Ordered Disruption of Subunit Interfaces during the Stepwise Reversible Dissociation of *Escherichia coli* Phosphofructokinase with KSCN†

Dominique Deville-Bonne, Gisèle Le Bras, Wolfgang Teschner, and Jean-Renaud Garel*

Laboratoire d'Enzymologie, CNRS, F-91190 Gif-sur-Yvette, France

Received July 5, 1988; Revised Manuscript Received October 5, 1988

ABSTRACT: The reversible inactivation and dissociation of the allosteric phosphofructokinase from *Escherichia coli* has been studied in relatively mild conditions, i.e., in the presence of the chaotropic agent KSCN. At moderate KSCN concentration, the loss of enzymatic activity involves two separated phases: first, a rapid dissociation of part of the tetramer into dimers, second, a slower displacement of the dimer–tetramer equilibrium upon further dissociation of the dimer into monomers. These two reactions can no longer be distinguished above 0.3 M KSCN since complete inactivation occurs in a single reaction. Different changes are observed for the fluorescence and the activity of the enzyme in KSCN: the fluorescence is not affected by the dissociation into dimers which is responsible for inactivation. The decrease in fluorescence reflects the change in environment of the unique tryptophan residue, Trp 311, during the dimer to monomer dissociation. This residue belongs to the interface containing the regulatory site, and its native fluorescence indicates that this interface is still present in the dimer. The substrate fructose 6-phosphate protects phosphofructokinase from inactivation by binding to the tetramer and prevents its dissociation into dimers. The presence of phosphoenolpyruvate prevents the slow dissociation of the dimer into monomers, which shows the ability of the dimer to bind the inhibitor. Two successive processes can be observed during reassociation of the protein upon KSCN dilution. First, a fast reaction ($k_1 = 2 \times 10^5$ $M^{-1} \cdot s^{-1}$) is accompanied by a fluorescence increase and results in the formation of the dimeric species. Then, dimers slowly associate into the active tetramer ($k_2 = 10^4$ $M^{-1} \cdot s^{-1}$). The rate of this reactivation step is slowed down by phosphoenolpyruvate, which indicates again the binding of the inhibitor to the dimer. Phosphofructokinase can be also reactivated by adding fructose 6-phosphate without dilution of KSCN. Reaction is also biphasic, but the presence of the salt decreases the rates of these two steps. This reactivation is due to the slow shift back toward the tetramer upon binding of fructose 6-phosphate to the native enzyme. With use of KSCN, conditions are found in which the enzyme accumulates mostly in a dimeric state. This dimer is the same whether obtained by dissociation of the tetramer or by association of two monomers, as judged from its native fluorescence and ability to bind phosphoenolpyruvate. This shows that the self-assembly of phosphofructokinase involves a specific order in the formation of the interfaces with a defined geometry in subunit pairing.

In *Escherichia coli*, phosphofructokinase (PFK)[1] catalyzes the committed step of the glycolytic pathway:

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

This tetrameric protein, composed of identical subunits (Hellinga & Evans, 1985), can be renatured after separation and complete unfolding of its chains (Martel & Garel, 1984). The simplest mechanism describing the formation of a tetrameric structure involves two consecutive bimolecular reactions:

$$4 \text{ monomers} \rightarrow 2 \text{ dimers} \rightarrow \text{tetramer}$$

Kinetic studies have shown that the reappearance of activity is controlled by a bimolecular reaction, but it is not known whether reactivation measures the formation of the dimer or the tetramer. In order to identify which of these two association steps is rate limiting for the overall renaturation, we have looked for conditions under which PFK could be partially dissociated. This strategy, which assumes that the last step(s) in assembly is (are) the first step(s) in disassembly, has been used in the case of lactate dehydrogenase to show that renaturation is rate limited by the dimer to tetramer step (Jaenicke et al., 1981; Zettlmeissl et al., 1982; Girg et al., 1983).

There is another interest in studying the dissociation of PFK into dimeric species. In the native tetramer the four subunits are arranged to form a dimer of dimers (with a *D*2 symmetry), as schematized in Figure 1. Each subunit makes contacts with two others along two different interfaces. One of these interfaces, the A (for active) interface, contains the binding site for the substrate Fru-6P, and the other, the R (for regulatory) interface, contains the binding site for the allosteric activator ADP (or GDP) or inhibitor PEP (Evans & Hudson, 1979). Therefore the dissociation of the tetramer can yield two different dimers depending on which interface is broken first. Similarly, the association of two monomers can also produce two different dimeric species according to the nature of the created interface. It is not known whether (i) the "dimeric intermediate state" of PFK is a single species or a mixture of the two possible structures and (ii) this dimeric intermediate state is the same in assembly and disassembly. A detailed

† This work was supported by the Centre National de la Recherche Scientifique and the Université Paris 6 (UER 58). W.T. is a recipient of an EMBO long-term fellowship.

[1] Abbreviations: Fru-6P, D-fructose 6-phosphate; Fru-1,6P_2, D-fructose 1,6-bisphosphate; Fru-2,6P_2, D-fructose 2,6-bisphosphate; Tris, tris(hydroxymethyl)aminomethane; PFK, phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate.

0006-2960/89/0428-1917$01.50/0

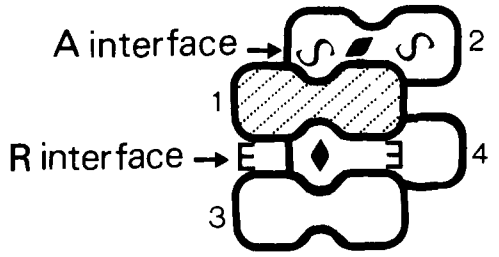

FIGURE 1: Simplified representation of the structure of PFK. Each of the four identical subunit is composed of two lobes. The hatched subunit 1 is in contact with the two open ones along two different interfaces A and R. The "horizontal" interface A with the upper back subunit 2 includes two active sites for the substrates S related by a local dyad axis (◆); the "vertical" interface R with the lower front subunit 3 includes two regulatory sites for the effectors E related by a local dyad axis (◆). There is almost no interaction between subunits 2 and 3. The interactions involving the lower back subunit 4 have not been shown for clarity.

study of the renaturation of PFK should not remain limited to the kinetics of formation or disappearance of this dimeric intermediate but should determine the geometry of this intermediate.

In the presence of guanidine hydrochloride, dissociation and unfolding of PFK take place together in an all-or-none process (preceding paper of this issue). Milder conditions are choosen in order to induce a stepwise dissociation of the enzyme. In the presence of moderate concentrations of KSCN, a solvent additive known to destabilize the interactions between proteins (Arakawa & Timasheff, 1982), tetrameric PFK dissociates rapidly into dimers and looses its activity. Further dissociation into monomers takes place, but more slowly so that dimeric PFK accumulates and can be characterized. Upon dilution of KSCN, monomeric PFK reassociates rapidly into dimers, then more slowly into tetramers with concomitant reactivation. The dimeric species which accumulates in both directions is able to bind the inhibitor PEP, and thus possesses a functional R interface (Figure 1). The unique tryptophan residue, Trp 311, which belongs to this R interface (Shirakihara & Evans, 1988), is still shielded from the aqueous medium in the dimeric species, also indicating the existence of the R interface. It appears that the dissociation of PFK involves an ordered disruption of the interactions between subunits, the A interface being broken first and the R interface last. Reciprocally, the self-assembly of PFK from its separated chains proceeds in two steps; the R interface is formed first and the A interface last. This conclusion, which is also reached in the preceding paper (Teschner & Garel, 1989), suggests that the renaturation of oligomeric proteins (with more than two subunits) may also involve a specific order in the formation of the interactions between polypeptide chains.

Experimental Procedures

Chemicals. All chemical reagents were of analytical grade. The auxiliary enzymes used in the PFK coupled assay (aldolase, triosephosphate isomerase, and glycerolphosphate dehydrogenase) as well as GDP and PEP were purchased from Boehringer Mannheim; ATP, DTT, Fru-6P, Fru-1,6P_2, and NADH were obtained from Sigma. All other reagents were analytical grade substances from Merck.

PFK Purification. PFK was prepared from *E. coli* K12, strain C600 (F, thr, leu, thi, lacY, supE, tonA) transformed with the plasmid pGE7 carrying the pfkA gene (Shimosaka et al., 1982). Alternatively, PFK was expressed in HE1 cells [pro-82, Δpfk B201, recA56, Δ(rha,pfkA)200, endA1,–hsd R17, supE44/F′:traD36, proAB$^+$, lacI^q, lacZ, ΔM15] transformed with the plasmid pHL1 (Lau et al., 1987), both kindly sent by F. Lau and A. Fersht (Imperial College, London). The plasmid pHL1 is a pEMBL8(+) vector where pfkA insert has been cloned in a *Bam*HI restriction site. The enzyme was prepared according to Kotlarz and Buc (1982) with the following modifications: cells were disrupted with alumina beads in P buffer (20 mM phosphate, pH = 7.6, containing 1 mM $MgCl_2$ and 7 mM mercaptoethanol). The cleared lysate was loaded on a Blue Dextran–agarose column, which was extensively washed with P buffer containing 1 M NaCl, after which the enzyme was eluted with a 0–1.2 mM ATP gradient in P buffer where mercaptoethanol was replaced by 2 mM DTT. The eluate was concentrated in an Amicon Diaflo cell (PM10 membrane) and dialyzed overnight against 0.1 M Tris-HCl, pH = 8.2, containing 1 mM $MgCl_2$, 2 mM DTT, 0.4 M NaCl, and 1 mM Fru-6P, then stored as a 65% $(NH_4)_2SO_4$ suspension at 4 °C. Homogeneity was demonstrated by the observation of a single band on SDS–polyacrylamide gel.

For enzyme assays, this suspension was dialyzed overnight against standard buffer (50 mM imidazole hydrochloride buffer, pH 7.0, containing 1 mM $MgCl_2$ and 2 mM DTT). Imidazole was used instead of the usual phosphate to avoid nonspecific stabilization by the buffer. The enzyme was incubated during 45 min at 27 °C in the presence of 2 mM ATP in order to eliminate any residual Fru-6P, then extensively dialyzed against the same buffer.

Protein concentration was determined either spectrophotometrically, with an extinction coefficient at 278 nm of 0.6 $L \cdot g^{-1} \cdot cm^{-1}$ (Kotlarz & Buc, 1982) or by the Bradford assay (Bradford, 1976) with immunoglobulin as a standard.

PFK Activity Measurement. The enzymatic activity of PFK was monitored as previously described (Blangy et al., 1968) with both substrates ATP and Fru-6P at 1 mM.

PFK was inactivated by incubation at 20 °C during 30 min in 0.5 M KSCN in standard buffer unless specified. The reactivation process was initiated by diluting 20:1 or 50:1 the inactivated enzyme in standard buffer, which sometimes included substrates or effectors. Alternatively, the reactivation process was initiated by Fru-6P addition. The extent of reactivation was measured at given time intervals by adding the reactivated enzyme into the standard assay mixture and following spectrophotometrically the NAD^+ production at 340 nm during 2 min at 27 °C. Low residual KSCN concentrations (less than 25 mM) do not interfere with the enzymatic assay.

Equilibrium Sedimentation. The molecular weight of the enzyme (0.8 mg/mL) incubated in 0.5 M KSCN in standard buffer (d = 1.026 g/mL) was determined by equilibrium sedimentation in an MSE-Centriscan M75 ultracentrifuge at 12 000 rpm, 20 °C, during 24 h, assuming a value of 0.73 mL/g for the protein specific volume.

Fluorescence Steady-State Measurements. Protein fluorescence was followed with a SPEX Fluorolog 2 fluorometer equipped with a SPEX DM1B spectroscopy laboratory coordinator. The equilibrium and kinetic fluorescence measurements were carried out in a buffer made of 0.1 M sodium phosphate, pH 7.0, containing 2 mM DTT and 1 mM $MgCl_2$. The changes in the fluorescence emitted by the protein when excited at 280 nm were monitored as described in the preceding paper in this issue.

Results and Discussion

Throughout all this work, the activity measured for PFK, whether total or partial, during inactivation or reactivation

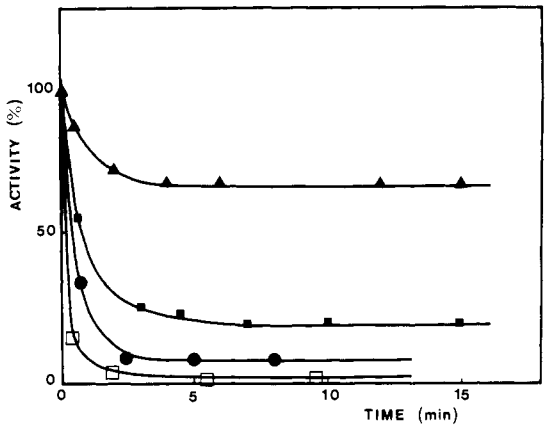

FIGURE 2: Time-courses of PFK inactivation in the presence of KSCN. The enzyme (15 μM in PFK subunits) is incubated at 20 °C in 50 mM imidazole hydrochloride buffer, pH 7.0, containing 1 mM $MgCl_2$, 2 mM DTT, and 0.15 (▲), 0.25 (■), 0.3 (●), or 0.4 M KSCN (□). The residual activity is measured as described under Experimental Procedures and expressed relative to the initial activity.

experiments, is as sensitive to allosteric effectors, i.e., activated by GDP or inhibited by PEP, as that of the native enzyme. The structure of PFK given in Figure 1 shows that such a regulated activity can be associated only with the native tetramer. Therefore, changes in activity directly measure the formation or disappearance of this species.

KSCN-Induced Inactivation of PFK. In the presence of KSCN at various concentrations, PFK looses rapidly (part of) its enzymatic activity. Higher KSCN concentrations result in faster inactivation and at a greater extent (Figure 2). The residual activity measured after 30 min, i.e., after completion of this rapid loss of activity, is given in Figure 3 as a function of KSCN: PFK retains its original activity up to 0.05 M KSCN and is completely inactivated above 0.3 M KSCN. A higher protein concentration results in a slight stabilization of the activity: a 10-fold higher PFK concentration causes an upward shift of the order of 0.1 M KSCN in the midpoint of the inactivation curve (compare Figures 3 and 5). Because of its small amplitude, this concentration dependence has not been studied in detail.

The residual activity obtained after incubating PFK in KSCN for a few minutes does not remain constant over a long time period and diminishes gradually, which shows that a second much slower reaction also leads to inactivation. After 20 h in KSCN, PFK is no longer active even at the lowest KSCN concentration, 0.05 M (not shown). This slow inactivation is not irreversible since PFK can be reactivated by dilution of KSCN (see below).

A simple mechanism describing the KSCN-induced inactivation of PFK involves two consecutive reactions:

$$N \xrightarrow{\text{faster}} D \xrightarrow{\text{slower}} M$$

in which native PFK N, is first converted rapidly into an inactive species D, which itself undergoes a slow conformational change to yield a second inactive species M. The molecular weight of M, as determined by sedimentation equilibrium, is 33 000 ± 2000; M is thus a monomeric form of PFK. The kinetic study of the reactivation of PFK (see below) indicates that D is a dimeric form of PFK. Up to 0.3 M KSCN, the dissociation of PFK shows two distinct phases: the faster corresponds to the dissociation of N into D and the slower to the slow displacement of the equilibrium between N and D upon the further dissociation of D into M. These two phases cannot be observed above 0.3 M KSCN because complete inactivation occurs in the faster reaction.

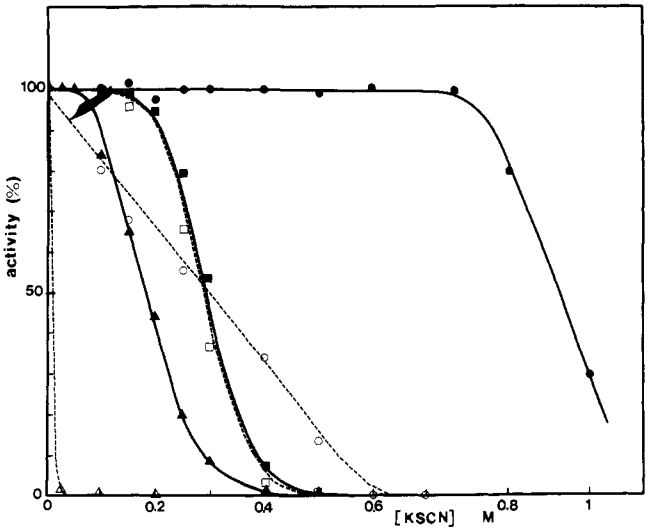

FIGURE 3: Effect of ligands on the inactivation of PFK by KSCN. The enzyme (6 μM in PFK subunits) in standard buffer (50 mM imidazole hydrochloride, 1 mM $MgCl_2$, 2 mM DTT) is incubated at 20 °C in various KSCN concentrations during 30 min (closed symbols, continuous line) or 20 h (open symbols, dashed line), in the absence of ligands (▲, △) or in the presence of 8 mM PEP (■, □) or 1 mM Fru-6P (●, ○). The control activity remaining after 20 h in the absence of KSCN is 85% of the initial value.

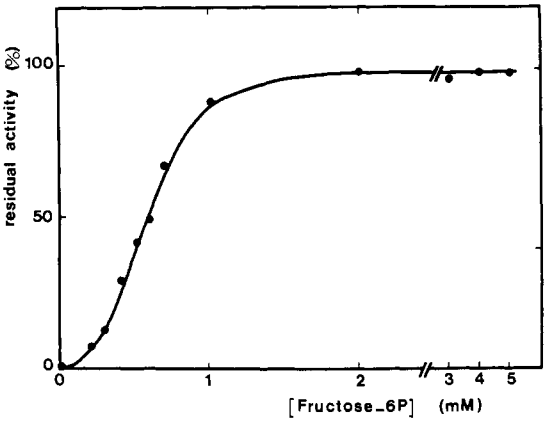

FIGURE 4: Protection of PFK by Fru-6P against the KSCN-induced inactivation. The enzyme (8 μM in subunits) is incubated during 0.5 h at 20 °C with 0.5 M KSCN and the indicated concentration of Fru-6P. The same curve is obtained for the saturation of native PFK by Fru-6P (Blangy et al., 1968). The cooperativity coefficient is n_H = 3.1 ± 0.3.

The presence of a nucleotide, either the substrate ATP or the allosteric activator GDP, has no influence on the extent of inactivation after 30 min in KSCN (not shown). No loss of activity is observed after 30 min in 0.7–0.8 M KSCN in the presence of a saturating concentration of the substrate Fru-6P. The extent of activity remaining after 30 min in 0.5 M KSCN follows the saturation of N by Fru-6P (Figure 4), suggesting that the complex between native PFK and Fru-6P is more stable toward dissociation into D than free PFK. The slow inactivation still occurs when Fru-6P is present, the activity measured after 20 h being lower than that after 30 min (Figure 3). Fru-6P seems to protect PFK by binding to N and preventing (or markedly slowing) its dissociation into D.

A slight protection by the allosteric inhibitor PEP is also obtained after 30 min in KSCN (Figure 3), which suggests that the binding of PEP to N slightly stabilizes it against dissociation into D. More surprising is that the same activity is measured after 30 min and 20 h in the same KSCN concentration when PEP is present (Figure 3). Therefore, the

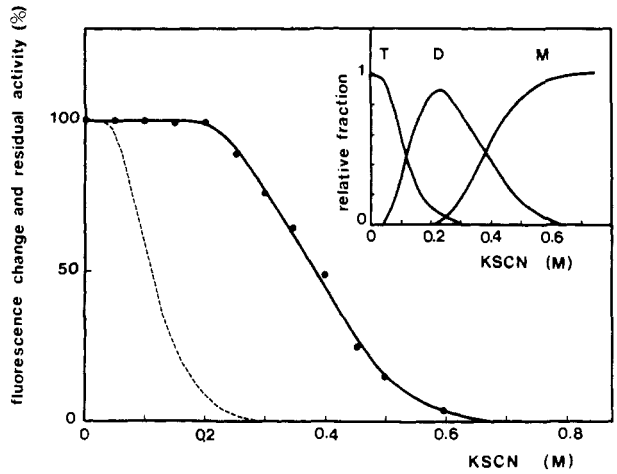

FIGURE 5: KSCN-dependent inactivation of PFK. Enzymatic activity (--) and relative fluorescence (—) of 0.5 μM PFK are measured after 30-min incubation at 20 °C in KSCN. The ratios of the fluorescence emitted at 330 and 310 nm (with an excitation wavelength of 280 nm) are 1.30 for native PFK and 0.80 for PFK in 0.75 M KSCN. Fluorescence and activity changes are expressed as fractions of the maximum values. (Inset) Relative fraction of the different oligomeric states of PFK present after 30 min at different KSCN concentrations. The amount of tetramer T is derived from the residual activity, the fluorescence change is related to the tetrameric plus dimeric (D) states. M: monomer.

presence of the allosteric inhibitor prevents completely the second slower phase of inactivation from taking place, showing not only that D can bind PEP but also that this binding stabilizes D and completely prevents it from dissociating into M.

KSCN-Induced Conformational Change of PFK As Detected by Fluorescence. The fluorescence spectrum emitted by native PFK upon excitation at 280 nm corresponds mainly to the unique tryptophan residue, Trp 311, which is shielded from the aqueous solvent (see Figure 1 of the preceding article). In 0.75 M KSCN, the emission spectrum of PFK is similar to that of the guanidine-unfolded protein and to that of a mixture of tyrosine and tryptophan in the 11:1 molar ration found in PFK. This indicates that Trp 311 is then exposed to the solvent. Upon transfer into 0.75 M KSCN, the fluorescence of PFK changes rapidly, in less than 1 min.

Figure 5 shows the KSCN dependence of the changes of the PFK fluorescence, as determined from spectra recorded 30 min after transferring the enzyme into the given KSCN concentration. Up to 0.2 M KSCN, the fluorescence of PFK, within 30 min, remains the same as that of the native protein. Between 0.2 and 0.6 M KSCN, the fluorescence decreases markedly showing that PFK undergoes a conformational transition which modifies the environment of Trp 311. Above 0.6 M KSCN the fluorescence of PFK is the same as that of the unfolded protein.

In the presence of Fru-6P, the fluorescence of PFK remains that of the native protein up to 0.6–0.7 M KSCN. Thus Fru-6P appears to stabilize the native enyzme against KSCN, heat (Le Bras & Garel, 1982), guanidine hydrochloride (Teschner & Garel, 1989), or urea (unpublished results). The inhibitor PEP does not have such a marked stabilizing effect. No measurement of the fluorescence of PFK can be performed in the presence of the substrate ATP or the activator GDP, which absorb at 280 nm.

Figure 5 also shows that, when measured after 30 min and at the same protein concentration, the loss of activity of PFK occurs at lower KSCN concentrations than those needed for its fluorescence change: the midpoints of the transition curves are respectively 0.15 M for activity and 0.4 M for fluorescence. This suggests the occurrence of at least two distinct processes. For instance, 30 min in 0.25 M KSCN inactivates almost completely PFK without altering its fluorescence. The dissociation of N into D, which is responsible for the loss of activity, is not accompanied by a change in fluorescence. The species N and D have therefore the same fluorescence corresponding to a buried position of Trp 311.

Upon transfer into 0.25 M KSCN the fluorescence of PFK remains constant for 30 min (Figure 5) but decreases on the time scale of hours. However, in the presence of PEP, PFK retains the same fluorescence after 30 min or 20 h in KSCN, which shows that the inhibitor suppresses this slow reaction. The fluorescence change in the absence of PEP thus follows the slow phase of inactivation, indicating that the dissociation of D into M causes the exposure of Trp 311 to solvent. It is not known whether M is completely unfolded or has retained some conformation, such as the monomeric intermediate described in the preceding article. Dissociation into correctly structured monomers has been reported for aspartate transcarbamylase in similar conditions (neutral pH, 1.25 M NaSCN) (Burns & Schachman, 1982).

The fraction of PFK which is present as native tetramer N, dimer D, or monomer M after 30 min at a given KSCN concentration can be determined from the data of Figure 5, considering that the activity measures the fraction of N and the fluorescence change that of M. The inset of Figure 5 shows that PFK is mostly in a dimeric state in 0.25 M KSCN and in a monomeric state in 0.5 M KSCN and above.

Reactivation and Reassociation of PFK upon Dilution of KSCN. After complete inactivation of PFK by KSCN, a simple dilution of the salt allows the enzymatic activity to reappear. This reactivation takes place even after 48 h in KSCN, showing the absence of irreversible damage such as thiocarbamylation of the protein (Stark, 1970).

Two different procedures can be used to inactivate PFK, either 30 min in 0.25 M KSCN, which leads mostly to D, or 30 min in 0.5 M KSCN, which leads mostly to M (Figure 5, inset). The time courses of reactivation from either D or M are shown in Figure 6. Reactivation from D is quantitative, while only 80% of the original activity is regained when starting from the completely dissociated enzyme. In either case reactivation is governed by a bimolecular reaction without any detectable lag phase. Under the same final conditions, the second-order rate constants are similar for the two sets of initial conditions, with a value of $(1.2 \pm 0.3) \times 10^4$ $M^{-1} \cdot s^{-1}$ when starting from D and of $(5 \pm 1.5) \times 10^3$ $M^{-1} \cdot s^{-1}$ when starting from M. The 2-fold difference between these rate constants is explained below.

When starting from M, the fluorescence change of PFK is much faster than its reactivation. The fluorescence of the native protein is regained so rapidly that its rate can be measured only at low final protein concentration, yielding a second-order rate constant of $(2 \pm 1) \times 10^5$ $M^{-1} \cdot s^{-1}$ at least.

These results are consistent with a simple mechanism involving the same species N, D, and M considered above for the inactivation pathway:

$$4M \xrightarrow{2 \times 10^5\ M^{-1} \cdot s^{-1}} 2D \xrightarrow{1.2 \times 10^4\ M^{-1} \cdot s^{-1}} N$$

in which the fluorescence change takes place in the step from M to D and the activity appears in the last step (D → N). The progress curve of formation of N calculated from this mechanism is, within experimental errors, the same as that given by a simple bimolecular reaction with a rate constant of 5×10^3 $M^{-1} \cdot s^{-1}$ and thus agrees with the observed kinetics of reactivation from M.

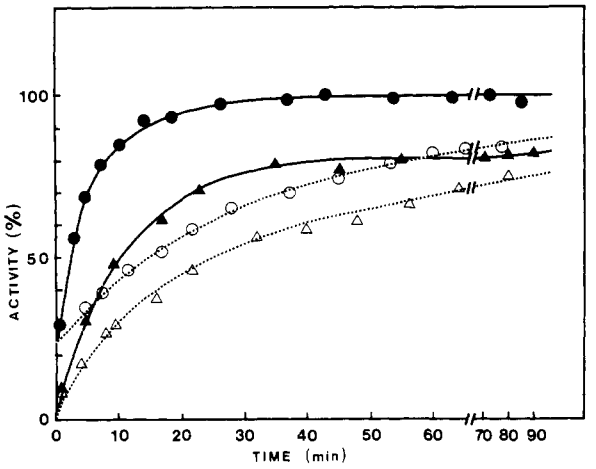

FIGURE 6: Reactivation of PFK upon dilution of KSCN. Kinetics of reappearance of the activity of PFK upon dilution of KSCN in standard buffer at pH 7, 20 °C, after inactivation in 0.25 or 0.5 M KSCN. The enzyme (9.6 μM) is incubated 30 min in 0.25 M (●, ○) or 0.5 M (▲, △) KSCN and then diluted to 0.48 μM in standard buffer in the absence (●, ▲) or in the presence of 8 mM PEP (○, △). During reactivation, PFK activity is assayed in standard conditions and expressed relative to that of native PFK at the same concentration in order to correct for the slight inactivation of native enzyme observed at concentrations below 1 μM.

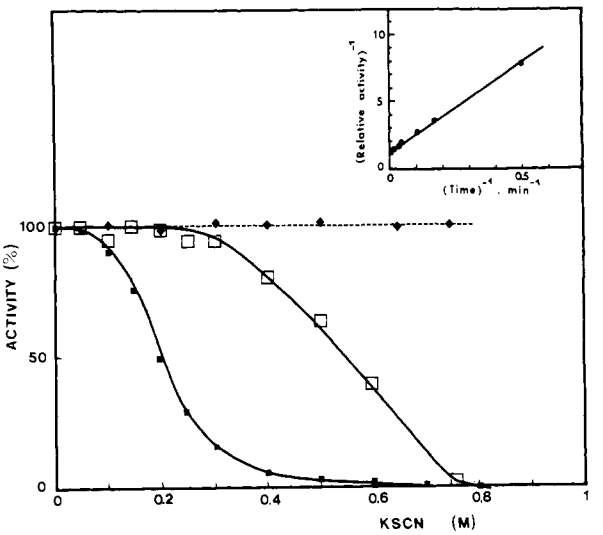

FIGURE 7: Reactivation of KSCN-inactivated PFK by Fru-6P addition. PFK is incubated 30 min at 20 °C with various KSCN concentrations (■) and then 3 h at 20 °C after 3 mM Fru-6P addition in each sample (□). (◆) represent control assays where Fru-6P is added together with KSCN. PFK activity is expressed relative to that of native enzyme at the same concentration (20 μM). (Inset) Second-order double-reciprocal plot of the kinetics of reactivation of PFK (7 μM) at 20 °C in 0.5 M KSCN upon 2 mM Fru-6P addition. The rate constant of the reactivation is here 250 $M^{-1}\cdot s^{-1}$ and refers to the monomer concentration.

The substrates of PFK, ATP, and Fru-6P and the activator GDP have no effect on the rate or extent of reactivation. The allosteric inhibitor PEP is found to decrease the rate of reactivation by a 2-fold factor (Figure 6). This effect of PEP is observed when starting from D or M, as expected since reactivation is mostly rate limited by the 2D → N reaction. This result strengthens the above observation that the dimeric species D can bind PEP, the effector binding site being created by the pairing of subunits through the R interface.

Nature of the Dimeric Species D. The dimeric species (or set of species) D observed here has the fluorescence of native PFK, showing that Trp 311 is not contact with the solvent, and is able to bind PEP, showing that its effector site is still functional. Only, the pairing of two subunits along the R interface can both shield Trp 311 from solvent and create an effector binding site (Figure 1). The first step in the dissociation of native PFK would be the disruption of the A interface which destroys the Fru-6P binding site so that D is inactive. The A interface buries a smaller solvent-accessible area between subunits than the R interface (Shirakihara & Evans, 1988) and should therefore be less stable (Chothia & Janin, 1975). It has also been proposed that the last step of the renaturation of guanidine-unfolded PFK is the formation of the A interface (preceding article). The dimer D in which the R interface is formed appears then as a compulsory intermediate between the native tetramer and the separated chains of PFK in both directions. Homotetrameric proteins are often built as a dimer of dimers: their dissociation and/or self-assembly should also involve a dimeric intermediate with a specific geometry, probably that with the largest interface between subunits.

Influence of Fru-6P on the KSCN-Induced Dissociation of PFK. Not only does Fru-6P stabilize PFK against KSCN-induced inactivation (Figure 3), but it can also reactivate PFK even in the presence of KSCN. Upon addition of saturating Fru-6P all the activity can be regained in 0.3 M KSCN, and significant reactivation is observed in up to 0.6 M KSCN (Figure 7), provided that PFK is inactivated for a short enough time so that its dissociation does not proceed completely to the monomer. The properties of this reactivation are as follows:

(1) When starting from the dimer D, after 30 min in 0.25 M KSCN, it follows bimolecular kinetics, with a second-order rate constant of 250 $M^{-1}\cdot s^{-1}$ in 0.25 M KSCN (Figure 7, inset), about 50 times lower than that without KSCN.

(2) In the same final conditions, 0.25 M KSCN, its rate is about three times lower ($\approx$80 $M^{-1}\cdot s^{-1}$) when starting from the monomer M than from the dimer D, but its yield is also much lower.

(3) In 0.5 M KSCN, its extent depends on Fru-6P concentration in the same way as the saturation of the tetramer or the protection against inactivation (Figure 4).

(4) It is specific for Fru-6P and does not occur with Fru, Fru-1P, Fru-1,6P_2, Fru-2,6P_2, or sucrose. All these results suggest that the reactivation is due to reversal of the dissociation reaction upon the binding of Fru-6P to native PFK:

$$4M \rightarrow 2D \rightarrow N \xrightarrow{+\text{Fru-6P}} \text{N–Fru-6P}$$

The fraction of PFK present as D can be more easily reactivated than the fraction of monomers M (compare Figures 5 and 7).

In native PFK, the binding site for Fru-6P lies across the A interface, with a subsite for the sugar moiety on one subunit and a subsite for the phosphate group on another subunit (Evans & Hudson, 1979). Bound Fru-6P holds these two subsites close to each other and makes this interface more difficult to break, thus explaining the increased stability of the PFK-Fru-6P complex toward dissociation into dimers.

CONCLUSIONS

The association–dissociation reactions of PFK in the presence of a moderate concentration of KSCN, up to 0.5 M, are described by the mechanism

$$N \rightleftharpoons 2D \rightleftharpoons 4M$$

in which the various steps depend on KSCN concentration. In particular conditions, D is found to accumulate, and some of its properties can be analyzed. Fru-6P binds only to N but PEP to both N and D. N and D have the same fluorescence.

Dissociation of tetrameric PFK occurs by disruption of the A interface (Figure 1), and yields dimers in which two subunits interact along the R interface. Similarly, association of two monomers into a dimer occurs along the R interface, while the A interface is formed only when two dimers associate into a tetramer. A unique dimeric species thus exists as a kinetic intermediate in both association and dissociation of PFK. The preceding article suggests that this dimeric species is also the intermediate in the self-assembly of PFK from its separated and unfolded chains (Teschner & Garel, 1989). Subunit association follows a specific pathway in which the various interactions between subunits are formed at different steps. First interactions along the R interface lead to the regulatory dimers, which then interact through the A interface to generate the native tetramer. PFK is one of the first cases for which each step of this pathway has been characterized: the interaction which implies the larger area of contact is formed first during renaturation–association and is broken last during dissociation.

In PFK, the regulatory site is though to exist under two conformations, one which binds the activator GDP and the other the inhibitor PEP (Blangy, 1971; Evans & Hudson, 1979). In the unique dimeric species, this site binds PEP and is apparently unable to bind GDP. The conformation of this site is thus different in the dimer and in the tetramer. The association of two dimers into a tetramer not only creates the A interface with the binding site for Fru-6P but also changes the whole protein structure so that the regulatory site becomes able to oscillate between its PEP binding and GDP binding, i.e., between the "inactive" and the "active" conformations. The specific order of formation of subunit interfaces determines the acquisition of both the catalytic and regulatory properties of the final native protein.

ACKNOWLEDGMENTS

We are grateful to Drs. P. Evans and Y. Shirakihara for communication of their results prior to publication, to Drs. M. Shimosaka and A. Kimura for sending the pGE7 overproducing strain, to Drs. F. Lau and A. Fersht for the gift of the plasmid pHL_1 and HE_1 cells, and to Dr. M. Goldberg for performing the equilibrium sedimentation.

Registry No. PFK, 9001-80-3; Fru-6P, 643-13-0; PEP, 138-08-9; KSCN, 333-20-0.

REFERENCES

Arakawa, T., & Timasheff, S. N. (1982) *Biochemistry 21*, 6545–6552.

Blangy, D. (1971) *Biochimie, 53*, 139–149.

Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 13–35.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Burns, D. L., & Schachman, H. K. (1982) *J. Biol. Chem. 257*, 8638–8647.

Chothia, C., & Janin, J. (1975) *Nature 256*, 705–708.

Evans, P. R., & Hudson, P. J. (1979) *Nature 279*, 500–504.

Girg, R., Rudolph, R., & Jaenicke, R. (1983) *FEBS Lett. 163*, 132–135.

Hellinga, H. W., & Evans, P. R. (1985) *Eur. J. Biochem. 149*, 363–373.

Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 5195–5201.

Jaenicke, R., & Rudolph, R. (1986) *Methods Enzymol. 131*, 218–250.

Jaenicke, R., Vogel, W., & Rudolph, R. (1981) *Eur. J. Biochem. 114*, 525–531.

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

Lau, F. T. K., Fersht, A. R., Hellinga, H. W., & Evans, P. R. (1987) *Biochemistry 26*, 4143–4148.

Le Bras, G., & Garel, J.-R. (1982) *Biochemistry 21*, 6656–6660.

Martel, A., & Garel, J.-R. (1984) *J. Biol. Chem. 259*, 4917–4921.

Shimosaka, M., Fukuda, Y., Murata, K., & Kimura, A. (1982) *J. Bacteriol. 152*, 98–103.

Shirakihara, Y., & Evans, P. R. (1988) *J. Mol. Biol.* (in press).

Stark, G. R. (1970) *Adv. Protein Chem. 24*, 261–308.

Teschner, W., & Garel, J.-R. (1989) *Biochemistry* (preceding paper in this issue).

Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1982) *Biochemistry 21*, 3946–32950.