and regulatory sites (Shirakihara & Evans, 1988). This step thus measures the formation of the native tetramer T, and its second-order kinetics show that it is limited by the association of two dimers D. The simpler scheme describing the renaturation of PFK is

$$4U \rightarrow 4M \rightarrow 2D \rightarrow T$$

The influence of a given ligand on a particular step can be related to the ability of some of these intermediates to bind this ligand. The increase of three to eight times in the rate of the 4M → 2D reaction in the presence of ATP indicates that the intermediate M is able to bind ATP. However, this increase is not sufficient to explain the 20-fold faster reactivation in the presence of ATP (Martel & Garel, 1984). Therefore, the rate of the 2D → T reaction is also higher when ATP is bound to PFK. That the monomeric species M is already able to bind ATP is compatible with the results of X-ray crystallography which show that ATP is the only ligand bound by a unique subunit, whereas the binding sites for F6P or the effector ADP involve residues from two adjacent subunits (Evans & Hudson, 1979; Shirakihara & Evans, 1988).

The U → M reaction involves the formation of two specific binding sites, one for ATP and the other for another M to dimerize into D. This fact implies that M is already a well-folded species. However, the U → M reaction is only accompanied by a small change in the fluorescence of PFK, indicating that Trp 311 is still largely exposed to the solvent in M. It is the 2M → D reaction which causes the major change in the fluorescence of PFK. This suggests that the dimerization step shields Trp 311 from the aqueous solvent. The three-dimensional structure of *E. coli* PFK shows that Trp 311 is located in one of the contact areas between two subunits, close to the effector binding site (Shirakihara & Evans, 1988). The finding that Trp 311 is exposed in M and buried in D indicates that the interface corresponding to the effector binding site is formed in D. D is indeed able to bind

phosphoenolpyruvate as shown by the slowdown of the reactivation process in the presence of this ligand (Deville-Bonne et al., 1989).

X-ray diffraction studies show that the binding site for the substrate F6P is also located at an interface between two subunits (Shirakihara & Evans, 1988). The presence of F6P has no effect on the rate of reactivation (Martel & Garel, 1984) or on that of fluorescence changes, suggesting that neither M nor D can bind F6P. This is consistent with the geometry proposed for the intermediate dimer D in which this interface does not yet exist and is formed only in the reaction $2D \rightarrow T$ in which PFK regains its activity. The second bimolecular step of the self-assembly of PFK thus implies the formation of all four sites for F6P.

The presence of 2 mM F6P markedly increases the stability of the native tetrameric state against unfolding by Gdn-HCl. The transition midpoint is shifted from 0.6 to 1.45 M denaturant for both fluorescence and activity. A marked stabilization of PFK is also observed against thermal denaturation (Blangy, 1971; Le Bras & Garel, 1982) or dissociation by KSCN (Deville-Bonne et al., 1989). Since only T can bind F6P, stabilization of the enzyme by F6P occurs at the level of its quaternary structure, by preventing the tetramer from dissociation into dimers.

The spontaneous self-assembly of PFK from its unfolded and dissociated chains is a sequential process in which several steps have been identified: an intrachain folding step which leads to a folded monomer M with a specific dimerization site and a binding site for ATP; a bimolecular step which leads to a dimer D in which the two chains are held together by interactions involving the C-terminal segment and which has an effector binding site; a second bimolecular step which leads to the native tetramer with all its catalytic and regulatory sites.

The main result of this work is that the use of several parameters cannot only unravel the kinetic mechanism of the renaturation of an oligomer such as PFK but also elucidate the geometry of the intermediate species, i.e., the order in time of the formation of the different interfaces between subunits.

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Registry No. PFK, 9001-80-3.

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