Urea-Induced Inactivation, Dissociation, and Unfolding of the Allosteric Phosphofructokinase from *Escherichia coli*†

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ABSTRACT: The influence of urea on the allosteric phosphofructokinase from *Escherichia coli* has been studied by measuring the changes in enzymatic activity, protein fluorescence, circular dichroism, and retention in size-exclusion chromatography. Tetrameric, dimeric, and monomeric forms of the protein can be discriminated by their elution from a high-performance liquid chromatography gel filtration column. Three successive steps can be detected during the urea-induced denaturation of phosphofructokinase: (i) the dissociation of the native tetramer into dimers which abolishes the activity; (ii) the dissociation of dimers into monomers which exposes the unique tryptophan, Trp-311, to the aqueous solvent; (iii) the unfolding of the monomers which disrupts most of the secondary structure. This pathway involves the ordered dissociation of the interfaces between subunits and supports a previous hypothesis (Deville-Bonne et al., 1989). Phosphofructokinase can be quantitatively renatured from urea solutions, provided that precautions are taken to avoid the aggregation of one insoluble monomeric state. The renaturation of phosphofructokinase from urea implies three steps: an initial folding reaction within the monomeric state is followed by two successive association steps. The faster association step restores the native fluorescence, and the slower regenerates the active enzyme. The renaturation and denaturation of phosphofructokinase correspond to the complex pathway: tetramer ⇌ dimer ⇌ folded monomer ⇌ unfolded monomer. It is found that the subunit interface which forms the regulatory site is more stable and associates 40 times more rapidly than the subunit interface which forms the active site.

In *Escherichia coli*, the flux through the glycolytic pathway is controlled by phosphofructokinase (PFK)[1] which catalyzes the conversion of Fru-6P into Fru-1,6P_2 using ATP. This control occurs via the allosteric regulation of the activity of PFK: the saturation of the enzyme by Fru-6P is highly cooperative, and the affinity for Fru-6P is increased by ADP (or GDP) and decreased by PEP (Blangy et al., 1968). The protein is a tetramer composed of four identical chains (PM = 34 817) (Hellinga & Evans, 1985) arranged as dimer of dimers with a *D2* symmetry, in which the subunits are paired along two different interfaces (Evans & Hudson, 1979); the binding site for Fru-6P belongs to one interface, the A (for active) interface, and the binding site for allosteric effectors belongs to the other, the R (for regulatory) interface (Evans et al., 1981; Shirakihara & Evans, 1988).

A dimeric form of PFK can be transiently obtained during the renaturation and self-assembly of the protein after unfolding and separation of the chains by guanidine hydrochloride (Teschner & Garel, 1989). A dimeric form of PFK can also be observed during the dissociation of native tetrameric PFK by the chaotropic salt KSCN (Deville-Bonne et al., 1989). The dimeric intermediates have the same properties in the two cases: they are both able to bind the allosteric inhibitor PEP and to shield the unique tryptophan residue, Trp-311, from the aqueous solvent. This suggests that a unique dimeric species, in which the two subunits are held together by interactions along the R interface, is a compulsory intermediate in both association and dissociation of PFK. The association between subunits when starting from guanidine-unfolded PFK as well as the dissociation of the tetramer induced by KSCN seems to follow the same specific order, in which the R interface is formed before and broken after the A interface. However, this conclusion has been reached by using different solvent additives, guanidine hydrochloride for association and KSCN for dissociation; also, these are both salts, and it is important to check that this order is the same in conditions which do not imply a high ionic strength.

In the present work, the influence of urea, a neutral compound, is examined on both the association and dissociation of PFK. In addition to the properties used previously such as enzymatic activity, fluorescence, and circular dichroism (Teschner & Garel, 1989), the protein is also characterized by its behavior on a molecular sieve in high-performance liquid chromatography (HPLC). This technique not only monitors the signficant changes in size and/or shape implied by protein association–dissociation at equilibrium but also allows kinetic measurements thanks to its rather short dead time (below 10 min in some cases). The dissociation of PFK induced by urea takes place in two steps, and the intermediate species can be separated by HPLC. The first step follows a time course identical with that of the loss of enzymatic activity and involves the dissociation of PFK from tetramer into dimers. The second step, which corresponds to the formation of monomeric species, can be observed even in conditions where one of these monomeric species shows a strong tendency toward aggregation.

Experimental Procedures

The origin of chemicals, the preparation of PFK, and the measurements of enzymatic activity, fluorescence intensities,

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[1] Abbreviations: PFK, phosphofructokinase [ATP:D-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.11)]; HPLC, high-performance liquid chromatography; CD, circular dichroism; Fru-6P, D-fructose 6-phosphate; Fru-1,6P_2, D-fructose 1,6-diphosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane.

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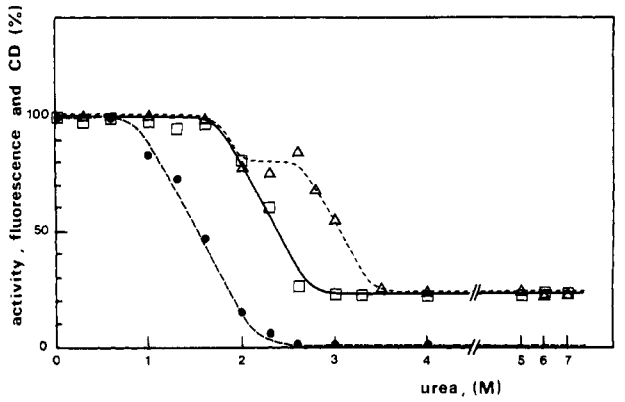

FIGURE 1: Urea-dependent deactivation and denaturation of PFK. Enzymatic activity (●), relative fluorescence (λ = 280 nm) (□), and circular dichroism (ellipticity at 222 nm) (△) of PFK are measured after 24 h at 22 °C in several urea concentrations in 0.1 M sodium phosphate buffer, pH 7.6, containing 1 mM magnesium acetate and 2 mM DTT (buffer P). These changes have been normalized relative to complete unfolding: the value of 100% corresponds to native PFK in the absence of any denaturant while the 0% value corresponds to the completely unfolded state of PFK as obtained in concentrated guanidine hydrochloride solutions (Teschner & Garel, 1989). Enzyme subunit concentration: 8 μM (activity and CD) and 520 nM (fluorescence).

circular dichroic spectra, etc. have been described previously (Teschner & Garel, 1989; Deville-Bonne et al., 1989).

HPLC Gel Filtration Analysis. The setting used for HPLC was composed of an LKB 2150 high-pressure pump, a Rheodyne 7125 injector with a 20-μL sample loop, an LKB 2138 UV detector equipped with a microcell of 8 μL, and a Merck-Hitachi D-2000 chromato-integrator. Alternatively, proteins were detected by a Schoeffel FS 970 fluorometer. Gel filtration of phosphofructokinase was carried out by using a TSK G 3000 SW column (0.75 × 30 cm) preceded by a TSK SWP guard column (0.75 × 7.5 cm). All buffers used for elution were first degassed and then filtered through a Millipore 0.22-μm GSWP filter. In a typical run, a phosphofructokinase sample of 20 μL (with 0.5–2 mg/mL protein) was injected and eluted with 0.2 M phosphate buffer, pH 7.0, containing 1 mM Mg^{2+}, urea, and sometimes Fru-6P; the flow rate was maintained at 1 mL/min with a pressure of 16–28 bar, depending on the urea concentration. The protein was detected by using its absorbance at 280 nm or its fluorescence at 330 nm (λ_{exc} = 280 nm).

Results

Loss of Enzymatic Activity, Exposure of Trp-311 to Solvent, and Destruction of Secondary Structure Take Place at Different Concentrations of Urea. Figure 1 shows the transition curves obtained after incubating native PFK for 24 h in different concentrations of urea, as determined by enzymatic activity, fluorescence, and circular dichroism. As the urea concentration increases, the first property to be modified is the activity: half of the initial activity is lost at 1.5 M urea, and PFK is completely inactivated above 3 M urea. The fluorescence also follows a monophasic cooperative curve with a midpoint of 2.3 M urea. Between 3.5 and 7 M urea, the fluorescence emission spectrum of PFK remains constant but is slightly different from that of the protein completely unfolded by guanidine hydrochloride (Teschner & Garel, 1989). The changes in circular dichroism are biphasic: at 1.8–2 M urea, a first smaller change corresponds to a relative amplitude of 20–25%, and a second larger occurs with a midpoint at 3 M urea for the remaining 75–80%. Here also, the circular dichroic spectrum measured above 4 M urea is not exactly that

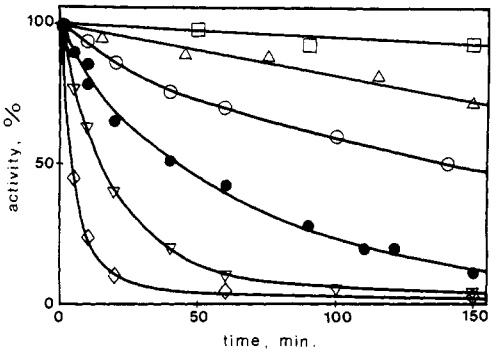

FIGURE 2: Kinetics of inactivation of PFK at different concentrations of urea. The enzyme free from Fru-6P was incubated at a concentration of 35 μM in PFK subunits for the indicated time in buffer P at 22 °C in the presence of (□) 1, (△) 2, (○) 3, (●) 4, (▽) 5, and (◇) 6 M urea; activity was then measured in standard conditions after a dilution of at least a 100-fold. It has been verified that no reactivation takes place during the assay (see text and Figure 4).

of the protein completely unfolded by guanidine hydrochloride (Teschner & Garel, 1989). Although some aggregated PFK was probably present in some of the CD samples (see below), the extent of aggregation did not interfere significantly with the analysis of the CD spectra.

Finding nonsuperimposable transition curves for different parameters shows that several intermediate forms of PFK are present at equilibrium depending on the urea concentration:

$$N \rightleftharpoons D \rightleftharpoons M \rightleftharpoons U$$

The N ⇌ D step is related to the inactivation of PFK, the D ⇌ M step involves the change in fluorescence as well as a small change in circular dichroism, and most of the change in circular dichroism occurs in the M ⇌ U step. Fluorescence measures the degree of shielding from solvent of Trp-311 (Brand & Witholt, 1967), and circular dichroism reflects the content of secondary structure (Adler et al., 1973). The influence of urea involves three successive steps: first, PFK loses its activity, then it exposes its Trp-311 to solvent, and finally its secondary structure disappears. Comparison of these data with previous data (Teschner & Garel, 1989; Deville-Bonne et al., 1989) suggests that D is the same dimeric species as found earlier (inactive with a native fluorescence), that M is a folded monomer (retaining most of the secondary structure), and that U is a largely unfolded monomeric state (but both its circular dichroism spectrum and its intrinsic fluorescence indicate that U has retained some residual structure, as compared to guanidine-unfolded PFK).

Urea-Induced Inactivation of PFK. Figure 2 gives the kinetics of the loss of activity of PFK at different urea concentrations: the rate of inactivation increases with the denaturant concentration. Previous reactivation studies (Martel & Garel, 1984; Teschner & Garel, 1989; Deville-Bonne et al., 1989), as well as results shown below in Figure 4, indicate that PFK does not regain a significant fraction of its activity during the time of the assay, even in the presence of saturating concentrations of its substrates. At any time, the enzyme which has been partially inactivated by urea shows the allosteric behavior of native PFK in terms of cooperative saturation by Fru-6P, activation by GDP, and inhibition by PEP. This is expected if the urea-induced inactivation occurs by the same dissociation as that found earlier in KSCN (Deville-Bonne et al., 1989).

The presence of the substrate Fru-6P protects markedly the enzyme against urea-induced inactivation (Figure 3), as it does against inactivation by heat (Le Bras & Garel, 1982), guanidine (Teschner & Garel, 1989), or KSCN (Deville-Bonne et

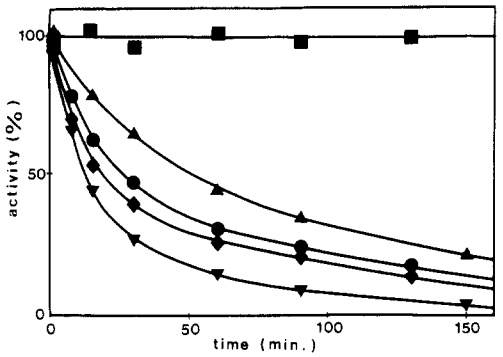

FIGURE 3: Kinetics of inactivation of PFK by 4 M urea in the presence of different ligands. The enzyme was incubated at a concentration of 35 μM for the indicated time in buffer P at 22 °C in the presence of 4 M urea and of (●) no ligand, (■) 2 mM Fru-6P, (♦) 1 mM ATP, (▲) 2 mM GDP, and (▼) 16 mM PEP; activity was then measured in standard conditions after a dilution of at least a 100-fold.

al., 1989). When PFK is saturated by Fru-6P, the activity remains constant for several hours in 4 M urea. The presence of the other substrate ATP has little effect on the rate of inactivation, even at a saturating concentration (Figure 3).

The presence of allosteric effectors has only a weak influence on the stability of PFK in 4 M urea: GDP seems to slightly decrease and PEP to slightly increase the rate of inactivation (Figure 3). This effect is similar to the KSCN-induced inactivation and different from the thermal inactivation of PFK, which is considerably slower in the presence of GDP (Le Bras & Garel, 1982).

One of the Species Involved in the Dissociation of PFK Has a Tendency To Aggregate at Intermediate Urea Concentrations. Incubation of PFK in urea can lead to the formation of aggregates. This aggregation prevents complete reactivation upon dilution of the denaturant and is responsible for an apparent hydrodynamic volume large enough to be excluded on a TSK G 3000 SW column (see below). The extent of aggregation depends on the time of incubation in urea, the concentration of urea, and the concentration of the protein. This indicates that one of the intermediate states formed during the dissociation–denaturation of PFK induced by urea is not very soluble and aggregates at intermediate urea concentrations.

Complete reactivation of PFK shows that no irreversible aggregation has occurred after 24 h in urea in the following conditions: (i) less than 0.5 μM in PFK subunits at any urea concentration; (ii) less than 10 μM in PFK subunits above 4 M urea; and (iii) less than 40 μM in PFK subunits above 6 M urea. Very limited aggregation can take place in conditions used for CD measurements (Figure 1), but the size and/or number of aggregates is small enough so that the signal-to-noise ratio remains correct. Also, when samples which give complete reactivation are subjected to size-exclusion chromatography, their elution profiles show sometimes that part of PFK elutes as a high molecular weight species. It could be that the matrix of the HPLC column is an environment which favors (or even triggers) aggregation. It could also be that such HPLC gel filtration detects some reversible aggregates which are not stable upon dilution of urea.

PFK Can Be Renatured and Reactivated by Dilution of Urea. All the properties of native PFK can be quantitatively recovered upon dilution of urea provided that the adequate precautions have been taken to avoid protein aggregation. Figure 4 shows the kinetics of the increase in fluorescence and of regain of activity upon dilution of PFK from 7.5 to 0.5 M urea. The initial part of the reaction has been enlarged to show

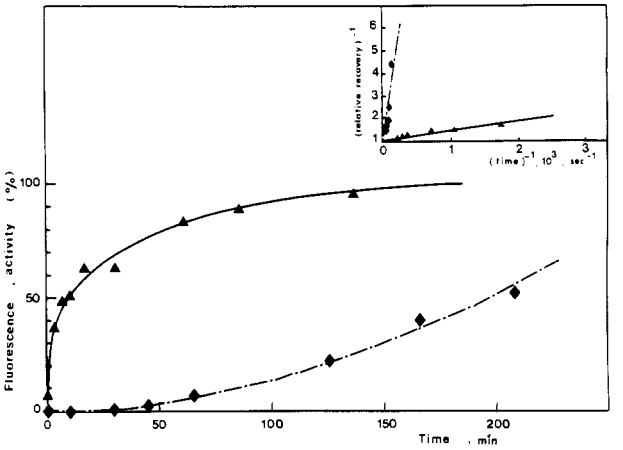

FIGURE 4: Reactivation of PFK upon dilution of urea: the early part of the kinetics of renaturation of PFK as measured by the reappearance of native fluorescence (▲) and activity (♦) upon dilution of urea after inactivation in 7.5 M urea. The enzyme (7.8 μM) is incubated 24 h in urea (7.5 M) and then diluted to 0.52 μM in buffer P at 22 °C. Inset: Second-order double-reciprocal plot of the complete kinetics of native fluorescence recovery (▲) and reactivation (♦) of PFK (0.52 μM) at 22 °C in residual 0.5 M urea. The fluorescence change is corrected from the rapid initial increase (15%). The observed rate constants are 4×10^3 $M^{-1}\cdot s^{-1}$ for the fluorescence change and 10^2 $M^{-1}\cdot s^{-1}$ for the reactivation. Only the points taken after 120 min and corresponding to a reactivation of at least 25% (and up to 80%) are shown in the inset (and not those of the early lag phase) and used for the extrapolation to an infinite renaturation time.

that the protein resumes its native fluorescence much faster than it recovers its activity. After an initial rapid increase, the fluorescence change follows a bimolecular reaction, with a second-order rate constant of 4×10^3 $M^{-1}\cdot s^{-1}$ (inset of Figure 4). The reactivation shows biphasic kinetics composed of a lag phase followed by a bimolecular reaction with a second-order rate constant of 10^2 $M^{-1}\cdot s^{-1}$. No activity is measured during several minutes, showing that reactivation of PFK during the assay is negligible. The length of this lag phase in reactivation is comparable to the half-time of the regain of native fluorescence, as if both correspond to the same reaction. Extrapolation to infinite time of the double-reciprocal plot given in the inset of Figure 4 shows that both the native fluorescence and enzymatic activity of PFK can be recovered to 100%. Such quantitative renaturation of PFK has also been obtained after unfolding by guanidine hydrochloride or KSCN (Martel & Garel, 1984; Teschner & Garel, 1989; Deville-Bonne et al., 1989). Results obtained at several PFK concentrations are in agreement with a two-step mechanism:

$$4 \text{ monomers} \xrightarrow{4 \times 10^3\ M^{-1}\cdot s^{-1}} 2 \text{ dimers} \xrightarrow{1.2 \times 10^2\ M^{-1}\cdot s^{-1}} \text{tetramer}$$

where the fluorescence change corresponds to the faster step, monomer → dimer, and the activity regain corresponds to the slower step, dimer → tetramer. The same scheme and kinetic analysis have been proposed previously for renaturation from guanidine hydrochloride (Teschner & Garel, 1989) or KSCN (Deville-Bonne et al., 1989), with transient accumulation of a dimeric form of PFK.

No lag phase is seen in the fluorescence change, indicating that a fast reaction within the monomeric state of PFK converts the largely unfolded U species, initially present in 7.5 M urea, into the folded M, able to dimerize.

Direct Measurement of the Urea-Induced Dissociation of PFK by HPLC. Figure 5 shows the elution diagram of PFK from a TSK G 3000 SW HPLC gel filtration column after various times of incubation in 4 M urea. The elution buffer also contains 4 M urea, and the protein remains in the same

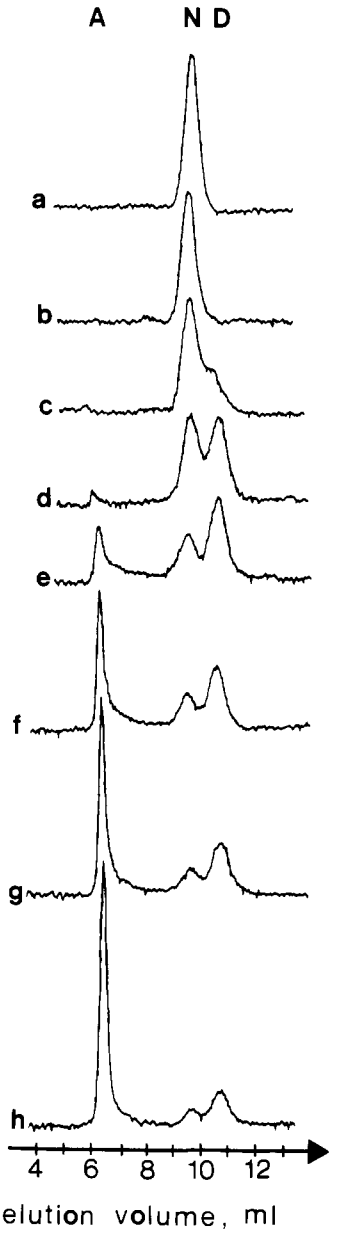

FIGURE 5: Changes in the HPLC elution diagram of PFK upon dissociation of the protein by 4 M urea. The enzyme free from Fru-6P was incubated at a concentration of 35 μM in buffer P with 4 M urea. At different times, a sample was withdrawn, injected into an HPLC gel filtration setting composed of a guard column and a TSK G 3000 SW column (30 × 0.75 cm), and eluted at a flow rate of 1 mL/min by buffer P containing 4 M urea. Temperature: 22 °C. Diagrams a and b are control experiments showing that PFK remains native after an incubation of 0 (a) or 180 (b) min when 2 mM Fru-6P is present in addition to buffer P and urea during both the incubation and elution. Diagrams c–h are obtained after incubating PFK in 4 M urea for 0 (c), 20 (d), 60 (e), 100 (f), 160 (g), and 220 (h) min. These times correspond only to the initial incubation and do not include the 6–11 min needed for the protein to elute.

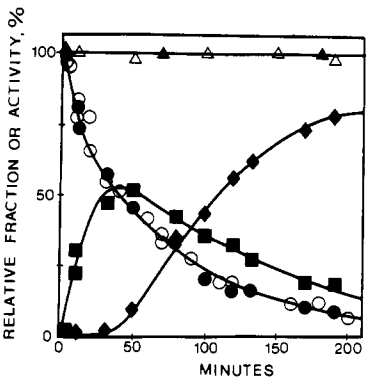

FIGURE 6: Kinetics of the changes in the structure and activity of PFK induced by 4 M urea. Experimental conditions as given in the legend of Figure 5. Activity (○) is expressed relative to that of the native enzyme before dilution in buffer P and urea. The relative amounts of the (●) N, (■) D, and (♦) A species were determined from elution diagrams such as those shown in Figure 5 by cutting and weighting the different peaks and are expressed as the ratio between the area of a given peak and the total area under all three peaks N, D, and A. Also shown are the results obtained for the activity (△) and the fraction of N (▲) in the presence of 2 mM Fru-6P in the incubation medium and elution buffer. The time indicated corresponds to the duration of the incubation plus 10 min to take the elution time into account.

medium before and during the chromatography. Inactivation of PFK by 4 M urea is slow, so the activity does not change markedly during the time of the HPLC run. A rather high concentration of 35 μM is used for PFK to allow good detection using the protein absorption at 280 nm. When Fru-6P is present, the elution diagram of PFK remains unchanged for several hours, with an elution volume of 9.6 mL corresponding to the native protein. In the absence of Fru-6P, the native protein, N, gradually disappears, while another species, D, with a slightly larger elution volume of 10.7 mL is formed. As shown below, D is the first inactive intermediate and is thus the same as the D species considered above. After about 30 min in 4 M urea, a third species, A, begins to appear with a smaller elution volume of 6.6 mL. This species A represents the largest fraction of PFK after 3 h (Figure 5), and all of it after 24 h. Increasing the time of incubation of PFK in 4 M urea changes only the peak heights and not their positions in the elution diagram, indicating a "slow-exchange" situation, i.e., a slow interconversion between the species N, D, and A.

The fraction of PFK present as N, D, or A after different times in 4 M urea is given in Figure 6. There is a good correlation in time between the disappearance of N, the formation of D, and the lag phase in the appearance of A; the disappearance of D and the formation of A are also correlated. This suggests that the ordered scheme:

$$N \rightarrow D \rightarrow A$$

accounts for the changes in the elution diagram. As expected, these two reactions are slow, with half-lives of about 30 and 70 min. Figure 6 also shows that the loss of enzymatic activity and the disappearance of N follow the same kinetics, and thus both D and A are inactive. Fru-6P at a concentration of 1 mM or above protects PFK against inactivation (Figure 3) and dissociation (Figure 6) in 4 M urea.

The elution volume of D is larger than that of N, indicating that it has a smaller size. However, the elution volume of A is not that expected for a monomer of PFK; rather, the elution of A corresponds to the exclusion volume of the HPLC column, suggesting that A is an aggregated form of PFK. The incubation of 35 μM PFK in 4 M urea leads indeed to extensive protein aggregation (see above). The total area corresponding to all three peaks, N, D, and A, of the elution profile remains approximately constant during 3 h (Figure 5), suggesting that no serious loss of protein occurs during the HPLC run. This indicates that A is an aggregated form of PFK which is still soluble in 4 M urea and does not stick to the TSK column. This aggregation is related to the low ionic strength of urea solutions, since (a) it is significantly reduced when 0.5 M NaCl is present in addition to 4 M urea in the initial incubation (unpublished observations from this laboratory) and (b) it does not occur when dissociation is induced by KSCN instead of urea (see below).

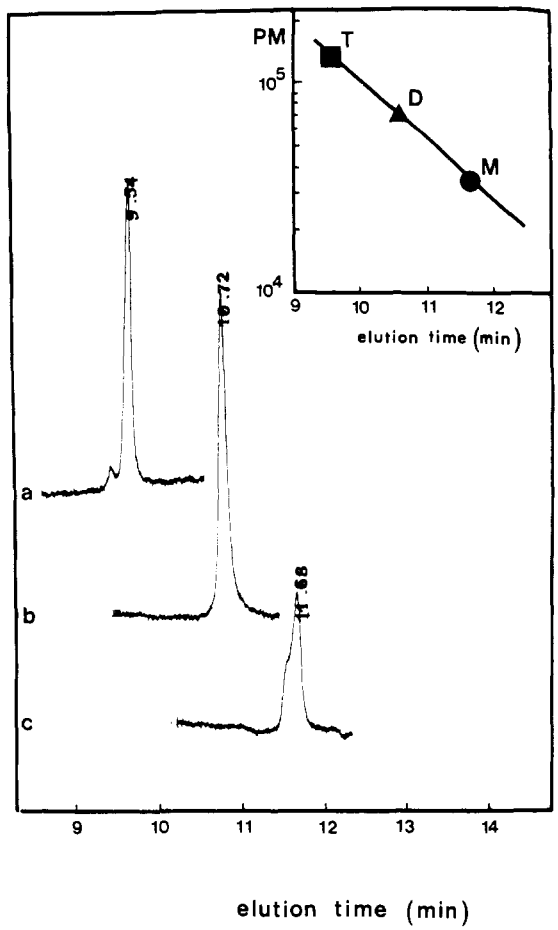

FIGURE 7: HPLC elution diagram of PFK upon dissociation by KSCN. The LKB TSK G 3000 SW column (30 × 0.75 cm) equipped with a precolumn (7.5 × 0.75 cm) was run at 1 mL·min^{-1} in buffer H (0.1 M sodium phosphate, pH 7.0, with 1 mM magnesium acetate and 0.25 M NaCl) containing also (a) 0.25 M KSCN and 2 mM Fru-6P, (b) 0.25 M KSCN, or (c) 0.5 M KSCN. PFK samples (20 μL at a protein concentration of 50 μM) were loaded after a 0.5-h incubation in the corresponding elution buffer. Protein was detected by using the fluorometer (λ_{exc} = 280 nm). Inset: Calibration of HPLC gel filtration.

The KSCN-induced dissociation of PFK has been previously studied by fluorescence and activity measurements (Deville-Bonne et al., 1989). The time dependence of the HPLC elution diagram of PFK in KSCN resembles that in urea and corresponds also to the interconversion between three species. Species N and D have the same elution volume as in urea, but the elution volume of the last species to be formed, M, is 11.7 mL instead of 6.6 mL for species A in urea. The HPLC column can be calibrated by using PFK itself: the protein is tetrameric in the presence of Fru-6P, dimeric after 30 min in 0.25 M KSCN, and monomeric after 30 min in 0.5 M KSCN (Deville-Bonne et al., 1989). Figure 7 shows that the elution volumes of 10.7 mL for D and of 11.7 mL for M correspond to dimeric and monomeric PFK, respectively. Comparison of the behaviors of PFK in urea and in KSCN suggests that it is the monomeric state of the protein which undergoes aggregation in urea.

Discussion

The present work shows that HPLC can be used to identify intermediate species during the association or dissociation of oligomeric proteins. By lowering the protein concentration, one can decrease the rate of bimolecular reactions so that they become slow as compared to the HPLC dead time, about a few minutes. Spectrophotometric or fluorometric detection is sensitive enough to be adapted to these low concentrations.

All the preceding results about the conformational changes of PFK in urea are compatible with the following mechanism:

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

where N is the native tetramer, D is the dimer where the "regulatory" interface is formed, and M and U are a (partially) folded and a largely unfolded monomer, respectively. N is the only active species; N and D both have a native fluorescence; N and D have a native and M a close-to-native CD spectrum; the association states of N, D, and M are those corresponding to their hydrodynamic volumes.

The presence of (at least) four species is established by the noncoincidence of the transition curves measured for three different parameters (Figure 1). The stoichiometry between these species is indicated by the kinetics of renaturation, which imply two successive bimolecular reactions (Figure 4). This mechanism is also in agreement with the following points: (i) The residual activity measured for PFK at any time is as sensitive to allosteric effectors as is the native enzyme. The three-dimensional structure of PFK is such that the tetramer N is the only form with both active and regulatory sites (Evans & Hudson, 1979; Shirakihara & Evans, 1988): indeed, loss of activity and disappearance of the tetramer occur at the same time (Figure 6). (ii) The fluorescence of PFK is mainly due to its single tryptophan residue, Trp-311. Trp-311 is located at one of the contact areas between subunits (Shirakihara & Evans, 1988) and is thus at the surface of an isolated monomer, whether folded as M or unfolded as U. (iii) The change in fluorescence takes place after the inactivation when PFK dissociates (Figure 1) and before the reactivation when the protein reassociates (Figure 4). This shows that the disappearance (or appearance) of N and the exposure to (or shielding from) solvent of Trp-311 occur in different steps. The same dimer D, with a buried Trp-311, is therefore an intermediate in the association and dissociation of PFK, independently of the denaturant, urea, guanidine hydrochloride, or KSCN (Teschner & Garel, 1989; Deville-Bonne et al., 1989).

According to the above mechanism, the quaternary structure of PFK is disrupted before the tertiary structure. The rates of dissociation are, however, different in the three denaturing agents: in both guanidine hydrochloride (Teschner & Garel, 1989) and KSCN (Deville-Bonne et al., 1989), which are polar, dissociation is rapid and occurs within minutes, whereas it is slower in the less polar agent urea. This could indicate that electrostatic interactions are involved in the association between subunits.

The pathway of reconstitution of the native PFK tetramer from monomers (whether partially folded or completely unfolded) is composed of two successive bimolecular reactions. The rate constants obtained for such renaturation from guanidine hydrochloride (Teschner & Garel, 1989), KSCN (Deville-Bonne et al., 1989), or urea are given in Table I. These rates can hardly be compared directly because of the differences in the renaturation conditions (pH, buffer, residual concentration of denaturant, etc.), but it seems that reassociation is slower in the case of urea. Table I also shows that the ratio between the rates of the two association steps is the same in the three cases: forming the dimer from two monomers is about 40 times faster than forming the tetramer from four monomers. This difference in rate is independent of the nature and residual concentration of denaturant (Table I), and therefore seems to reflect a difference in the interactions between PFK subunits rather than a difference in the interactions between subunits and solvent. The first association step leading

Table I: Reassociation of PFK by Dilution after Inactivation by Several Dissociating Agents

dissociation by	residual denaturant during reassociation (M)	second-order rate constant ($M^{-1}\cdot s^{-1}$) for regain of native fluorescence (M → D)	second-order rate constant ($M^{-1}\cdot s^{-1}$) for reactivation (M → T)	ref
7.5 M urea[a]	0.5[a]	4×10^3	10^2	this paper
2 M Gdn-HCl[a]	0.02[a]	4×10^4	10^3	*c*
0.5 M KSCN[b]	0.025[b]	2×10^5	5×10^3	*d*

[a] 0.1 M phosphate buffer, pH 7.6, containing 1 mM Mg^{2+} and 2 mM DTT. Dissociation time = 24 h. [b] 50 mM imidazole buffer, pH 7.0, containing 1 mM Mg^{2+} and 2 mM DTT. Dissociation time = 0.5 h. [c] Teschner and Garel (1989). [d] Deville-Bonne et al. (1989).

to D involves a larger area of contact between subunits than the second association step leading to N, which explains the greater stability of the R (regulatory) interface as compared to the A (active) interface (Shirakihara & Evans, 1988). It is probable that the 40-fold difference found here in the rates of subunit association is also related to the fact that these two steps do not decrease the protein surface accessible to solvent by the same account.

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REFERENCES

Adler, A., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675–735.

Blangy, D., Buc, H., & Monod, J. (1968) *J. Mol. Biol. 31*, 15–35.

Brand, L., & Witholt, B. (1967) *Methods Enzymol. 11*, 776–856.

Deville-Bonne, D., Le Bras, G., Teschner, W., & Garel, J.-R. (1989) *Biochemistry 28*, 1917–1922.

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

Evans, P. R., Farrants, W. G., & Hudson, P. J. (1981) *Philos. Trans. R. Soc. London, B 293*, 53–62.

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

Lau, F. T.-K., & Fersht, A. R. (1987) *Nature (London) 326*, 811–812.

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

Shirakihara, Y., & Evans, P. (1988) *J. Mol. Biol. 204*, 973–994.

Teschner, W., & Garel, J.-R. (1989) *Biochemistry 28*, 1912–1916.

Dissection of the Effector-Binding Site and Complementation Studies of *Escherichia coli* Phosphofructokinase Using Site-Directed Mutagenesis†

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ABSTRACT: A systematic study by site-directed mutagenesis has been conducted on the effector site of phosphofructokinase from *Escherichia coli* to delineate the role of side chains in binding the allosteric activator, GDP, and inhibitor, PEP, and to search for key residues in the allosteric transtion. Target residues were identified from the crystal structure of the enzyme–nucleoside diphosphate complex. It is found that both activator and inhibitor bind to the same set of amino acid side chains. Deletion of positively charged groups (Arg21, Arg25, Arg54, Arg154, and Lys213 mutated to alanine) weakens binding of both effectors by 2–3 kcal/mol, consistent with the disruption of charged hydrogen bonds. Residue Glu187, which is known from the crystal structure to bind the coordinated Mg^{2+} ion of GDP, is found to have a unique behavior on mutation and appears to be crucial in triggering the allosteric transition. All other residues mutated simply weaken binding of both PEP and GDP in a parallel manner. However, mutation of Glu → Ala187 reverses the roles of GDP and PEP, causing GDP to become an allosteric inhibitor and PEP an activator. Mutation of Glu → Gln187 has only a small effect on the binding of PEP, and both PEP and GDP are inhibitors. Studies are described in which mutations in different subunits of a tetrameric complex complement each other. The effector site is composed of residues from two subunits. In particular, Arg21 and Lys213 in each site are from different subunits. Mutations of either one of these residues abolishes activation by GDP of the homotetramer. However, the hybrid Arg → Ala21/Lys → Ala213 is activated by GDP and its kinetic properties consistent with a heterotetramer being formed in the expected statistical distribution. Assuming this distribution, the kinetic data may be adequately described by the MWC concerted mechanism.

Phosphofructokinase catalyzes the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. The major form of phosphofructokinase in *Escherichia coli* is pfk 1, which represents about 90% of the phosphofructokinase activity in the cells. It is a tetramer of four identical subunits. The kinetic properties of pfk 1 from *E. coli* have been studied extensively by Blangy et al. (1968) and Blangy (1971). The native enzyme is allosterically regulated by its effectors, being inhibited by phosphoenolpyruvate (PEP) and activated by adenosine di-

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