Nativelike Intermediate on the Unfolding Pathway of Pig Kidney Fructose-1,6-bisphosphatase†

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ABSTRACT: The unfolding and dissociation of the tetrameric enzyme fructose-1,6-bisphosphatase from pig kidney by guanidine hydrochloride have been investigated at equilibrium by monitoring enzyme activity, ANS binding, intrinsic (tyrosine) protein fluorescence, exposure of thiol groups, fluorescence of extrinsic probes (AEDANS, MIANS), and size-exclusion chromatography. The unfolding is a multistate process involving as the first intermediate a catalytically inactive tetramer. The evidence that indicates the existence of this intermediate is as follows: (1) the loss of enzymatic activity and the concomitant increase of ANS binding, at low concentrations of Gdn·HCl (midpoint at 0.75 M), are both protein concentration independent, and (2) the enzyme remains in a tetrameric state at 0.9 M Gdn·HCl as shown by size-exclusion chromatography. At slightly higher Gdn·HCl concentrations the inactive tetramer dissociates to a compact dimer which is prone to aggregate. Further evidence for dissociation of tetramers to dimers and of dimers to monomers comes from the concentration dependence of AEDANS-labeled enzyme anisotropy data. Above 2.3 M Gdn·HCl the change of AEDANS anisotropy is concentration independent, indicative of monomer unfolding, which also is detected by a red shift of MIANS-labeled enzyme emission. At Gdn·HCl concentrations higher than 3.0 M, the protein elutes from the size-exclusion column as a single peak, with a retention volume smaller than that of the native protein, corresponding to the completely unfolded monomer. In the presence of its cofactor Mg^{2+}, the denaturated enzyme could be successfully reconstituted into the active enzyme with a yield of ∼70−90%. Refolding kinetic data indicate that rapid refolding and reassociation of the monomers into a nativelike tetramer and reactivation of the tetramer are sequential events, the latter involving slow and small conformational rearrangements in the refolded enzyme.

The reaction catalyzed by fructose-1,6-bisphosphatase

$$\text{Fru-1,6-P}_2 + \text{H}_2\text{O} \rightarrow \text{Fru-6-P} + \text{P}_i$$

is a fundamental regulatory step in the gluconeogenic pathway, the metabolic pathway going from nonglycolytic precursors to intracellular glucose. Therefore, fructose-1,6-bisphosphatase plays a major role in the regulation of gluconeogenesis in mammalian cells. The activity of the enzyme is modulated negatively by AMP and Fru-2,6-P_2[1] which, in a reciprocal fashion, affect positively the activity of phosphofructokinase, a control point in glycolysis (for a review, see ref *1*). The inhibition of fructose-1,6-bisphosphatase by AMP is at an allosteric site (*2*−*5*), while Fru-2,6-P_2 is a competitive inhibitor of all known forms of the enzyme, and kinetic and structural evidence indicates that the sugar bisphosphate binds to the enzyme active site (*4*, *6*). The inhibition by Fru-2,6-P_2 is synergistic with AMP modulation serving as a fine-tuning mechanism for the regulation of fructose-1,6-bisphosphatase.

The pig kidney fructose-1,6-bisphosphatase is a tetrameric protein composed of identical subunits, each of 337 amino acid residues and a molecular weight of about 36500 (*7*, *8*). The molecular and crystal structures of this enzyme complexed with various ligands have been solved (*5*, *6*, *9*, *10*). In the native tetramer the subunits are paired along two different interfaces, with a D_2 symmetry. The crystallographic studies established two quaternary conformations for the protein, the R and T forms, which differ by a 17° rotation of the lower dimer C3C4 relative to the upper dimer C1C2 (*5*, *9*, *11*−*13*). These observations indicate that in the native tetramer the four subunits are arranged structurally and functionally as a dimer of dimers. Within each monomer the allosteric site lies 28 Å from the nearest active site (*5*, *10*, *12*). Kinetic and structural results show that the binding of AMP alone at the allosteric site is sufficient to lock the enzyme into the T form, while binding of substrate and inhibitors (such as Fru-2,6-P_2 and Fru-6-P) to the active site in the R form does not alter the quaternary structure to the T form. One of the interfaces contains the binding site for the substrate Fru-1,6-P_2, while the other is adjacent to the binding site for the allosteric inhibitor AMP. Therefore, the dissociation of the tetramer can yield two different dimers

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[1] Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; Gdn·HCl, guanidine hydrochloride; ANS, 1-anilinonaphthalene-8-sulfonate; I-AEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; NBS_2, 5,5′-dithiobis(2-nitrobenzoic acid).

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depending on which interface gets broken first. Similarly, the association of two monomers in refolding experiments can also produce two different dimeric species according to the nature of the created interface. Furthermore, pig kidney fructose-1,6-bisphosphatase does not contain tryptophan residues, but each monomer has one reactive thiol group, Cys128, located in the interface of the active site which can be used to attach fluorescent reporter groups (*14*). All of the above studies were undertaken with a common central goal to explore structure−function relationships in the enzyme molecule. However, basic information needed to establish how individual subunits of fructose-1,6-bisphosphatase fold into a catalytically active conformation following their biosynthesis is still scant.

Understanding the folding/unfolding and self-assembly processes of oligomeric proteins remains a major problem. Equilibrium denaturation studies of such proteins provide important information on the relationship of folding and oligomerization processes and on the influence of quaternary structure on protein stability (*15*, *16*). The fructose-1,6-bisphosphatase provides an attractive system for investigating the transition between the folded (native) and denaturated states, but with only two exceptions (*17*, *18*) denaturation and refolding studies on this enzyme have not been made. Jiang and Tsou (*17*) have reported that inactivation of snake muscle fructose-1,6-bisphosphatase precedes loss of allosteric properties during Gdn·HCl denaturation, suggesting that unfolding takes place sequentially. On the contrary, it has been reported (*18*) that inactivation and dissociation of the snake muscle enzyme occur simultaneously. Since the snake muscle enzyme is virtually unique among fructose-1,6-bisphosphatases, because it is the only one which can be activated by AMP (*19*), and there is no evidence in the literature that the mammalian fructose-1,6-bisphosphatase tetramer can be dissociated and re-formed, we have investigated the dissociation and unfolding of pig kidney fructose-1,6-bisphosphatase in Gdn·HCl solutions using the following probes: (i) enzyme activity, to sense disruption of the active site region; (ii) intrinsic fluorescence emission intensity and thiol group exposure, to monitor global structural changes induced by the denaturant; (iii) ANS binding, to detect the appearance of hydrophobic patches in the protein during the unfolding; (iv) size-exclusion chromatography, to detect dissociation steps at intermediate denaturant concentrations; and (v) fluorescence emission wavelength and fluorescence anisotropy of the reporter groups, MIANS and AEDANS, introduced on Cys128 of the enzyme, to monitor conformational changes and enzyme dissociation. The data indicate that unfolding and refolding of pig kidney fructose-1,6-bisphosphatase are multistate processes involving a catalytically inactive tetrameric intermediate during the course of the reaction. In addition, we report that this enzyme can be denatured to unfolded monomers in Gdn·HCl and subsequently refolded to its catalytically active native state. The inactivation and dissociation as well as the reassociation and reactivation of the enzyme are described as sequential events, and the reactivation is shown to involve slow and small conformational rearrangements in the refolded molecule.

EXPERIMENTAL PROCEDURES

Materials. The preparation of purified fructose-1,6-bisphosphatase was as described previously (*20*). ANS and MIANS were obtained from Molecular Probes. Gdn·HCl, DTT, and auxiliary enzymes were purchased from Sigma. All other chemicals were of analytical grade and were obtained mostly from Sigma and Merck.

Spectrophotometric Assay of Fructose-1,6-bisphosphatase Activity. The fructose-1,6-bisphosphatase enzyme activity was determined by measuring spectrophotometrically the release of fructose 6-phosphate during hydrolysis of fructose 1,6-bisphosphate (*14*). One unit of activity is defined as 1 μmol of fructose 6-phosphate generated/min at 30 °C, and the specific activity of the purified enzyme was 35−38 units/mg of protein when assayed in the presence of 150 mM K^+. At the usual protein concentration used in the assays during Gdn·HCl-dependent deactivation experiments (0.2 μg/mL), nonlinear progress curves were seen, indicating that significant reactivation of the protein occurred in these conditions. To prevent this reactivation, trypsin (20 μg of protein/mL) was added to the assay mixture (*21*). Control experiments showed that this concentration of trypsin prevents further reactivation of fructose-1,6-bisphosphatase in the assay media (presumably by degrading reactivation-competent unfolded intermediates) without affecting the activity of native (or folded) fructose-1,6-bisphosphatase or auxiliary enzymes. Under these conditions the assays followed linear kinetics for at least 5 min.

Gdn·HCl Treatment. For enzyme denaturation studies, the fructose-1,6-bisphosphatase was diluted into 0.1 M Hepes/NaOH buffer, pH 7.5, containing 0.1 mM EDTA, 5 mM DTT, and 2 mM $MgSO_4$ and Gdn·HCl at the desired concentration. The solutions were allowed to stand for 4 h at 15 °C to reach equilibrium. Aliquots were removed for enzyme activity assays and for fluorescence measurement. The concentration of the Gdn·HCl stock solution was determined by refractometry according to Pace (*22*). Since unfolding and refolding reactions are strongly affected by the temperature, all of the experiments were performed at 15 °C to compare the results obtained by the methods described below.

Fluorescence Determinations. Fluorescence spectra were determined at 15 °C using a Perkin-Elmer LS-50B spectrofluorometer, equipped with filter polarizers. For spectra, excitation and emission slits were set to 5 nm. For intrinsic tyrosine fluorescence, 278 nm excitation was used; emission spectra were collected from 290 to 355 nm. For MIANS fluorescence, the excitation wavelength was set to 327 nm, and emission was determined from 350 to 550 nm. Spectra were corrected for the buffer background. For the anisotropy measurements of AEDANS-labeled enzyme, the excitation wavelength was set to 380 nm, and emission was detected at 475 nm, using excitation and emission slits of 5 and 20 nm, respectively. For each sample anisotropy was measured four times, and the values were averaged.

Light Scattering. Light scattering was measured with a Perkin-Elmer LS-50B spectrofluorometer. The excitation and emission wavelengths were set at 400 nm and excitation and emission slit widths at 2.5 nm.

ANS Binding. The binding of ANS to pig kidney fructose-1,6-bisphosphatase was studied by fluorescence at 15 °C as a function of Gdn·HCl concentration (0−3.0 M). The excitation wavelength was set at 400 nm, and the ANS emission was monitored at 474 nm. The concentration of ANS was 50 μM. The protein samples containing Gdn·HCl

were allowed to equilibrate for 4 h before the addition of ANS and measurement of ANS binding.

Spectrophotometric Titration of Thiol Groups by NBS_2. The reactivity of thiols in fructose-1,6-bisphosphatase was measured by monitoring at 412 nm the time course of the reaction of the protein with NBS_2 (180-fold excess over the enzyme subunit). Aliquots of 5 mM NBS_2, enough to attain a final concentration of 0.25 mM, were added into two cells: a reference cell containing solvent only (50 mM Hepes/NaOH, 0.1 mM EDTA, 2 mM $MgSO_4$, pH 7.5) and a test cell containing the same solvent and 50 μg/mL fructose-1,6-bisphosphatase. Gdn·HCl at different concentrations was added to both cells. The absorbance at 412 nm was monitored at 15 °C until all accessible sulfhydryl groups have been titrated by the reagent. Control experiments with cysteine and β-mercaptoethanol have shown that Gdn·HCl did not interfere with the measurements. The molar absorption coefficient at 412 nm was taken as 13600 M^{-1} cm^{-1} (*23*).

Size-Exclusion HPLC. HPLC was run on a Shimadzu LC-4A chromatograph equipped with a SIL-1A sample injector, a SPD-2AM spectrophotometric detector, a C-R3A recorder (Shimadzu), and a TSK-G3000 SW_{XL} column (0.78 × 30 cm; TosoHaas). All buffer solutions prepared for HPLC were passed through a 0.45 μm pore size nylon filter. Fructose-1,6-bisphosphatase samples at different Gdn·HCl concentrations in 50 mM Hepes/NaOH, pH 7.5, at 15 °C, containing 0.1 mM EDTA, 5 mM DTT, 2 mM $MgSO_4$, and 100 mM Na_2SO_4, were prepared 4 h before injection onto the column which was preequilibrated at 15 °C with the same concentration of Gdn·HCl in the same buffer. The rate of flow through the column was maintained at 0.8 mL/min, and the absorbance at 280 nm of the effluent was recorded. Twenty micrograms of protein was used in each chromatographic run. The following proteins were used as molecular mass standards: RNase A (14 kDa), chicken egg ovalbumin (43 kDa), BSA (67 kDa), γ-globulin (158 kDa), and thyroglobulin (monomer 335 kDa; dimer 663 kDa).

Labeling with Fluorescent Probes. The native fructose-1,6-bisphosphatase was reacted with MIANS at 4 °C in 50 mM Hepes/NaOH buffer, pH 7.5, 2 mM $MgSO_4$, and 0.1 mM EDTA, with MIANS being added in a 3:2 molar ratio to the enzyme subunit. After 30 min the reaction was stopped by addition of 10 mM DTT, and the excess reagent was removed by gel filtration onto Sephadex G-25. The protein concentration in the eluate was determined by the procedure of Bradford (*24*), and the incorporation of MIANS was estimated using an $\epsilon^{327nm} = 17000$ M^{-1} cm^{-1} (*25*). It was estimated that 1.02 mol of MIANS/mol of enzyme subunit was incorporated into the native enzyme. The modified enzyme was stable for at least 1 month at −20 °C.

For AEDANS labeling, the native fructose-1,6-bisphosphatase was reacted with I-AEDANS at 30 °C in 50 mM Tris-HCl buffer, pH 7.5, 0.1 mM EDTA, and 0.44 mM AMP, with I-AEDANS being added in a 2:1 molar ratio to the enzyme subunit. After 25 min the reaction was stopped by addition of 10 mM DTT, and the excess reagent was removed as described for labeling with MIANS. The incorporation of AEDANS was determined using an $\epsilon^{339nm} = 6100$ M^{-1} cm^{-1} for AEDANS (*25*). It was estimated that 0.83 mol of AEDANS/mol of enzyme subunit was incorporated.

Refolding Studies. For the kinetic determination of the renaturation rate, fructose-1,6-bisphosphatase was first denatured in 4 M Gdn·HCl in 0.1 M Hepes/NaOH buffer, pH 7.5, 0.1 mM EDTA, 2 mM $MgSO_4$, and 5 mM DTT for 3 h at 15 °C. The denatured enzyme was diluted in the same buffer without denaturant at 15 °C to attain a final concentration of 0.1 M Gdn·HCl. At indicated times, aliquots were removed for enzyme activity assays. To follow reactivation after treatment at different concentrations of Gdn·HCl, the protein was incubated at the indicated concentrations of denaturant for 4 h at 15 °C; then it was diluted to a final concentration of 0.2 M Gdn·HCl and different concentrations of protein. The recovery of enzyme activity was measured after further incubation at 15 °C for enough time to ascertain that it attained a stable final value.

RESULTS

Changes in Enzyme Activity, Intrinsic Protein Fluorescence, ANS Binding, and SH Group Exposure Take Place at Different Concentrations of Gdn·HCl. Figure 1 shows the transition curves obtained after native fructose-1,6-bisphosphatase was incubated for 4 h at 15 °C in different concentrations of Gdn·HCl, as determined by enzymatic activity, intrinsic fluorescence, ANS binding, and titration of protein SH groups. The enzymatic activity of fructose-1,6-bisphosphatase as a function of the denaturant concentration shows an initial enhancement (maximum ~30%) between 0 and 0.5 M Gdn·HCl (Figure 1A). However, at Gdn·HCl concentrations higher than 0.6 M a sharp decrease in enzymatic activity occurred (Figure 1A). No residual activity was observed above 1.0 M Gdn·HCl. The midpoint for Gdn·HCl-based inactivation was 0.75 M. Neither the initial activity enhancement nor the activity loss is due to an effect of Gdn·HCl in the assay medium (data not shown). At enzyme concentrations ranging over 1−25 μg/mL, the inactivation curve did not change significantly. These observations suggest that low Gdn·HCl concentrations perturb the active site region and also that inactivation of the enzyme is not coincident with dissociation of the protein.

ANS, a hydrophobic fluorophore, can be used as an external probe for the unfolding of fructose-1,6-bisphosphatase. This fluorescent probe has a low emission in a polar environment, such as aqueous solutions, but its fluorescence emission is dramatically increased in nonpolar environments in such a way that the changes in ANS fluorescence are related to the increase in accessible hydrophobic surface upon protein unfolding. We found that native fructose-1,6-bisphosphatase binds ANS in the absence of Gdn·HCl (Figure 1B). AMP displaces 50% of the bound fluorescent probe from the enzyme (data not shown). The addition of low denaturant concentrations does not affect ANS fluorescence, but between 0.6 and 0.9 M Gdn·HCl there is a sharp rise in ANS fluorescence and thus in ANS binding. This transition is coincident with the loss of activity; the midpoint for this change is about 0.75 M Gdn·HCl. The ANS emission spectrum (following excitation at 390 nm) shows a maximum at 487 nm in the presence of the native enzyme, which is shifted to 474 nm at 0.9 M Gdn·HCl, demonstrating binding of the probe to the newly exposed hydrophobic areas of the protein as denaturation occurs. Furthermore, we were unable to detect an effect of the protein concentration, between 5 and 50 μg/mL, on the normalized ANS fluorescence (data

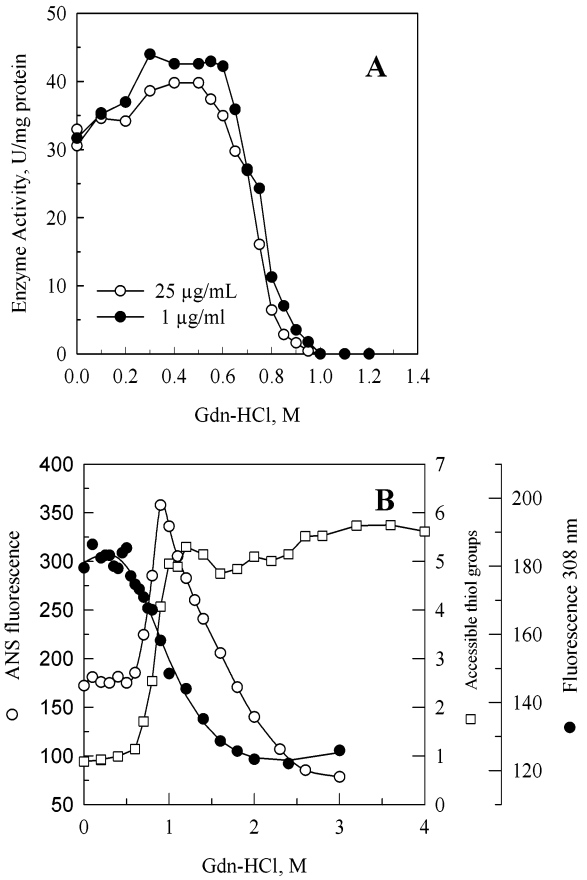

FIGURE 1: Gdn·HCl-dependent deactivation and denaturation of fructose-1,6-bisphosphatase. The enzyme was incubated in the indicated concentrations of Gdn·HCl as described in Experimental Procedures. (A) Fructose-1,6-bisphosphatase activity changes at protein concentrations of 1 μg/mL (●) and 25 μg/mL (○). Enzyme activity was measured at 50 μM substrate after the enzyme was incubated for 4 h at 15 °C at the Gdn·HCl concentration indicated. (B) Effects of Gdn·HCl denaturation on fluorescence emission (●), ANS binding (○), and accessibility of thiol groups to NBS_2 (□). Fluorescence measurements were done after the enzyme was maintained for 4 h at 15 °C in different concentrations of denaturant. Enzyme concentrations were 10 μg of protein/mL for intrinsic fluorescence and 50 μg of protein/mL for ANS binding determinations. Thiol titration of denatured fructose-1,6-bisphosphatase (50 μg of protein/mL) by NBS_2 was performed at 15 °C as described in Experimental Procedures.

not shown). This suggests that the exposure of hydrophobic areas is not related with tetramer dissociation. Beyond 0.9 M Gdn·HCl the ANS binding shows a gradual decrease, reflecting the disappearance of the hydrophobic patches where ANS binds.

The fluorescence spectrum emitted by native fructose-1,6-bisphosphatase upon excitation at 278 nm is characterized by a maximum emission wavelength of 308 nm (data not shown). This fluorescence corresponds to the 14 tyrosine residues of the enzyme, since pig kidney fructose-1,6-bisphosphatase does not contain tryptophan residues (*7*, *8*). Upon dissociation and unfolding by Gdn·HCl, the fluorescence at 308 nm is reduced by about 30% of the native fluorescence intensity (Figure 1B), without altering the maximum emission wavelength. This change probably is indicative of the exposure of the tyrosine residues from the interior of the protein to the aqueous solvent. A single transition is obtained, with a midpoint at 1.0 M Gdn·HCl.

To monitor changes in cysteine environment, we titrated accessible thiol groups with NBS_2. It is known that, in the native tetrameric protein, one thiol group (Cys128) among the six present is quite prone to sulfhydryl blocking (*14*, *26*). The remaining five groups were progressively accessible to NBS_2 above 0.6 M Gdn·HCl, and the maximum value of six thiol groups titrated was obtained at 1.2−1.4 M Gdn·HCl (Figure 1B). This titration curve, as a function of denaturant concentration, exhibits a midpoint value of 0.95 M.

Direct Measurement of the Gdn·HCl-Induced Dissociation of Fructose-1,6-bisphosphatase by Size-Exclusion High-Performance Liquid Chromatography. The noncoincidence of the transition curves (Figure 1), as measured by different probes, is consistent with a mechanism involving intermediate states (*27*, *28*). Size-exclusion chromatography can be applied to the study of protein unfolding, since it is able to resolve changes in the hydrodynamic properties along the denaturation pathway and to detect the presence of intermediate states provided they are kinetically stable within the time scale of the chromatographic run (*29*).

Elution profiles of pig kidney fructose-1,6-bisphosphatase in various concentrations of Gdn·HCl were obtained on a TSK-G3000 SW_{XL} HPLC size-exclusion column preequilibrated at 15 °C with the respective solvents. Figure 2 shows that the elution profiles of the protein have a complex behavior. Between 0 and 0.9 M Gdn·HCl the enzyme elutes as a single peak centered at 10.2 min, which was slightly higher than the elution time of 9.7 min observed for the protein marker γ-globulin (M_r 158000). As the molecular mass of native tetrameric fructose-1,6-bisphosphatase is 146 kDa, these observations indicate that native enzyme under the conditions studied is tetrameric. At 1.0−1.2 M Gdn·HCl a shoulder at higher elution volume appears in the elution patterns. The apparent molecular weight of the protein form corresponding to this peak (elution time 11.0 min) was estimated to be about 70000. Since the molecular weight of the monomer is 36500, we concluded that a significant portion of the tetrameric fructose-1,6-bisphosphatase molecule is converted to a relatively compact dimer, which persists up to about 1.8 M denaturant. At concentrations beyond 1.3 M Gdn·HCl most of the protein exhibits an apparent molecular weight higher than the tetramer. The elution profiles indicate the presence of aggregated species in the Gdn·HCl concentration range of 1.3−3.0 M. Since blue dextran elutes at 6.9 min (void volume of the column), most of the aggregates must have an apparent molecular mass less than 300 kDa. Effectively, light scattering measurements of protein samples (50 μg of protein/mL) incubated with various concentrations of Gdn·HCl, in a L-type spectrofluorometer set at 400 nm and with excitation and emission slit widths of 2.5 nm, did not indicate the presence of large aggregates. At concentrations beyond 2.7 M Gdn·HCl an elution peak around 9.2 min becomes predominant, and at denaturant concentrations over 3.4 M the protein elutes as a single sharp peak with an elution time smaller than that of the native tetramer (8.8 min), indicative of an increase in the apparent Stokes radius, which is expected for the complete unfolding of the monomer. Hence, the peak at retention time 8.8 min observed for 3.9 M Gdn·HCl corresponds to the fully unfolded enzyme. Similarly, a retention volume smaller than that of the native tetramer has

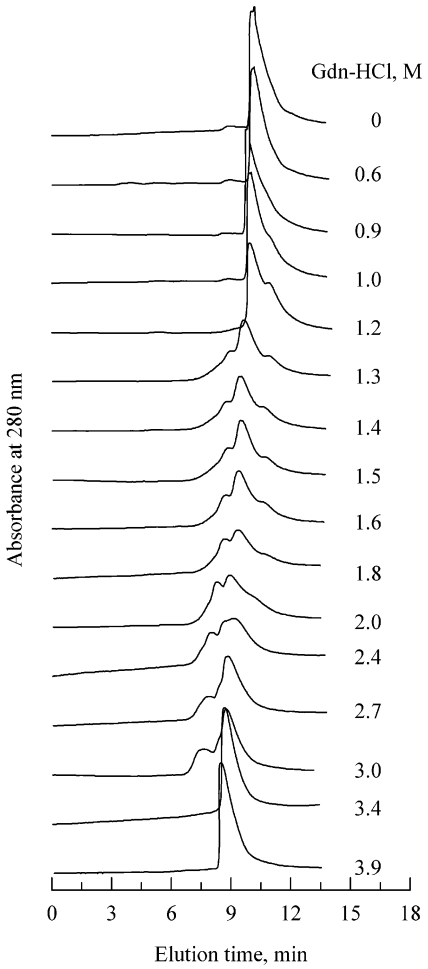

FIGURE 2: Dissociation and unfolding of fructose-1,6-bisphosphatase as monitored by size- exclusion HPLC in the presence of Gdn·HCl. Elution profiles of 20 μg samples of pig kidney fructose-1,6-bisphosphatase each at the indicated concentration of Gdn·HCl are shown. The chromatographic runs were done at 15 °C after the protein was equilibrated at the same denaturant concentration for at least 3 h as described in Experimental Procedures.

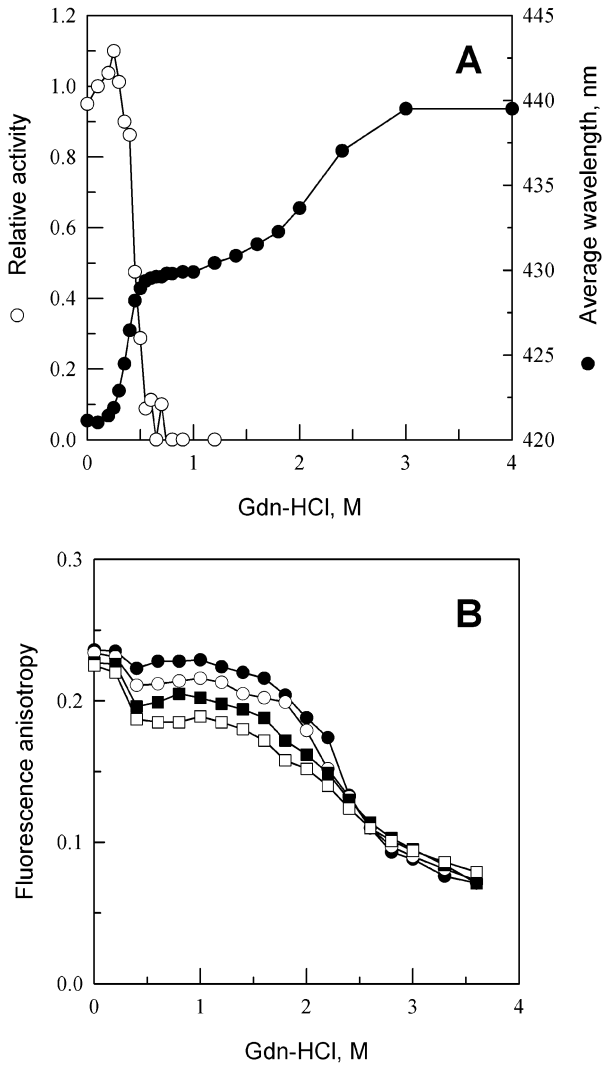

FIGURE 3: Inactivation, dissociation, and unfolding by Gdn·HCl of fructose-1,6-bisphosphatase with fluorescent probes attached to the reactive cysteine residue. Labeling of the protein with MIANS and AEDANS was done as described in Experimental Procedures. (A) Loss of enzyme activity (○) and perturbation of the fluorescent probe (●) were determined after incubation of MIANS−fructose-1,6-bisphosphatase (10 μg of protein/mL) with Gdn·HCl for 4 h at 15 °C. To monitor MIANS fluorescence, the probe was excited at 327 nm, and intensity-averaged emission wavelengths were calculated in the range of 370−500 nm. (B) Fluorescence anisotropy of AEDANS-labeled fructose-1,6-bisphosphatase in Gdn·HCl. The protein was equilibrated for 4 h with the indicated concentrations of Gdn·HCl, and fluorescence anisotropy was determined at 15 °C with excitation and emission wavelengths of 380 and 475 nm, respectively. The protein concentrations were 5 μg/mL (□), 10 μg/mL (■), 20 μg/mL (○), and 50 μg/mL (●).

been reported for the unfolded monomer of duck δ2-crystallin (*30*). The presence of compact monomeric intermediates that should appear at 12.2 min was not observed in the elution profiles.

Fluorescence Properties Associated with Denaturation in Gdn·HCl of Modified Fructose-1,6-bisphosphatase. An alternative approach to obtain information regarding the unfolding of the enzyme is to introduce a reporter group. Taking advantage of the fact that highly specific modification of Cys128 of fructose-1,6-bisphosphatase generates derivatives which retain most of the catalytic properties of native enzyme (*14*, *20*), we introduced MIANS as an extrinsic fluorescent probe at this residue. The reactive Cys128 is located near the active site in such a way that changes in the environment of the probe should reflect perturbations in the structure of the active site region. Moreover, the fluorescent probe should allow a study of the dissociation and unfolding process at higher (>1.5 M) denaturant concentrations. The modification reaction generates an enzyme derivative that is more prone to inactivation by Gdn·HCl (Figure 3A). The emission of MIANS-labeled fructose-1,6-bisphosphatase in the absence of Gdn·HCl is blue shifted, with a maximum at 410 nm (data not shown). As expected, the spectrum shifts significantly to longer wavelengths as denaturant concentration increases, and there is an overall decrease in the fluorescence intensity. The red shift in emission as a function of Gdn·HCl concentration was determined by calculating the intensity-averaged emission wavelength (*31*). Our data show that Gdn·HCl induces a stepwise increase in accessibility of the probe; the transition curve exhibits two phases, separated by a plateau. The first transition is centered at 0.4 M and paralleled the transition characterized by inactivation of the modified enzyme. A second transition centered at 2.2 M was detected. Between 0.7 and 1.4 M Gdn·HCl the averaged emission wavelength

Table 1: Conditions for Reversible Unfolding−Refolding of Pig Kidney Fructose-1,6-bisphosphatase

condition[a]	[Gdn·HCl] (M) initial	[Gdn·HCl] (M) final	% recovery
control	0	0	100
dialysis[b]	6.0	0.008	12
no additive	4.0	0.1	58.7
2 mM $MgSO_4$	4.0	0.1	67.1
5% glycerol	4.0	0.1	67.1
75 mM K_2SO_4	4.0	0.1	61.0
2 mM $MgSO_4$, 5% glycerol	4.0	0.1	67.3
2 mM $MgSO_4$, 75 mM K_2SO_4	4.0	0.1	66.0
2 mM $MgSO_4$, 5% glycerol, 75 mM K_2SO_4	4.0	0.1	66.0
2 mM $MgSO_4$, 0.1 mM Fru-1,6-P_2	4.0	0.1	58.0

[a] Unfolding−refolding experiments were performed with 25 μg of protein/mL in 0.1 M Hepes/NaOH, pH 7.5, 0.1 mM EDTA, and 5 mM DTT at 15 °C (control) plus the indicated additive. The protein was allowed to unfold at the indicated Gdn·HCl concentration for 3 h and refold for at least 6 h. [b] The unfolded protein (500 μL) at 10 μg of protein/mL was equilibrated in 1 L of buffer containing 50 mM Hepes/NaOH, pH 7.5, 0.1 mM EDTA, and 5 mM DTT at 15 °C. Aliquots were taken to measure enzyme activity and Gdn·HCl concentration.

does not change, suggesting the presence of an intermediate state during the unfolding of this enzyme form.

To determine whether the transition steps detected in the modified fructose-1,6-bisphosphatase correspond to dissociation of the protein, anisotropy studies were carried out with AEDANS-labeled enzyme. The AEDANS group was also introduced on Cys128. This fluorescent group is suitable for these anisotropy studies mainly because of its relatively long lifetime (*32*, *33*). An average lifetime of 17.9 ns was determined for AEDANS−fructose-1,6-bisphosphatase using multifrequency phase fluorometry (data not shown).

As shown in Figure 3B, the anisotropy value for the AEDANS-labeled enzyme (50 μg/mL) decreased with the denaturant concentration in a biphasic fashion, indicating an increase in the mobility of the fluorophore. The first transition occurred between 0.2 and 0.4 M Gdn·HCl and was coincident with the inactivation of the enzyme derivative, and the second transition occurred between 1.6 and 3.3 M Gdn·HCl approximately. These transitions are coincident with those observed for MIANS-labeled enzyme. Interestingly, between 0.4 and 2.3 M Gdn·HCl there was a marked effect of protein concentration on the denaturation curve, suggesting that tetramer and dimer dissociation was occurring.

Reactivation and Reassociation of Fructose-1,6-bisphosphatase upon Dilution of Gdn·HCl. Refolding of denatured fructose-1,6-bisphosphatase was monitored by the recovery of enzyme activity upon dilution of Gdn·HCl (Table 1). In preliminary experiments just 12% of the activity was recovered after extensive dialysis of Gdn·HCl from the solution of inactivated protein. However, 59% of the activity was regained upon a 40-fold rapid dilution of the denatured protein (25 μg of protein/mL) into refolding buffer. The presence of DTT was mandatory to get significant refolding; in its absence just 22% of activity was regained (not shown). Additives such as enzyme substrates, cofactors, or effectors (*15*) or the presence of the stabilizing agent glycerol (*34*) may enhance refolding of proteins into their native conformation. Thus, refolding of denatured fructose-1,6-bisphosphatase was performed by rapid dilution in the presence of different additives. Neither the enzyme substrate (Fru-1,6-P_2) nor the effectors AMP, Fru-2,6-P_2, and K^+ gave a significant improvement in activity recovery, but in the presence of Mg^{2+}, cofactor of the reaction, 67% of the enzyme activity was recovered. Combinations of $MgSO_4$ with glycerol, K_2SO_4, or Fru-1,6-P_2 in the refolding mixture did not enhance the recovery of activity further than the effect of $MgSO_4$ alone (Table 1). On the other hand, as expected, the renaturation yield was strongly dependent on the protein concentration. The reactivation of the enzyme yields 84 ± 8% of the initial activity at a low protein concentration (5−15 μg of protein/mL) and 2 mM Mg^{2+}, and the renatured enzyme was indistinguishable from native pig kidney fructose-1,6-bisphosphatase with respect to catalytic and physicochemical properties (data not shown). The renaturation yield reaches just 42% at 90 μg of protein/mL.

We also studied the kinetics of the refolding process, to assess if in a general way the reactivation pathway can be described as a simple reversal of the denaturation reactions. After complete unfolding of fructose-1,6-bisphosphatase by incubation with 4 M Gdn·HCl, a simple dilution of the salt allows the enzymatic activity to reappear. The rate of reactivation as well as the increase in intrinsic fluorescence at 308 nm was determined. The final concentration of Gdn·HCl was 0.1 M. The fluorescence intensity at 308 nm of the renaturing protein increased too fast to resolve the rate; the intensity change was complete within the time required for manual mixing (≤15 s; not shown). The species formed is enzymatically inactive although it is largely folded, as judged by fluorescence. The rate of recovery of the enzyme activity was considerably slower, taking about 120 min to reach final values (Figure 4A). Interestingly, the reactivation is governed by a monomolecular step with a rate constant of 4.54×10^{-4} s^{-1}; no lag phase was observed. Furthermore, the rate of reactivation does not depend on enzyme concentration in the range from 1 to 25 μg of protein/mL, at both 15 °C (Figure 4A) and 4 °C (not shown). These observations indicate that the rate-limiting step in the refolding of the enzyme does not depend on the pairing of subunits to yield dimeric or tetrameric intermediates. More probably, enzyme reactivation is mostly rate limited by a rearrangement on the tetrameric protein molecule.

Effect of Gdn·HCl Concentration on Enzyme Reactivation. When native pig kidney fructose-1,6-bisphosphatase was equilibrated at 15 °C with different concentrations of Gdn·HCl and then diluted to a final concentration of 0.2 M Gdn·HCl, the yield of reactivation was strongly influenced by the concentration of Gdn·HCl used (Figure 4B). At concentrations below 0.8 M Gdn·HCl in the denaturation step, the reversibility of the unfolding of the bisphosphatase was nearly 100%. Between 1.2 and 2.4 M Gdn·HCl the recovery of catalytic activity was low; and finally, partial reversibility was observed in samples incubated at higher concentrations of Gdn·HCl (2.8−6.0 M) (Figure 4B). Furthermore, the percentage of renaturation decreases as the protein concentration increases from 1 to 25 μg/mL, at Gdn·HCl concentrations higher than 1.4 M, suggesting that aggregation takes place during the unfolding incubation period. On the other hand, it is also apparent from Figure 4B that a higher protein concentration favors the enzyme reactivation in the range of 0.8−1.4 M Gdn·HCl, suggesting that the equilibrium in this concentration range is governed by at least one reassociation step. Accordingly, the size-exclusion chromatography

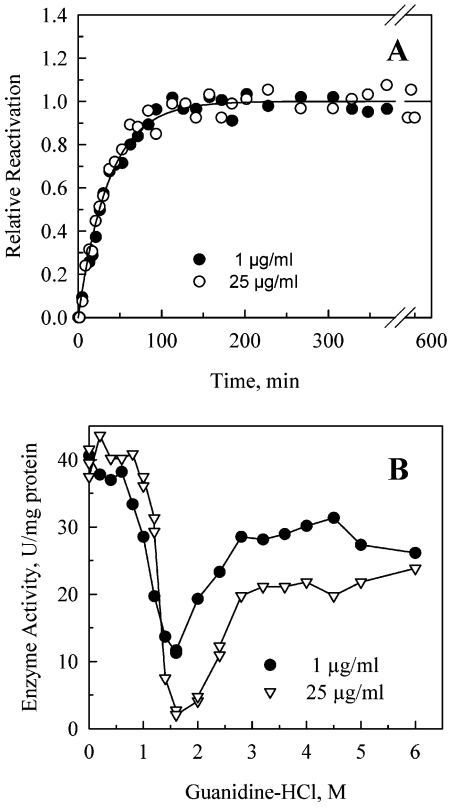

FIGURE 4: Refolding of fructose-1,6-bisphosphatase after complete denaturation in Gdn·HCl. (A) Kinetics of the reactivation at varying enzyme concentrations after 3 h denaturation by 4 M Gdn·HCl. The protein concentrations were 1 μg/mL (●) and 25 μg/mL (○). Reactivation starts from zero activity. The results are expressed relative to the final yield (88% and 55% for the experiment done at 1 and 25 μg/mL, respectively), as determined after up to 6 h of reconstitution. The solid line was calculated according to a first-order reaction with $k = 4.54 \times 10^{-4}$ s^{-1}. (B) Reactivation of the enzyme after incubation into varying concentrations of Gdn·HCl. The protein was maintained in the concentrations of Gdn·HCl indicated for 4 h at 15 °C before being diluted to a final Gdn·HCl concentration of 0.2 M and protein concentrations of 1 μg/mL (●) or 25 μg/mL (▽). The enzyme recovery was measured after further incubation of the enzyme in these conditions at 15 °C for 6 h. The specific activity of the fructose-1,6-bisphosphatase before the incubation with Gdn·HCl (native enzyme) was 38 units/mg of protein.

experiments show that in these conditions the protein is found as a tetramer and compact dimers (Figure 3).

DISCUSSION

Our results clearly indicate that the equilibrium unfolding of fructose-1,6-bisphosphatase in Gdn·HCl is a multistep process involving several intermediate species, as demonstrated by the noncoincidence of the unfolding transition curves measured by different techniques (*28*). The progressive addition of Gdn·HCl to pig kidney fructose-1,6-bisphosphatase leads first to the disappearance of the enzymatic activity by a partial unfolding of the subunits, second to the dissociation of the tetramer to dimers, third to further dissociation of the dimer to intermediate species, and fourth to a conformational change of the protein to fully unfolded monomers.

The evidence which indicates that inactivation of the enzyme precedes the dissociation of the tetramer is as follows: (1) the enzymatic activity of fructose-1,6-bisphosphatase is lost at low concentrations of Gdn·HCl (lower than 0.9 M) and is protein concentration independent (Figure 1A); (2) the size-exclusion experiments reveal that at 0.9 M Gdn·HCl fructose-1,6-bisphosphatase remains in a tetrameric state while enzyme activity has been lost completely (Figure 2); and (3) in the same Gdn·HCl concentration range where enzymatic activity is lost ANS binding increases, resulting in a 2-fold enhancement in ANS fluorescence. Accordingly, we were not able to detect a protein concentration dependence in the steep ascending part of the ANS binding curve (not shown). Thus, a disturbance of the active site spatial conformation seems to be responsible for the loss of enzymatic activity before dissociation and gross conformational changes can be detected.

The existence on snake fructose-1,6-bisphosphatase of an inactive tetrameric state in the presence of Gdn·HCl has already been suggested (*17*). However, it has also been reported that inactivation of snake muscle fructose-1,6-bisphosphatase and dissociation by 0.4 M Gdn·HCl occur simultaneously (*18*).

For many oligomeric enzymes, the first step of denaturation upon exposure to appropriate amounts of denaturants is the dissociation to monomers, which is usually connected with the loss of enzymatic activity (*35*−*38*). In these cases, it is believed that the secondary forces responsible for monomer association are weak compared to the forces between the domains in a monomer. In contrast, inactivation prior to dissociation has been reported for glutathione transferase (*39*), creatine kinase (*40*), organophosphorus hydrolase (*41*), and triosephosphate isomerase (*42*). For these enzymes a disturbance of the active site obliterates the catalytic activity, in some cases before significant structural changes can be detected. Our results indicate the occurrence of a conformational change of pig kidney fructose-1,6-bisphosphatase at the tetramer level, which causes inactivation, probably by a distortion of the active site. This conformational change also involves the exposure of hydrophobic surfaces, as detected by ANS binding (Figure 1B).

On the other hand, the initial enhancement of enzymatic activity between 0 and 0.5 M Gdn·HCl (Figure 1A) can be explained by an increase of conformational flexibility at the active site, to bind the substrate. A similar effect has been observed for adenylate kinase (*43*) and for δ2-crystallin (*30*). At the denaturant concentrations where the enzymatic activity is enhanced, no changes of intrinsic fluorescence, ANS binding, or exposure of protein thiol groups were detected (Figure 1B). These results are in line with the suggestion of Tsou (*44*), that the active sites of enzymes may display more conformational flexibility than the enzyme molecules as a whole. Interestingly, the activity enhancement at low Gdn·HCl concentrations reported for snake muscle fructose-1,6-bisphosphatase (*17*, *18*) has been interpreted as a consequence of the effect of the guanidinium cations (*18*). In these studies the enzymatic activity was measured in the presence of the same denaturant concentration as used during the equilibrium denaturation. However, our results cannot be explained by this mechanism, since Gdn·HCl was not added to the enzymatic assay medium, and the small amounts contained in the enzyme sample had a negligible effect.

We found that fructose-1,6-bisphosphatase in the native state binds a significant amount of ANS. It has frequently been suggested that ANS binds to nucleotide sites on proteins

(*45*, *46*) and is displaced by the addition of nucleotides (*45*, *47*−*50*). This assumption is based on the observation that on addition of the nucleotide the fluorescence enhancement of the probe is reduced. Thus, the displacement of ANS by AMP indicates that ANS binds to the nucleotide site. Likewise, ANS binding to native enzyme could be due also to an interaction with a novel allosteric site for anilinoquinazolines, which has been recently described (*51*). These compounds are not substrate mimics nor AMP analogues, and they have been found to bind at a specific site at the subunit interface of fructose-1,6-bisphosphatase.

We have introduced in pig kidney fructose-1,6-bisphosphatase two extrinsic fluorescent probes on the reactive cysteine residue (Cys128). Because this residue is located near the active site, we expected that these probes would allow us to monitor the perturbation of the active site region during the unfolding process. The modification of the reactive cysteine residue of the enzyme destabilizes the active site region, as detected by its loss of enzyme activity at lower Gdn·HCl concentrations than for unmodified fructose-1,6-bisphosphatase. The inactivation was accompanied by the perturbation of MIANS fluorescence in the same denaturant range, suggesting that the probe becomes more exposed to the aqueous solvent.

The dissociation of the tetramer to a compact dimer was detected by size-exlusion chromatography beyond 1 M Gdn·HCl. This dissociation is accompanied by changes in tertiary structure, as revealed by a decrease of the intrinsic protein fluorescence due to tyrosine residues and an increase of thiol group accessibility. The sharp drop in denaturation reversibility between 1 and 1.5 M Gdn·HCl (Figure 4B) suggests that the dimer is prone to aggregate irreversibly. This interpretation is in agreement with the chromatographic results which show that at no Gdn·HCl concentration the dimer becames a dominant state. Probably monomers formed by dimer dissociation also aggregate. A similar aggregation of unfolding intermediates has been previously reported (*38*, *42*).

Further evidence for dissociation of tetramers to dimers and of dimers to monomers comes from the concentration dependence of AEDANS anisotropy data in the range of 0.4−2.3 M Gdn·HCl (Figure 3B). According to these results, tetramer dissociation of AEDANS−fructose-1,6-bisphosphatase begins inmediately after the conformational change which inactivates the enzyme derivative (0.2−0.4 M Gdn·HCl). Both phenomena occur at a lower denaturant concentration than tetramer dissociation of the unmodified enzyme. As Cys128 is located near the C1C2 subunit interface, the introduction of a hydrophobic group on it may induce some energetically unfavorable interactions at this interface. The physical separation of the C1C2 and C1C3 interfaces, evident in the crystal structure (*9*), should allow the later to remain unaffected. In the 2.3−3.3 M Gdn·HCl range the change of AEDANS anisotropy was concentration independent, indicative of a first-order process, such as monomer unfolding. The final unfolding of the monomers was detected coincidentally by a red shift of MIANS−fructose-1,6-bisphosphatase emission, which becomes completely exposed to the polar aqueous solvent (Figure 3A).

On the other hand, the absence of any compact folded monomeric intermediate suggests that intersubunit interactions play an important role in the stability of the protein. Since only partial reversibility was observed when unfolding was carried out at Gdn·HCl concentrations higher than 2.0 M (Figure 4B), it is plausible to think that the dissociation of the dimer yields a monomeric state (a partially folded intermediate) that may aggregate. Accordingly, HPLC experiments show the presence of aggregates at these denaturant concentrations.

In conclusion, the unfolding of native fructose-1,6-bisphosphatase can be described by the scheme

$$\begin{array}{ccccccccc} T & \rightarrow & T^* & \rightarrow & 2\,D & \rightarrow & 4\,M^* & \rightarrow & 4\,U \\ & & & & \downarrow & & \downarrow & & \\ & & & & A & & A & & \end{array}$$

where T and T* correspond to the active and the inactive tetrameric enzymes, respectively; D corresponds to a dimer; M* and U correspond to a monomeric intermediate and unfolded monomer, respectively; and A corresponds to aggregates.

Upon unfolding and refolding of fructose-1,6-bisphosphatase, at 25 μg/mL, 59% of the initial activity was regained (Table 1) because part of the protein becomes trapped in a "wrong" structure, which is refractory to regain activity and aggregates. As would be expected, when the refolding experiments were carried out in the presence of Mg^{2+}, an increase of the recovery of enzyme activity was seen. The reasons for the effect of Mg^{2+} are currently unknown but must be related to the particular interactions between this cofactor and the protein. Indeed, questions remain about the role of the Mg^{2+} in the catalytic mechanism (*10*). It is possible that the binding of the metal ion triggers a compaction of the metal binding region, which favors the formation of native tertiary structure, as reported for the effect of Zn^{2+} and Co^{2+} on the refolding of carbonic anhydrase (*52*). In principle, Mg^{2+} can affect the mechanism mainly by stabilizing intermediates or the final product, thus shifting the equilibrium toward the native state.

The time-dependent recovery of the native structure after maximal unfolding by Gdn·HCl was monitored by fluorescence and activity measurements. Immediately after native conditions have been reestablished by diluting the denaturant, the fluorescence returned to native values, proving that structured intermediates are formed within the time of manual mixing (<15 s). However, the regain of activity was a lengthy process of 2 h, with a half-time of about 25 min. For several oligomeric proteins, it has been observed that the reappearance of activity during folding followed biphasic kinetics: a first-order reaction preceding another first-order or a second-order reaction as rate-determining steps. The first reaction, which yields an inactive species, therefore produces a lag phase. It is believed to be a folding step occurring within a single polypeptide chain (*53*, *54*). The folded monomer subsequently associates into the active dimer and oligomer. Therefore, the absence of this lag phase in the case of fructose-1,6-bisphosphatase indicates that there is a single rate-limiting step in expressing activity, and all preceding and subsequent steps must be rapid. Moreover, this limiting step is a monomolecular process. The three-dimensional structure of fructose-1,6-bisphosphatase is such that only dimeric or tetrameric species can have functional active sites, since the Fru-1,6-P_2 binding site involves residues from two adjacent subunits (*5*, *6*). This implies that the slow refolding

step could be a rearrangement at the dimer or tetramer level. HPLC experiments favor the later possibility because the formation of tetramer during the refolding was faster than the recovery of activity (Reyes et al., unpublished observations). Therefore, we propose that the rate-limiting step corresponds to the formation of the active tetramer from an inactive tetrameric species.

All of the preceding data are thus compatible with the association mechanism:

$$4U \rightarrow 4M' \rightarrow (4M \rightarrow 2D) \rightarrow T^* \rightarrow T$$

where M′ is a partially structured monomer, M a compact monomer, and the other symbols are as defined previously. This mechanism agrees with the folding pattern of many oligomeric proteins (*15*); however, more experimental data on the folding pathway are needed to draw a parallel between the unfolding and refolding pathways of fructose-1,6-bisphosphatase.

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