Tetramer−Dimer Equilibrium of Phosphofructokinase and Formation of Hybrid Tetramers†

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Received June 13, 1995; Revised Manuscript Received August 7, 1995®

ABSTRACT: Moderate concentrations of KSCN inactivate the allosteric phosphofructokinase from *Escherichia coli* by dissociating the subunit interface that contains the binding site for the substrate fructose-6-phosphate. At a given KSCN concentration, the activity varies with the concentration of protein as expected from a simple equilibrium between active tetramers and inactive dimers. The equilibrium constants for the dissociation of a tetramer into dimers have been determined in 0.4 M KSCN for the wild-type enzyme and the noncooperative mutant T125S, the hypercooperative mutant E148A-R152A, and the inactive mutant D127S. The stability of the tetrameric structure is decreased by the mutations E148A-R152A that are in the interface and increased by the mutation T125S that does not belong to it. There could be an inverse correlation between the cooperativity of the saturation by fructose-6-phosphate (in absence of any effector) and the stability of the interface that contains its binding site. Hybrid tetramers can be formed upon reassociation of a dimer from an active phosphofructokinase (wild-type, T125S, or E148-R152A) with a dimer from the inactive D127S mutant, and their stability and cooperativity toward fructose-6-phosphate have been measured without purifying them. The results indicate that the formation of a hybrid interface involves some flexibility of the two dimers and that the allosteric coupling between distant sites could be related to the plasticity and instability of the interactions across this interface.

Phosphofructokinase (PFK;[1] ATP:D-fructose-6-phosphate 1-phosphotransferase, E.C. 2.7.1.11) catalyzes the phosphorylation of fructose-6-phosphate (Fru-6P) by ATP. The enzyme from *Escherichia coli* shows a highly cooperative saturation by Fru-6P with Hill coefficient $n_H = 3.8$ for 4 sites and is allosterically activated by ADP or GDP and inhibited by phosphoenolpyruvate (PEP) (Blangy et al., 1968). The steady-state kinetics of *E. coli* PFK have been interpreted according to the concerted allosteric mechanism (Monod et al., 1965) in which two conformational states of the protein, R and T, having different affinities for Fru-6P and effectors, are in equilibrium (Blangy et al., 1968). In the concerted model, the structural transition between the two states R and T does not only modify the binding sites for the ligands but also the areas of contact between subunits (Monod et al., 1965). The sequential model of allosteric regulation also emphasizes the importance of conformational changes in the interfaces between subunits (Koshland et al., 1966). X-ray crystallography has indeed confirmed that changes in the quaternary structures of a few proteins such as hemoglobin and aspartate transcarbamylase are indeed involved in the interactions that couple distant sites (Perutz, 1990; Evans, 1991).

The X-ray structure of *E. coli* PFK shows that the protein is built as a dimer of dimers, in which a given subunit is in contact with two others along two different interfaces. One of these interfaces contains the active site and the other the regulatory site. The areas of contact between subunits are quite different for these two interfaces, being ca. 1200 Å^2 for the "active" interface and 1800 Å^2 for the "regulatory" interface (Shirakihara & Evans, 1988; Evans, 1992). These interfaces also have different stabilities toward dissociation: the "active" interface is less stable than the "regulatory" interface and is thus broken first during dissociation of PFK and formed last during self-assembly. Moderate concentrations of KSCN, a chaotropic solvent additive that destabilizes the interactions between proteins (Arakawa & Timasheff, 1982), causes a specific reversible disruption of the "active" interface and yields an equilibrium between tetrameric PFK and the dimeric species in which two subunits are still associated by interactions along the "regulatory" interface (Deville-Bonne et al., 1989). This partial dissociation is accompanied by inactivation.

In the present work, the reversible inactivation upon mild dissociation by 0.4 M KSCN is studied for wild-type PFK and three of its mutants that have altered catalytic and/or regulatory properties: the D127S mutant which is the least active mutant, with a catalytic activity reduced by more than 10^3-fold (Hellinga & Evans, 1987), the T125S mutant that does no longer show any cooperativity for Fru-6P saturation (Auzat et al., 1994), and the E148A-R152A double mutant which is hypercooperative (Auzat et al., 1995b). In the mild dissociating conditions corresponding to 0.4 M KSCN, the

† This work was supported by Grant No. EP079 from the CNRS, Grant No. 9270300 from the University Paris 6, and the BSN-Danone group. The cost of publication of this article was defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC Section 1734 solely to indicate this fact.

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® Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: PFK, ATP:D-fructose-6-phosphate 1-phosphotransferase (E.C. 2.7.1.11); Fru-6P, D-fructose-6-phosphate; PEP, phosphoenolpyruvate; GDP, guanosine 5′-diphosphate; BSA, bovine serum albumin; D127S, T125S, and E148A-R152A, mutants of PFK with respectively a serine instead of an aspartate at position 127, a serine instead of a threonine at position 125, and two alanines instead of a glutamate and an arginine at positions 148 and 152.

0006-2960/95/0434-13203$09.00/0

protein is in equilibrium between active tetramers and inactive dimers. The displacement of this equilibrium toward association (or dissociation) upon increasing (or lowering) protein concentration can be followed through the changes in activity that reflect the changes in the tetrameric fraction, and the value of the equilibrium constant in these conditions can be determined. This value is directly related to the strength of the interactions across the "active" interface that maintain the tetrameric structure of PFK.

It is also observed that the mass action law influences a mixture of two different PFKs in 0.4 M KSCN as expected from a dimer−tetramer equilibrium in which two different dimers can form a hybrid tetramer by interacting along the "active" interface. In this way, we show that hybrid tetramers can be formed from dimers of (a) wild-type PFK and the inactive D127S mutant, (b) the inactive D127S mutant and the noncooperative T125S mutant, (c) the inactive D127S mutant and the hypercooperative E148A-R152A double mutant, and (d) the noncooperative T125S mutant and the hypercooperative E148A-R152A double mutant. Because of the different properties of wild-type PFK and its three mutants, these hybrid tetramers can be partially characterized without separating them from the other species present at equilibrium. A hybrid tetramer of PFK can indeed reveal a property that none of its "parents" has: Some GDP activation can be measured after complete denaturation followed by simultaneous renaturation and reassociation of two mutants that are both insensitive to GDP activation (Lau & Fersht, 1989). This appearance of GDP activation shows the formation of a hybrid tetramer, and the new quaternary interactions that exist within the hybrid lead to the formation of a functional regulatory site, in a sort of "intracistronic complementation" between these two mutants insensitive to GDP (Lau & Fersht, 1989). In the present work, the comparison between the hybrid tetramers and the "parental" homologous tetramers is focused on two properties: the stability of the tetrameric structure, which is a local property of the "active" interface, and the cooperativity of Fru-6P saturation, which is an overall property of the whole structure.

MATERIALS AND METHODS

Wild-type and active mutant PFKs were prepared, stored, and assayed as described previously (Auzat, 1993; Auzat et al., 1994, 1995b). The standard affinity chromatography on Dextran blue-Sepharose was used to purify the inactive D127S mutant screening the fractions for their protein content instead of their PFK activity (Lainé et al., 1992). The activity of PFK was measured by a coupled assay (Kotlarz & Buc, 1982) in the native assay buffer composed of 0.1 M Mes, 0.051 M *N*-ethylmorpholine, and 0.051 M diethanolamine, at pH 8.2 (Ellis & Morrison, 1982). Protein concentrations were determined according to Bradford (1976).

In a buffer composed of 50 mM imidazole, 0.4 M KSCN, 1 mM magnesium chloride, and 2 mM dithiothreitol, at pH 7, called buffer I, the dissociation of wild-type PFK or its mutants was rapid, and the equilibrium between active tetramers and inactive dimers was reached within a few minutes (Deville-Bonne et al., 1989). Wild-type PFK or one of its mutants was incubated in buffer I for 30 min at a variable protein concentration until the tetramer−dimer equilibrium was reached, and the residual activity was then measured. This residual activity is expressed relative to the total activity determined for the same amount of protein but prior to the dilution in KSCN-containing buffer I. In order to study the tetramer−dimer equilibrium when two different proteins are present, we used a procedure which ensured that (i) all the proteins remained in the same buffer (buffer I), (ii) the concentration of one protein remained fixed, and (iii) the only variable was the concentration of the other protein. A first sample of wild-type PFK, the T125S mutant, or the E148A-R152A mutant was allowed to dissociate for 10 min in buffer I at 25 °C. During the same time, a second sample of the other protein [the D127S or the E148A-R152A mutant or bovine serum albumin (BSA)] was also incubated and dissociated for 10 min in buffer I. The two samples were then mixed so that the final concentration of the protein from the first sample was always fixed at 0.3 mg/mL, which is 8.6 μM in polypeptide chains of PFK, and the final concentration of the protein from the second sample ranged between 0 and 130 μM, which is a molar excess up to 15-fold. The mixture of the two proteins in the same buffer I (this mixing does not involve any change in buffer) was allowed to equilibrate between the dimeric and tetrameric species equilibrium for 20−30 min at 25 °C, and the residual activity was measured.

Measuring the residual activity present in a given tetramer−dimer equilibrium involves the transfer of a protein sample from the dissociating buffer I to the native assay buffer. Slowing down reassociation of dimers into tetramers and minimizing reactivation during the assay has been achieved by using a total PFK concentration below 1.5×10^{-7} M (or 5 μg/mL) in the assay, since the second-order rate constant for the association of two PFK dimers into a tetramer is of the order of 10^4 s^{-1} M^{-1} in the absence of KSCN (Deville-Bonne et al., 1989). It was indeed checked that little reassociation occurs after this change in buffer during the 1−2 min of the assay itself. Unless otherwise specified, the activity of various samples of PFK was measured at saturating substrate concentrations of 1 mM for ATP and 1 mM for Fru-6P, except for samples with the E148A-R152A mutant which were assayed with 3 mM Fru-6P. The residual activity is often expressed relative to the total activity expected if all the PFKs present in the sample were active.

The saturation by Fru-6P of each PFK sample was determined in the native assay buffer at 1 mM ATP and variable Fru-6P concentration. The half-saturating Fru-6P concentration $(Fru\text{-}6P)_{1/2}$ and cooperativity coefficient n_H were obtained by fitting the experimental curve to the Hill equation using the Kaleidagraph 3.0 software (Biosoft, U.K.).

RESULTS AND DISCUSSION

Tetramer−Dimer Equilibrium of Wild-Type PFK. Upon incubation of *E. coli* PFK in various concentrations of KSCN, the enzymatic activity measured at saturating Fru-6P and ATP decreases rapidly until it remains at a constant residual level, indicating that the protein has reached an equilibrium between active and inactive forms (Deville-Bonne et al., 1989). The activity of samples incubated in 0.4 M KSCN is measured immediately after dilution of at least 100-fold into the assay mixture (see Materials and Methods). When measured at a given PFK concentration, the level of residual activity decreases with increasing KSCN (Figure 1). The relative residual activity remaining at a given KSCN is higher

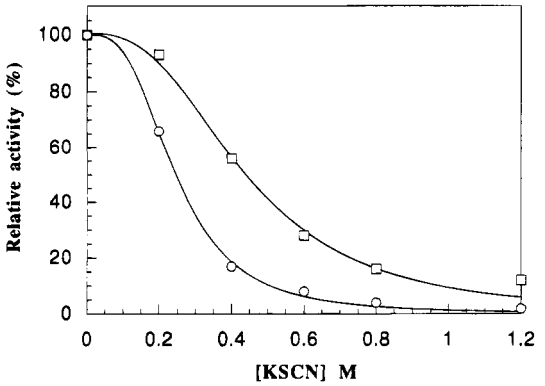

FIGURE 1: Relative residual activity of wild-type PFK equilibrated at 0.3 mg/mL (8.6 μM in polypeptide chains) (○) or 3 mg/mL (86 μM in chains) (□) in the presence of a variable concentration of KSCN for 20−30 min. Beside KSCN, the dissociation buffer is composed of 50 mM imidazole, 1 mM magnesium chloride, and 2 mM dithiothreitol, at pH 7. Activities are determined as described in Materials and Methods using 1 mM Fru-6P and 1 mM ATP. The relative residual activity corresponds to the ratio between the activity measured for a given amount of PFK after the tetramer−dimer equilibrium has been reached in the KSCN-containing buffer and the activity measured for the same amount of PFK before the addition of KSCN when all the enzyme is tetrameric and active (see text).

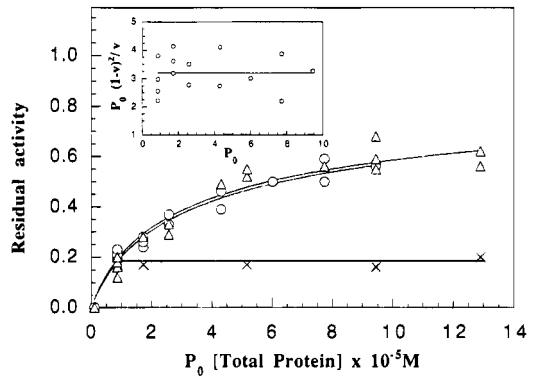

FIGURE 2: Variation of the relative residual activity v with the *total* protein concentration P_0 present in 0.4 M KSCN. The total protein concentration is made of wild-type PFK at a concentration of 0.3 mg/mL (8.6 μM in chains) and a variable concentration of wild-type PFK (○), of the inactive D127S mutant (△), or of BSA (×). The curve corresponds to the theoretical dependence of the fraction of tetramers v on the P_0 calculated from eq 1: $K_{eq} = P_0(1 - v)^2/v$, using the same value $K_{eq} = 3.2 \times 10^{-5}$ M for the dissociation equilibrium constant of the wild-type PFK tetramer and the hybrid tetramer between wild-type PFK and the D127S mutant. Inset: Plot of $P_0(1 - v)^2/v$ as a function of P_0 corresponding to the results on wild-type PFK (○) given in the figure.

when PFK is more concentrated (Figure 1), as expected if the loss of activity of PFK in the presence of KSCN is due to a dissociation of active tetramers into inactive dimers (Deville-Bonne et al., 1989). The fraction of PFK that is still active corresponds to the relative fraction of PFK chains that are still associated in a tetrameric active species. The relative residual activity is not increased by addition of BSA, indicating that an unrelated protein does not influence the tetramer−dimer equilibrium of PFK (Figure 2).

The increase of the relative residual activity measured upon increasing PFK concentration indicates that the number of subunits associated into active tetrameric species increases faster than the total number of subunits present. The dependence of the relative residual activity on the concentration of PFK present in 0.4 M KSCN is shown in Figure 2.

Table 1: Half-Saturating Concentrations [(Fru-6P)$_{1/2}$], Cooperativity Coefficients (n_H), and Relative Activity Determined for Native PFK, PFK Incubated for 20−30 min in 0.4 M KSCN at a Low (8.6 μM or 0.3 mg/mL) or High (130 μM or 4.5 mg/mL) Concentration, and PFK Incubated at 8.6 μM in 0.4 M KSCN in the Presence of a 15-Fold Molar Excess of Either BSA or the D127S Mutant[a]

PFK species	(Fru-6P)$_{1/2}$ (mM)	n_H	relative activity[b] (%)
native wild-type	0.35	3.9	100
wild-type in 0.4 M KSCN at			
4.5 mg/mL protein	0.40 ± 0.1	4.3 ± 0.6	59
0.3 mg/mL protein	0.35 ± 0.1	4.5 ± 0.7	18
0.3 mg/mL+ 15-fold D127S	0.40 ± 0.1	3.8 ± 0.5	64
0.3 mg/mL+ 15-fold BSA	0.60 ± 0.1	3.8 ± 0.6	17

[a] The concentration of ATP is 1 mM, and the values of (Fru-6P)$_{1/2}$ and n_H are obtained by fitting the saturation to the Hill equation. [b] Activity is expressed relative to the total amount of PFK present in the sample.

In the case of a simple tetramer−dimer equilibrium T ↔ 2D, the relative residual activity v is given by $v = 4T/P_0$ and the equilibrium dissociation constant K_{eq} by:

$$K_{eq} = D^2/T = (P_0 - 4T)^2/4T = P_0(1 - v)^2/v \quad (1)$$

with T and D being respectively the concentrations of tetramer and dimer and P_0 the total concentration of PFK expressed in polypeptide chains. The inset of Figure 2 shows that indeed $P_0(1 - v)^2/v$ does not vary with P_0 and gives the value of the equilibrium dissociation constant: $K_{eq} = (3.2 \pm 0.4) \times 10^{-5}$ M. The theoretical curve in Figure 2 has been calculated with this value of $K_{eq} = 3.2 \times 10^{-5}$ M and is in good agreement with the experimental points. Therefore the observed dependence of the residual activity on the total concentration of PFK is satisfactorily described by a simple tetramer−dimer equilibrium, T ↔ 2D.

The same half-saturating Fru-6P concentrations (Fru-6P)$_{1/2}$ and cooperativity coefficients n_H are obtained for the saturation by Fru-6P after incubation in KSCN of PFK at high or low concentration (Table 1), indicating that changing the protein concentration influences only the relative amount of active tetramers. Also, the presence during the incubation in KSCN of an inactive protein, either unrelated like BSA or very similar like the D127S mutant (see below), does not modify the saturation by Fru-6P (Table 1).

Tetramer−Dimer Equilibrium of the T125S and E148A-R152A Mutants. The dependences of the relative residual activity on protein concentration have been also measured at saturating Fru-6P and ATP for two other mutants of PFK, the noncooperative T125S single mutant (Auzat et al., 1994) and the hypercooperative E148A-R152A double mutant (Auzat et al., 1995b), and are shown in Figure 3. The K_{eq} of the dissociation of the tetramer into dimers has a value of $K_{eq} = (1.1 \pm 0.3) \times 10^{-5}$ M for the T125S mutant and $(1.9 \pm 0.4) \times 10^{-4}$ M for the E148A-R152A mutant. These values show that, as compared to wild-type PFK, the interaction between dimers is destabilized by the two mutations E148A and R152A in the interface, whereas it is stabilized by the active site mutation T125S. The lower stability of the E148A-R152A double mutant could be due to a packing defect of the interface and/or to the loss of some electrostatic interaction(s) (Shirakihara & Evans, 1988). It is more difficult to explain how the T125S mutation, which is not in the interface, can increase the stability all the more

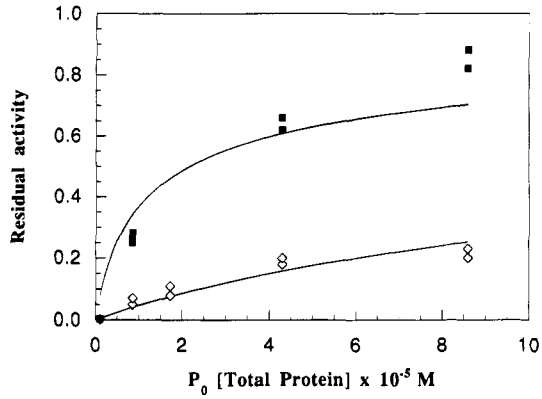

FIGURE 3: Dependence of the relative residual activity v on the protein concentration P_0 measured in 0.4 M KSCN for the T125S (■) and E148A-R152A (◇) mutants. The solid lines correspond to the theoretical dependences of v on P_0 calculated from eq 1 with values of K_{eq} of 1.1×10^{-5} M (■) and 1.9×10^{-4} M (◇), respectively. The activities are measured using Fru-6P concentrations of 1 mM for the T125S mutant and 3 mM for the E148A-R152A mutant and an ATP concentration of 1 mM for both.

since another mutation also in the active site, D127S, does not seem to have any effect (see below). It is possible that some mutations remote from the "active" interface cause not only minor local perturbations but also conformational changes that spread over (a large part of) the entire subunit and affect the stability of the interface. Such changes would affect the connection(s) between the active sites and the interfaces between subunits and could thus alter the allosteric interactions. Although they modify appreciably the enzymatic properties and the tetramer stability, the small changes due to some mutations are not detected by structural measurements: For instance, the fluorescence of the T125S mutant (Auzat, 1993) and its far-UV circular dichroism (Garel and Auzat, unpublished results) are identical with those of wild-type PFK. A study of several mutant PFKs is in progress and indicates that a 2-fold difference in K_{eq} can be measured, corresponding to a difference of less than 1 kJ/mol in the relative stabilities of the "active" interface in their quaternary structures.

The interactions between the two dimers across the "active" interface must at the same time maintain the tetrameric structure and participate in the allosteric conformational change(s). The value of K_{eq} reflects the strength of these interactions in terms of tetramer stability, and the value of n_H reflects their efficiency in coupling distant sites. The results obtained here with wild-type PFK and its two active mutants suggest that the stability of the tetramer and its cooperativity might be inversely correlated, with an increase (respectively decrease) in cooperativity being associated with a destabilization (respectively stabilization) of the "active" interface. Measurements of the residual activity in the presence of KSCN that can estimate the relative strength of the interaction between dimers are now being extended to a variety of other mutants of PFK, in order to check whether or not this correlation between tetramer stability and cooperativity of Fru-6P saturation is real. The PFK from *Bacillus stearothermophilus* would respect such a correlation, as this PFK is at the same time more stable than that from *E. coli* (Hengartner & Harris, 1975) and noncooperative in its saturation by Fru-6P in the absence of PEP (Valdez et al., 1989; Byrnes et al., 1994).

Hybrid between the Inactive D127S Mutant and Wild-Type PFK. In order to show that hybrid tetramers could be formed from two different dimers, we have used an inactive mutant as one of the partners, hoping that it could influence the tetramer−dimer equilibrium through the mass action law but without contributing directly to the total activity. The least active mutant of PFK is the D127S mutant that lacks a key catalytic group in its active site, namely, the carboxylate acting as a base to activate the OH group at the C1 position of Fru-6P during phosphoryl transfer (Hellinga & Evans, 1987; Lainé et al., 1992). Incubating together in 0.4 M KSCN a fixed concentration of wild-type PFK with increasing concentrations of this inactive D127S mutant results in an increase in the relative residual activity measured at saturating Fru-6P and ATP (Figure 2). If there were no interaction between wild-type PFK and the D127S mutant, the addition of an inactive protein should have no effect on the relative residual activity as in the case of BSA (Figure 2). The increase in relative residual activity shows that adding more and more D127S mutant increases the proportion of wild-type subunits that belong to a tetrameric structure and are active, even though the total number of wild-type subunits remains constant. This shows that an interaction exists between wild-type and mutant subunits that probably leads to a hybrid tetramer composed of one wild-type dimer and one D127S dimer interacting along the "active" interface with only two functional active sites (Figure 4).

The addition of the inactive D127S mutant to wild-type PFK in 0.4 M KSCN increases the relative residual activity as much as the addition of wild-type PFK itself: The fraction of PFK that exists as an active tetramer shows the same dependence on the concentration of wild-type PFK or the inactive D127S mutant added in order to displace the tetramer−dimer equilibrium (Figure 2). This striking similarity suggests that (i) the dissociation constant of the tetramer−dimer equilibrium for the interface between a wild-type and D127S dimer is the same as that for the wild-type homologous interface, $K_{eq} = 3.2 \times 10^{-5}$ M (and this value can probably be extrapolated to the dissociation of the homologous D127S interface), and (ii) a functional active site in the hybrid tetramer has the same specific activity as an active site in wild-type PFK. The wild-type dimer is inactive *per se* and reveals its potential activity only upon forming a tetramer (Figure 4), but a wild-type dimer has the same potential specific activity when a moiety in a hybrid tetramer formed with an inactive D127S dimer, as when a moiety in a homologous wild-type tetramer. The catalytic rate constant of the hybrid D127S/wild-type tetramer is found to be ca. 60 s^{-1}, i.e., about one-half that of wild-type PFK.

The equilibrium constant for the dissociation of the tetramer into dimers has the same value $K_{eq} = 3.2 \times 10^{-5}$ M for homologous wild-type and hybrid wild-type D127S tetramers, so that the ratio between hybrid and homologous tetramers will reflect the ratio between the amounts of wild-type and mutant subunits. With a 15-fold molar excess of D127S mutant over wild-type PFK, a wild-type dimer has a 1/16 = 6.25% probability of associating with another wild-type dimer into an homologous tetramer and a 15/16 = 93.3% probability of associating with a mutant dimer into a hybrid tetramer. Therefore, the wild-type subunits present in tetramers that are active will be distributed among 2 × 6.25% = 12.5% in homologous tetramers (with two wild-type dimers) and 87.5% in hybrid tetramers. The overall

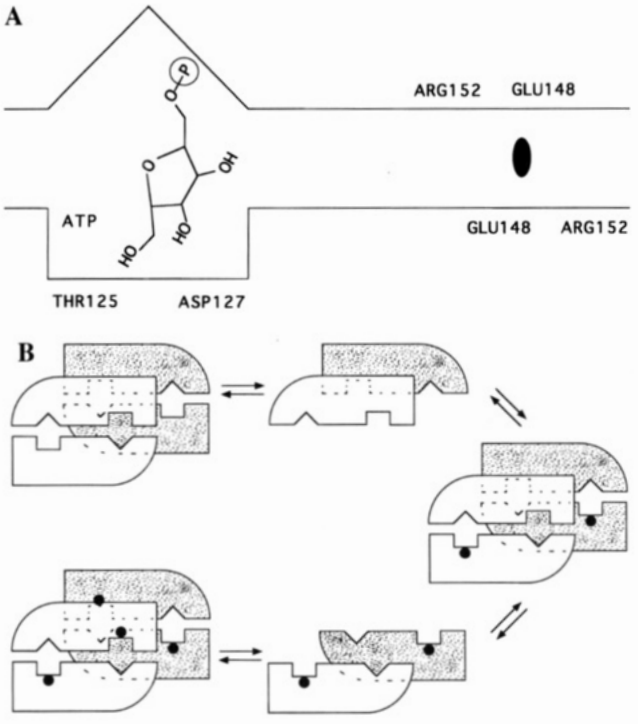

FIGURE 4: Simplified view of the structure of PFK inspired from the results of X-ray diffraction (Evans & Hudson, 1979; Shirakihara & Evans, 1988). (A) Part of the "active" interface with the residues E148 and R152 close to the 2-fold symmetry axis and the binding site for Fru-6P. The residues T125 and D127 do not strictly belong to the interface but to the subsite for the fructose moiety (shown as a square shape) of Fru-6P where the chemical reaction takes place. The subsite for the 6-phosphate group (shown as a triangle shape) is located on the opposite subunit across the interface. (B) Structures of tetrameric PFK with its four active sites and of dimeric PFK produced by the dissociation of the "active" interface. These structures explain why dissociation is accompanied by inactivation. Also shown are the structures of tetrameric and dimeric D127S inactive mutant (the mutation is represented by the black dot) and the structure of a hybrid tetramer that has only two functional active sites.

activity measured for wild-type subunits in the presence of a 15-fold molar excess of inactive D127S subunits is mostly due (at 87.5%) to the wild-type subunits present in hybrid tetramers. The $(Fru\text{-}6P)_{1/2}$ or the n_H is not modified by the presence of a 15-fold excess of D127S mutant (Table 1), which shows that wild-type and hybrid tetramers have the same saturations by Fru-6P. Hybrid tetramers have only two functional active sites (Figure 4), but they are as cooperative as homologous wild-type tetramers which have four active sites (Shirakihara & Evans, 1988). This indicates that the positive interactions between active sites that result in a cooperative saturation by Fru-6P do not require that catalysis takes place at all sites. The D127S mutation that drastically impairs catalysis does not alter the "active" interface with a wild-type dimer whether measured by its stability or its ability to couple distant sites. The same inhibition of 80% by 10 mM PEP is measured in the absence and presence of a 10-fold molar excess of the inactive D127S mutant, which shows that the hybrid tetramer can also undergo the same allosteric transition, from the R into the T state, as wild-type PFK.

Hybrid between the Noncooperative T125S Mutant and the Inactive D127S Mutant PFKs. As in the case of wild-type PFK, the changes in relative residual activity measured at saturating Fru-6P and ATP show that a hybrid tetramer can form between the noncooperative T125S mutant (Auzat et al., 1994) and the inactive D127S mutant (Hellinga & Evans, 1987), with a $K_{eq} = (1 \pm 0.3) \times 10^{-5}$ M. This value shows that the "active" interface formed between different dimers in this hybrid tetramer is as stable as that in the homologous T125S tetramer ($K_{eq} = 1.1 \times 10^{-5}$ M) and is more stable than that supposed in the homologous D127S tetramer ($K_{eq} = 3.2 \times 10^{-5}$ M). The effect of the T125S mutation on stability is not proportional to the number of mutated subunits, since stabilization of the tetrameric structure is already achieved with only two T125S chains per tetramer.

Table 2: Half-Saturating Concentrations $[(Fru\text{-}6P)_{1/2}]$ and Hill Cooperativity Coefficients (n_H) of the Fru-6P Saturation Determined for the Active Species Present at Equilibrium in 0.4 M KSCN When the T125S and E148A-R152A Mutants Are Either Alone or in the Presence of a 10-Fold Molar Excess of Inactive D127S Mutant[a]

PFK mutant	$(Fru\text{-}6P)_{1/2}$ (mM)	n_H
T125S	0.14 ± 0.04	1.0 ± 0.2
T125S + 10-fold D127S	0.21 ± 0.06	1.1 ± 0.2
E148A-R152A	1.6 ± 0.4	6.6 ± 0.6
E148A-R152A + 10-fold D127S	0.9 ± 0.3	4.3 ± 0.5

[a] The T125S mutant and the E148A-R152A double mutant are at the same concentration of 0.3 mg/mL (8.6 μM in polypeptide chains), and the D127S mutant is at 3 mg/mL (86 μM in chains). The concentration of ATP is 1 mM, and the values of $(Fru\text{-}6P)_{1/2}$ and n_H are obtained by fitting the saturation to the Hill equation.

The saturation by Fru-6P of the active T125S mutant is almost the same in the absence and presence of a 10-fold molar excess of inactive D127S mutant (Table 2), when more than 80% of the activity is due to hybrid tetramers. The saturation by Fru-6P of the hybrid tetramer remains similar to that of the homologous tetramer and is not cooperative, even though there could be a slight increase in the $(Fru\text{-}6P)_{1/2}$ (Table 2). The T125S mutation does not only abolish the cooperativity of Fru-6P saturation but also suppresses the inhibition by PEP (Auzat et al., 1994). The presence of a 10-fold excess of inactive D127S mutant does not restore any inhibition by PEP up to 10 mM PEP, indicating that the hybrid tetramer is as insensitive to PEP inhibition as the homologous T125S tetramer.

Therefore, the apparent behavior of the inactive D127S dimer depends on its partner within a hybrid tetramer: It is noncooperative and noninhibited by PEP when associated with a T125S dimer but can maintain both cooperativity and PEP inhibition when associated with a wild-type dimer.

Hybrid between the Hypercooperative E148A-R152A Mutant and the Inactive D127S Mutant PFKs. In the same way as above for wild-type PFK and the T125S mutant, the changes in relative residual activity measured at saturating Fru-6P and ATP show that a hybrid tetramer can form between one dimer from the hypercooperative E148A-R152A double mutant (Auzat et al., 1995b) and one dimer from the inactive D127S mutant (Hellinga & Evans, 1987), with a $K_{eq} = (8 \pm 3) \times 10^{-5}$ M. This value shows that the interaction between the two different dimers in this hybrid tetramer has an intermediate stability between that found for the less stable homologous E148A-R152A tetramer ($K_{eq} = 1.9 \times 10^{-4}$ M) and that supposed for the more stable homologous D127S tetramer ($K_{eq} = 3.2 \times 10^{-5}$ M).

The presence of a 10-fold molar excess of inactive D127S mutant modifies the saturation by Fru-6P of the active

E148A-R152A mutant. The saturation by Fru-6P measured when more than 80% of the activity is due to hybrid tetramers corresponds to values of $(Fru\text{-}6P)_{1/2}$ and n_H that are both lower than those of the homologous E148A-R152A tetramer (Table 2). Hybridization of a E148A-R152A dimer with an inactive D127S dimer leads to the formation of two functional active sites (Figure 4) with an increase of almost 2-fold in apparent affinity and a decrease of one-third in cooperativity coefficient (Table 2). Table 1 shows that the cooperativity of this hybrid tetramer between E148A-R152A and D127S still remains comparable to that of wild-type PFK and to that of the hybrid between wild-type PFK and D127S which also has two functional sites (Table 1).

Hybrid between the Hypercooperative E148A-R152A Mutant and the Noncooperative T125S Mutant PFKs. Up to now, we have only monitored the formation of hybrids between an active moiety (from wild-type, T125S, or E148A-R152A PFKs) and the inactive D127S moiety. These hybrids have been detected and partly characterized without purifying them because the inactive species displaces the equilibrium between active tetramers and inactive dimers through the mass action law but does not contribute to the overall activity that remains entirely due to the tetrameric fraction of the active partner.

In order to study the formation of a hybrid tetramer from two different active dimers, we have chosen two PFK mutants, T125S and E148A-R152A, that have so different saturations by Fru-6P that we anticipate one of them to appear as inactive at low Fru-6P concentration. Both mutants are fully active at 3 mM Fru-6P, but at 0.3 mM Fru-6P, the noncooperative T125S mutant still has 70% of its maximum activity (Table 2; Auzat et al., 1994), whereas the E148A-R152A mutant is essentially inactive, with less than 0.0015% activity, because of its hypercooperativity and rather high $(Fru\text{-}6P)_{1/2}$ (Table 2; Auzat et al., 1995b). The specific activity of the T125S mutant is ca. 35% of that of the other mutant (Auzat et al., 1994, 1995b), and it seems indeed unlikely that it could be enhanced by hybridization with a E148A-R152A dimer. Indeed, no activation of the T125S mutant is observed even with the allosteric activator of PFK, GDP (Auzat et al., 1994). Figure 5 shows the changes in activity measured at either 3 mM Fru-6P (Figure 5A) or 0.3 mM Fru-6P (Figure 5B) after incubating either separately or together in 0.4 M KSCN the T125S mutant at a fixed concentration of 0.3 mg/mL (or 8.6 μM in chains) and the E148A-R152A mutant at a variable concentration between 0 and 3 mg/mL (or 86 μM in chains).

When measured at 3 mM Fru-6P where both mutants are saturated, the activity obtained after incubation of the two mutants together is markedly higher than the sum of the activities obtained when each mutant is incubated separately. The T125S mutant alone gives a rather low activity because only 27% of the chains belongs to tetramers at the concentration used (Figure 3), and they have a lower specific activity (Auzat et al., 1994). The E148A-R152A mutant alone also gives a rather low activity because the instability of its tetrameric structure in 0.4 M KSCN ($K_{eq} = 1.9 \times 10^{-4}$ M) is such that only 20% of the chains is present as tetramers at the highest concentration used (Figure 3), even though this corresponds to a protein concentration 10 times higher than that of T125S. There is a substantial increase in activity upon incubating the two mutants together (Figure 5A), which shows the presence of hybrid tetramers, and we will admit

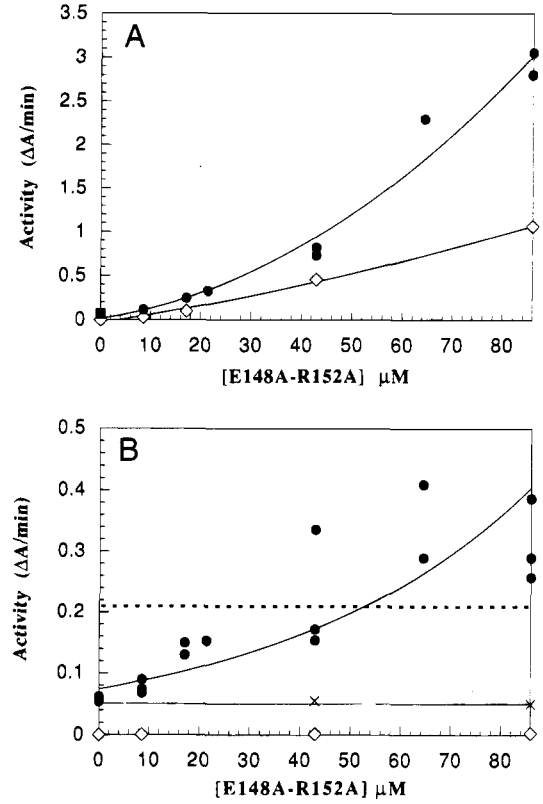

FIGURE 5: Activity measured for a variable concentration of the E148A-R152A mutant in equilibrium in 0.4 M KSCN when either alone (◇) or in the presence of a fixed concentration of 0.3 mg/mL (8.6 μM in chains) T125S mutant (●). Note that the activity here is expressed in absolute units of ΔA_{340nm}/min and not as a fraction relative to the total protein present as in Figures 1−3. The data for the E148A-R152A mutant correspond to those expressed in relative activity given by (◇) in Figure 3. (A) Activities are measured at a saturating Fru-6P concentration of 3 mM. The symbol (■) indicates the activity of the T125S mutant alone which corresponds to 27% relative to total T125S (Figure 3). The highest activity of the E148A-R152A mutant alone corresponds to 20% relative to total E148A-R152A (Figure 3). (B) Activities are measured at a Fru-6P concentration of 0.3 mM. The dashed line shows the activity expected if 100% of the T125S mutant present was tetrameric and active. The actual activity of the T125S mutant alone corresponds to the same tetramer fraction of 27% as that in panel A. Also shown is the activity measured for the T125S mutant in the presence of a 10-fold molar excess of BSA (×), which has no influence on the fraction of tetramers.

that this increase reflects an increase in the amount of tetramers rather than an increase in the specific activity of the T125S moiety (see above).

The activity due to the T125S subunits can be increased by an excess of E148A-R152A mutant because the fraction of T125S dimers that belongs to tetramers can be increased, just as an excess of inactive D127S dimers increases the activity of another PFK species (see above). This increase will take place as long as the hybrid tetramer is more stable than the homologous E148A-R152A tetramer ($K_{eq} = 1.9 \times 10^{-4}$ M) and even if it is less stable than the homologous T125S tetramer ($K_{eq} = 1.1 \times 10^{-5}$ M). Similarly, the activity due to the E148A-R152A mutant could also increase if the hybrid tetramer is more stable than the homologous E148A-R152A tetramer ($K_{eq} = 1.9 \times 10^{-4}$ M), so that a fraction larger than 20% of E148A-R152A dimers will belong to tetrameric structures and be active. It is likely that the value of K_{eq} for the hybrid between E148A-R152A and T125S lies between 8×10^{-5} and 1×10^{-5} M, which corresponds to

the hybrids between E148A-R152A and D127S and between T125S and D127S, respectively, so that the formation of hybrids is beneficial to both mutants and increases their fractions present as active tetramers.

Because the two "parents" are active as homologous tetramers, the actual value of the K_{eq} for the hybrid tetramer composed of one T125S and one E148A-R152A dimer cannot be derived solely from activity measurements. A quantitative analysis of the amounts of free dimers, homologous tetramers, or hybrid tetramers present in 0.4 M KSCN requires direct measurements of dimeric and tetrameric fractions, such as those done previously by calibrated high-performance liquid chromatography (Le Bras et al., 1989).

At 0.3 mM Fru-6P, the homologous E148A-R152A tetramer is 0.0015% saturated and thus appears as completely inactive (Auzat et al., 1995b), while the homologous T125S tetramer is ca. 70% saturated (Auzat et al., 1994). Figure 5B shows that, when activities are measured at 0.3 mM Fru-6P, incubating these two mutants together in 0.4 M KSCN also results in a substantial increase in activity as compared to incubating them separately. The level of activity exceeds that expected if 100% of the T125S dimers present were active (Figure 5B), showing that the increase in activity is not only due to an increase in the tetrameric fraction of the T125S mutant. Part of this increase must therefore be due to the E148A-R152A moiety present in hybrid tetramers with one E148A-R152A and one T125S dimer. Therefore, an E148A-R152A moiety belonging to a hybrid tetramer expresses a higher activity at 0.3 mM Fru-6P than the same E148A-R152A moiety belonging to a homologous tetramer. This higher activity at low Fru-6P of the E148A-R152A moiety can be explained by an increase in the affinity for Fru-6P and/or a decrease in the cooperativity of the E148A-R152A moiety upon hybridization with a T125S dimer. It has indeed been found above that both $(\text{Fru-6P})_{1/2}$ and n_H could be decreased; hybridization of an E148A-R152A dimer with an inactive D127S dimer lowers $(\text{Fru-6P})_{1/2}$ from 1.6 to 0.9 mM and n_H from 6.6 to 4.3 (Table 2). These values of $(\text{Fru-6P})_{1/2} = 0.9$ mM and $n_H = 4.3$ are however still too high to account for the marked increase in activity at low Fru-6P (Figure 5B), since the Hill equation predicts less than 1% saturation at 0.3 mM Fru-6P for the E148A-R152A moiety present in the hybrid. Rather, crude calculations suggest that the E148A-R152A moiety should be ca. 10% saturated at 0.3 mM Fru-6P in order to account for the observed activity and thus that $(\text{Fru-6P})_{1/2}$ could be as low as 0.5 mM with n_H remaining equal to 4.3 or as low as 2 with $(\text{Fru-6P})_{1/2}$ remaining equal to 0.9 mM. The apparent affinity and/or cooperativity of an E148A-R152A dimer seems thus to be modified to a larger extent upon hybridization with a noncooperative T125S dimer than with an inactive D127S dimer. This indicates that the same E148A-R152A moiety in a hybrid tetramer has different properties with a different partner, T125S or D127S.

Cooperative Saturation by Fru-6P of the Hybrid Tetramer between One Dimer from the Hypercooperative E148A-R152A Mutant and One Dimer from the Noncooperative T125S Mutant PFKs. Figure 6 shows that, up to 0.3 mM Fru-6P, the E148A-R152A mutant alone at a concentration of 3 mg/mL (86 μM in chains) has no or very little activity, and the T125S mutant alone at 0.3 mg/mL (8.6 μM in chains) gives the hyperbolic saturation expected from Table 2 with a value for $(\text{Fru-6P})_{1/2}$ of 0.15−0.2 mM. When the two

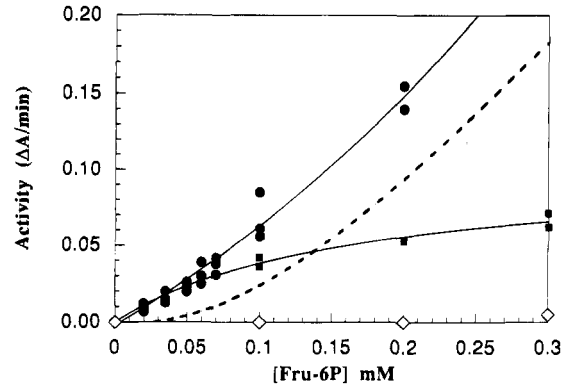

FIGURE 6: Low substrate part of the saturation by Fru-6 measured for the T125S and the E148A-R152A mutants after equilibrating them in 0.4 M KSCN either separately or together; (■) the T125S mutant alone at a concentration of 0.3 mg/mL (8.6 μM in chains), (◇) the E148A-R152A mutant alone at a 10-fold higher concentration of 3 mg/mL (86 μM in chains), and (●) the two mutants together with the same 10-fold molar excess of the E148A-R152A mutant (at 3 mg/mL) over the T125S mutant (at 0.3 mg/mL). The curve for the T125S mutant alone corresponds to a hyperbolic saturation with a $(\text{Fru-6P})_{1/2}$ of 0.17 mM. The dashed line corresponds to the difference between the curves obtained for the two mutants together and for the T125S mutant alone (see text).

mutants are together in the same 10 to 1 ratio of E148A-R152A to T125S, the overall Fru-6P saturation can be decomposed into the sum of a hyperbolic saturation similar to that of the T125S mutant alone and a sigmoidal saturation (Figure 6). Under these conditions, the only active species are the noncooperative homologous T125S and the hybrid tetramers. Although the relative proportions of the homologous and hybrid tetramers cannot be calculated without knowing the K_{eq} for the hybrid (see above), it is likely that the sigmoidal Fru-6P saturation reflects a cooperative saturation by Fru-6P of the hybrid tetramer having one T125S and one E148A-R152A dimer. However, the origin of this sigmoidal Fru-6P saturation cannot be attributed only to the E148A-R152A moiety of this hybrid tetramer and could also be due to its T125S moiety. Indeed, it cannot be exluded that the T125S dimer has acquired some cooperativity upon hybrization with an E148A-R152A dimer, but it can be concluded that the E148A-R152A moiety has not lost all its cooperativity upon hybridization with a T125S dimer.

CONCLUSIONS

The present results show that different dimers can pair along the "active" interface into hybrid tetramers, even when residues are mutated in this interface. Homologous tetramers possess a 2-fold symmetry (Shirakihara & Evans, 1988), and this symmetry is perturbed in hybrid tetramers and especially in those with an E148A-R152A moiety (and a D127S or T125S dimer) that are asymmetric in the area of contact itself (Figure 4). These asymmetric hybrids between E148A-R152A and D127S or T125S are more stable than the symmetric homologous E148A-R152A tetramer, indicating that stability is not related to symmetry. Within a hybrid tetramer, the more stable dimer stabilizes the least stable, so that the stability of the hybrid is intermediate between those of its "parents".

The properties of the hybrid tetramers show that a given dimer can exhibit different properties when hybridized with different partners. The inactive D127S dimer behaves as

noncooperative and noninhibited by PEP when associated with a T125S dimer but supports the cooperativity of an E148A-R152A dimer and can maintain both cooperativity and PEP inhibition of a wild-type dimer. Similarly, the hybridization of an E148A-R152A dimer with either a T125S or a D127S dimer, which should have the same "active" interface, leads to a different decrease in $(Fru\text{-}6P)_{1/2}$ and/or n_H. That a given dimer can "adapt" to different partners suggests that the interaction across the "active" interface is not rigid but flexible. Such flexibility of the interface might be crucial for the allosteric interactions that couple distant sites and could explain the possible negative correlation noted above between the strength of the interactions across the "active" interface and the cooperativity Hill coefficient. A stable and rigid interface would give a solid tetramer but would prevent any structural perturbation within a subunit from spreading, whereas a flexible and deformable interface would give a fragile tetramer but would be sensitive to and transmit minor changes occurring in one of the subunits. If cooperativity depends upon some flexibility, then "improving" a protein by increasing its stability could be detrimental to its function and affect its regulatory properties. It has also been noted recently that increasing the stability of T4 phage lyzozyme could decrease its catalytic efficiency (Shoichet et al., 1995).

Using the inactive D127S mutant is the easiest way to monitor the formation of hybrid tetramers and to determine some of their properties such as stability and Fru-6P saturation. When the two "parental" homologous tetramers are active, activity measurements by themselves cannot measure the stability or the parameters of Fru-6P saturation of the hybrid. Additional data are needed to determine the K_{eq} of the hybrid tetramer and the apparent affinites $(Fru\text{-}6P)_{1/2}$ and cooperativity coefficients n_H that can be attributed to each of the moieties. However, in combination with measurements of ligand binding by equilibrium dialysis or fluorescence (Berger & Evans, 1991; Deville-Bonne & Garel, 1992), of the tetramer−dimer equilibrium by HPLC (Le Bras et al., 1989), of the rate of ligand-induced conformational changes (Auzat et al., 1995a), and of steady-state activity, the characterization of hybrid tetramers between wild-type and/or mutant PFKs could be a valuable tool in a quantitative study of the long-range allosteric interactions in an enzyme.

ACKNOWLEDGMENT

The authors are grateful to Dr. Phil R. Evans for his generous gift of the inactive and useful D127S mutant and for many stimulating discussions on PFK.

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BI9513160