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ARTICLE *in* PROTEIN SCIENCE · AUGUST 1994

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Reversible unfolding of fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase

NOBUAKI TOMINAGA,[1] DAVID M. JAMESON,[2] AND KOSAKU UYEDA[1]

[1] Research Service of the Department of Veterans Affairs Medical Center and Biochemistry Department of The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75216
[2] Department of Biochemistry and Biophysics, University of Hawaii at Manoa, Honolulu, Hawaii 96822

(RECEIVED January 7, 1994; ACCEPTED May 2, 1994)

Abstract

Reversible unfolding of rat testis fructose 6-phosphate,2-kinase:fructose 2,6-bisphosphatase in guanidine hydrochloride was monitored by following enzyme activities as well as by fluorescence methodologies (intensity, emission maximum, polarization, and quenching), using both intrinsic (tryptophan) and extrinsic (5((2-(iodoacetyl)amino) ethyl)naphthalene-1-sulfonic acid) probes. The unfolding reaction is described minimally as a 4-state transition from folded dimer → partially unfolded dimer → monomer → unfolded monomer. The partially unfolded dimer had a high phosphatase/kinase ratio due to preferential unfolding of the kinase domain. The renaturation reaction proceeded by very rapid conversion (less than 1 s) of unfolded monomer to dimer, devoid of any enzyme activity, followed by slow (over 60 min) formation of the active enzyme. The recovery rates of the kinase and the phosphatase were similar. Thus, the refolding appeared to be a reversal of the unfolding pathway involving different forms of the transient dimeric intermediates. Fluorescence quenching studies using iodide and acrylamide showed that the tryptophans, including Trp-15 in the N-terminal peptide, were only slightly accessible to iodide but were much more accessible to acrylamide. Fructose 6-phosphate, but not ATP or fructose 2,6-bisphosphate, diminished the iodide quenching, but all these ligands inhibited the acrylamide quenching by 25%. These results suggested that the N-terminal peptide (containing a tryptophan) was not exposed on the protein surface and may play an important role in shielding other tryptophans from solvent.

Keywords: fluorescence quenching studies; fructose 6-phosphate,2-kinase:fructose 2,6-bisphosphatase; liver; muscle; reversible unfolding

A bifunctional enzyme, Fru 6-P,2-kinase:Fru 2,6-Pase, catalyzes the synthesis (Fru 6-P + ATP ⇆ Fru 2,6-P_2 + ADP) and the degradation (Fru 2,6-P_2 → Fru 6-P + P) of Fru 2,6-P_2. Several isozymic forms of the enzyme from mammalian tissues have been characterized (reviewed in Uyeda, 1991). These isozymes are all homodimers consisting of subunits with M_r ranging from 54,000 to 60,000. The Fru 6-P,2-kinase domain resides in the N-terminal half of the protein, whereas the Fru 2,6-Pase domain is in the C-terminal region. The amino acid sequences of the catalytic domains of all known isozymes are well conserved (Rider et al., 1987; Algaier & Uyeda, 1988; Lively et al., 1988; Crepin et al., 1989; Sakata & Uyeda, 1990; Sakata et al., 1991). However, the extreme N- and C-terminal peptides differ considerably and are the regions that determine the differences in the relative activities of the kinase and phosphatase. For example, the liver and the skeletal muscle enzymes are the same gene products except for the N-termini (Crepin et al., 1989; Kitamura et al., 1989). The muscle enzyme lacks 22 amino acids from the N-terminus, and the first 9 amino acids from the N-terminus are different from those of the liver enzyme. The ratios of the kinase to phosphatase activities of the liver and muscle enzymes are 1.2 (Sakakibara et al., 1984) and 0.4 (Kitamura et al., 1989), respectively. Recently, similar values for the ratios of kinase/phosphatase for the recombinant liver and muscle enzymes, 2 and 0.2, respectively, were reported (Kurland et al., 1993). Moreover, phosphorylation sites for various protein kinases are located in either N- or C-termini of some of the isozymes, and phosphorylation of these sites regulates the relative activities of the kinase and the phosphatase. For example, phosphorylation of the N-terminus of the liver enzyme results in decreased kinase and increased phosphatase activity (van Schaftingen et al., 1981; el-Maghrabi et al., 1982; Furuya et al., 1982), but phosphorylation of the C-terminus of the heart isozyme leads to the opposite effect on enzyme activity (Kitamura & Uyeda, 1987; Kitamura et al., 1988). Such regulation of opposite enzyme ac-

Reprint requests to: Kosaku Uyeda, Department of Veterans Affairs Medical Center, 4500 S. Lancaster Road, Dallas, Texas 75216.

Abbreviations: Fru 6-P,2-kinase:Fru 2,6-Pase, fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase; Fru 2,6-P_2, fructose 2,6-bisphosphate; IAEDANS, 5((2-(iodoacetyl)amino)ethyl)naphthalene-1-sulfonic acid; GdnHCl, guanidine hydrochloride; WT, wild type.

tivity in reciprocal manner most likely involves communication between catalytic domains and is manifested via intra- and intersubunit interactions. Improved understanding of the regulatory mechanism requires elucidation of these interactions.

As an initial step toward that goal, we assessed the role of the N-terminal peptide of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase by constructing deletion mutants that produced enzymes lacking 24 and 30 amino acids from the N-terminus. Their kinetic properties and stability under a variety of conditions were investigated (Tominaga et al., 1993). These deletion mutant-derived enzymes have lower Fru 6-P,2-kinase but much higher Fru 2,6-Pase activity than the enzyme obtained from wild-type enzyme. Similar results were obtained by Kurland et al. (1993) with the liver enzyme lacking the N-terminal 22 amino acids. In addition, our deletion mutant-derived enzymes are much more sensitive to thermal inactivation, denaturation in urea, and protein concentration-dependent dissociation (Tominaga et al., 1993). These results suggest the importance of the N-terminal peptide in stabilizing the enzyme, possibly by strengthening the subunit–subunit interaction and conserving the native conformation.

Dissociation/denaturation and renaturation studies can provide useful information regarding subunit interactions. In this study we investigated the guanidine hydrochloride-induced unfolding and refolding characteristics of the rat testis Fru 6-P,2-kinase:Fru 2,6-Pase. GdnHCl rather than urea was used because recovery of the enzyme activity and the structural integrity was significantly better in the former denaturant. Because the rat testis enzyme contains 4 tryptophan residues, Trp-15 at the N-terminus, Trp-64 in the kinase domain, and Trp-299 and Trp-320 in the phosphatase domain, we utilized the intrinsic tryptophan fluorescence as a spectroscopic probe of conformational dynamics.

Results

Loss of enzyme activities in GdnHCl

Figure 1A shows the effect of increasing GdnHCl concentrations on Fru 6-P,2-kinase and Fru 2,6-Pase activities. The loss of Fru 6-P,2-kinase activity was nearly linear with increasing GdnHCl and was complete above 1 M GdnHCl. This decrease in the kinase activity was not due to inhibition by GdnHCl (data not shown). Fru 2,6-Pase activity, however, was stable up to 0.6 M GdnHCl but decreased rapidly above this denaturant concentration, yet retained about 10% of the original activity between 1 and 2 M GdnHCl. The loss of both enzyme activities was half-maximal at approximately 0.8 M GdnHCl. It was verified that there was no reactivation of the enzyme during the assay period, which was typically 5–10 min. These results contrast with the inactivation reactions in urea (Tominaga et al., 1993). In the presence of up to about 2 M urea, both the kinase and the phosphatase are activated 20% and 60%, respectively. The inactivation of both activities occurs half-maximally at 2.7 M urea, which is higher than the inactivation concentration of GdnHCl.

Fluorescence properties associated with denaturation in GdnHCl

Fluorescence spectra of the enzyme in varying GdnHCl concentrations showed that the tryptophan emission intensity at 335 nm (excitation = 295 nm) decreased only slightly from 0 to 0.6 M GdnHCl. In the presence of 0.6–0.8 M denaturant, however, a large drop in the intensity and a shift in fluorescence maximum to 340 nm was observed (data not shown). Further increase in GdnHCl resulted in an additional red shift of the fluorescence maximum to 350 nm. Upon excitation at 280 nm, very similar trends in the fluorescence spectra were observed (data not shown).

A fractional unfolding, F_u, was calculated using the equation $F_u = 1 - (R_o - R_d)/(R_n - R_d)$, where R_o is the observed fluorescence intensity, and R_d and R_n are the intensities in the presence and absence of 4 M GdnHCl, respectively. The plot of F_u for unfolding (Fig. 1B, solid lines) as the function of GdnHCl concentration shows 2 transitions at half-maximal concentration, C_m, of approximately 0.75 M and 2 M. The first transition involved not only the sharp decrease in the intensity but also a wavelength shift. These changes were accompanied by loss of the kinase and phosphatase activities.

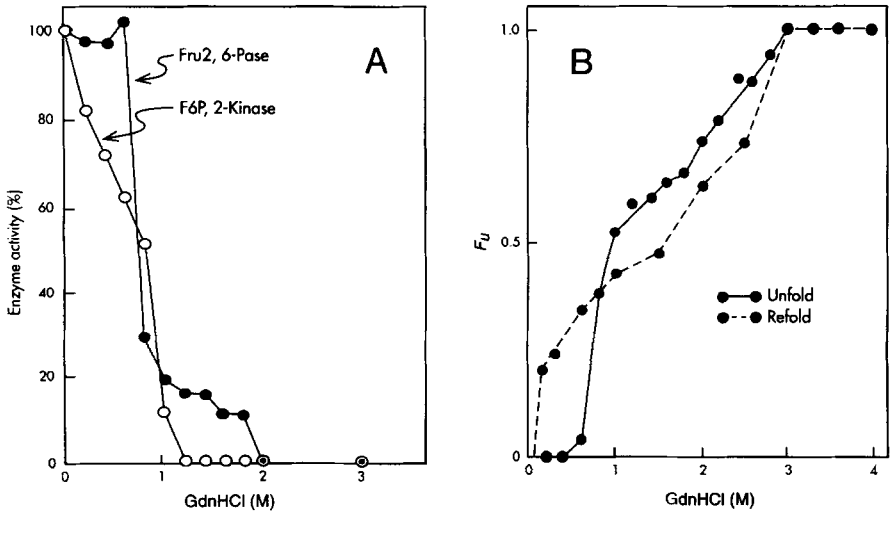

Fig. 1. Inactivation (**A**) and unfolding and refolding curves (**B**) of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase in GdnHCl. ○, Fru 6-P,2-kinase; ●, Fru 2,6-Pase. The enzyme was incubated in the indicated concentrations (M) of GdnHCl as described in the Materials and methods. The emission spectra were determined at 295 nm excitation. The fractional denaturation (F_u) was calculated as described in the text from the emission spectra. The experimental conditions for unfolding and refolding are described in the Materials and methods. Solid lines, unfolding; dotted lines, refolding.

Fluorescence polarization in GdnHCl

To ascertain if the first transition step corresponds to dissociation of the dimer, fluorescence polarization studies were carried out. As shown in Figure 2, the polarization value for the AEDANS-labeled enzyme (0.9 μM) in the absence of GdnHCl was 0.282, and this value persisted until a GdnHCl concentration above 0.6 M. The polarization then decreased in a biphasic manner; the first transition occurred between 0.6 and approximately 2 M GdnHCl and the second transition between 2 and 3.5 M GdnHCl. When the initial enzyme concentration was decreased 10-fold (0.09 μM), the biphasic profile was more pronounced. One notes that whereas the first transition of the denaturation curve was dependent upon the protein concentration, the second transition was not. Dilution of the final enzyme/GdnHCl solution back to the level of 0.78 and 0.33 and dialyzed to 0 M GdnHCl resulted in an increase in the polarization values to 0.276, 0.244, and 0.226 (□), respectively, close to the values obtained by the initial increase in GdnHCl concentration.

In contrast to the AEDANS results, the polarization of the intrinsic fluorescence of the WT enzyme increased initially in the 0–0.4 M GdnHCl range, then generally plateaued until 1 M (Fig. 3). The increased polarization in the dilute GdnHCl corresponded to the conditions in which the inactivation of Fru 6-P,2-kinase occurred, suggesting conformational changes in the kinase domain. Dilution of the enzyme in 4 M GdnHCl back to 0.8 M resulted in an increase in the polarization to the initial value at the same GdnHCl concentration (Fig. 3, □).

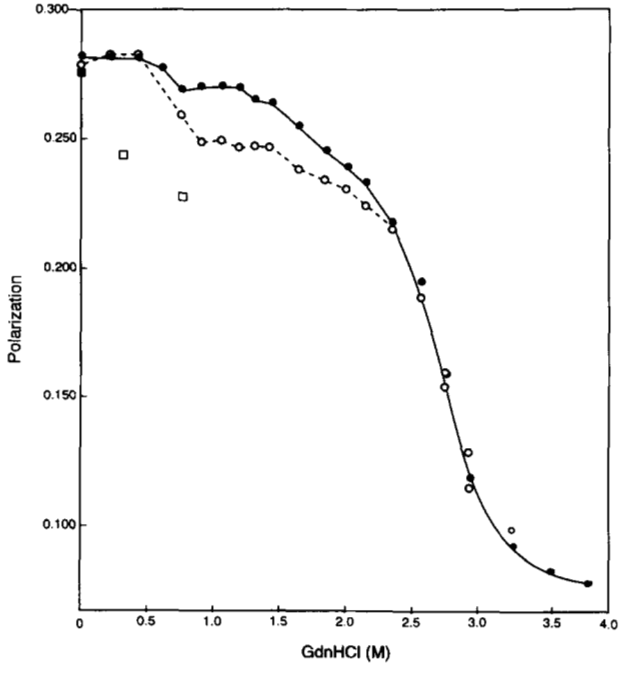

Fig. 2. Fluorescence polarization of AEDANS-labeled rat testis Fru 6-P,2-kinase:Fru 2,6-Pase in GdnHCl. To the enzyme (0.9 μM [●] or 0.09 μM [○]) in 120 μL buffer A were added 5-μL aliquots of 5 M GdnHCl solution. As controls, comparable amounts of H_2O, instead of GdnHCl, were added and no significant decrease in polarization was found. Fluorescence polarization was determined after diluting the enzyme in 4 M GdnHCl to 0.78 M and 0.33 M (□) within 10 min and to 0 (■) by overnight dialysis.

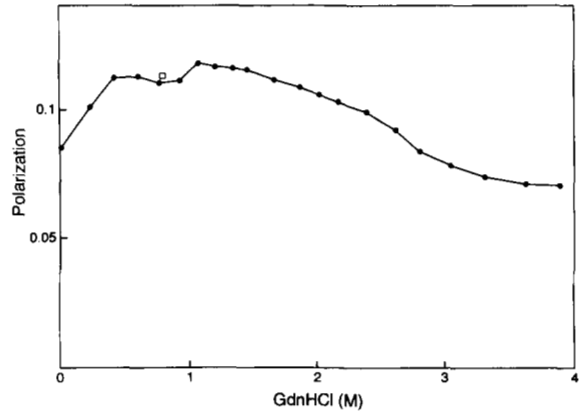

Fig. 3. Fluorescence polarization of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase in GdnHCl. The enzyme (0.2 mg/mL, 1.8 μM) in 110 μL buffer A was excited at 280 nm and emisson at >300 nm using a Schott KV320 filter. Addition of GdnHCl and the fluorescence measurements were the same as in Figure 2. Fluorescence polarization was determined after diluting the enzyme in 4 M GdnHCl to 0.78 M (□).

Renaturation

In order to study the refolding process, which may not be a simple reversal of the denaturation reactions, the enzyme was denatured in 3 M GdnHCl, followed by dilution of the denaturant in the buffer mixture as described in the Materials and methods. The final concentration of GdnHCl in the highest dilution was 0.38 M. The rates of the reactivation of the kinase and the phosphatase activities as well as increase in relative fluorescence at 338 nm were determined. The fluorescence intensity at 334 nm of the renatured enzyme increased too rapidly to resolve the rate (Fig. 4). The fluorescence maximum of the renatured protein was 340 nm immediately after the renaturation reaction, but a blue shift to 338 nm occurred slowly after 90 min (at which time both enzyme activities were fully recovered). The polarization of the AEDANS-labeled enzyme in 2.5 M GdnHCl was 0.064 but increased to 0.238 immediately upon dilution of the GdnHCl to 0.62 M. The latter value is comparable to that found in the same concentration of GdnHCl during the unfolding process (Fig. 2). These results suggest that the renatured enzyme was either a dimer or folded monomers and that its formation was extremely rapid. However, the rates of recovery of both enzyme activities are considerably slower than that of the associated intrinsic fluorescence at 334 nm, taking approximately 90 min to reach the maximum values.

The recoveries of the enzyme activities and the native fluorescence at 334 nm were 60% and 65%, respectively. These incomplete recoveries appeared to be due to irreversible aggregation of the protein and suggested that one of the intermediate states formed during the renaturation has a strong tendency to aggregate.

Effect of ligands on renaturation

ATP and Fru 6-P in excess enhanced the rate and extent of renaturation of GdnHCl-denatured Fru 6-P,2-kinase activity by

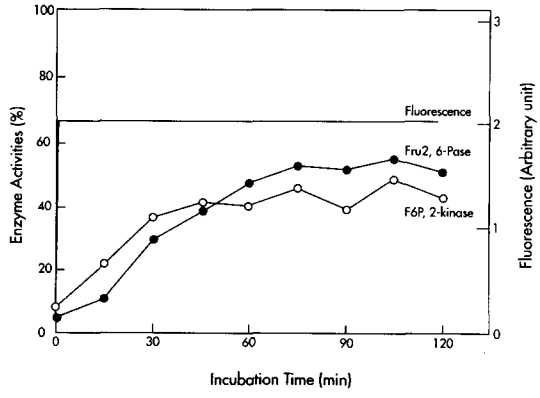

Fig. 4. The rate of refolding of unfolded Fru 6-P,2-kinase:Fru 2,6-Pase. The enzyme was incubated in buffer A containing 3 M GdnHCl for 1 h at 25 °C. The refolding reaction was initiated by diluting the enzyme solution with addition of an appropriate volume of buffer A to bring the final GdnHCl concentration to 0.38 M. Fluorescence was determined by excitation at 295 nm and emission at 334 nm. Fru 6-P,2-kinase and Fru 2,6-Pase in the refolding reaction mixture were determined as described in the Materials and methods.

about 10–20% (Fig. 5). Fru 2,6-P_2 also increased the rate and degree of the recovery of Fru 2,6-Pase activity, but ATP had little effect. The results demonstrated that each substrate facilitated formation of its specific catalytic domain and suggested that the kinase and phosphatase domains refold to the native conformation independently.

Iodide quenching

Iodide ions can generally quench surface-exposed tryptophans facilely but do not effectively penetrate into the interior of a protein and, hence, iodide quenching can be useful in estimating the extent of tryptophan exposure on the surface of a protein (Lehrer, 1971; Eftink & Ghiron, 1976). In order to determine whether the N-terminal peptide (containing Trp-15) of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase is exposed, iodide quenching of the WT enzyme was compared to that of an N-terminal deletion mutant (Del 24) that lacks the Trp residue. The Stern-Volmer equation, ($F_o/F = 1 + K_{SV}[Q]$) was used to analyze the data, where F_o and F are the fluorescence intensities in the absence and the presence, respectively, of a given concentration of Q quencher, and K_{SV} is the Stern-Volmer quenching constant (Lehrer & Leavis, 1978). As shown in Figure 6 inset, the WT enzyme yielded a nonlinear Stern-Volmer plot, indicating a heterogeneous quenching system, whereas the plot of the N-terminal deletion mutant enzyme was linear within the experimental error, indicating a homogeneous quenching system. The figure also shows that the slopes of WT and Del 24 quenching curves were similar up to 0.1 M KI, but this slope decreased in the WT at higher KI. This increased solvent accessibility of Trps in Del 24 but not in WT may be a result of the N-terminal peptide which blocks and shields Trps from solvent in the WT enzyme.

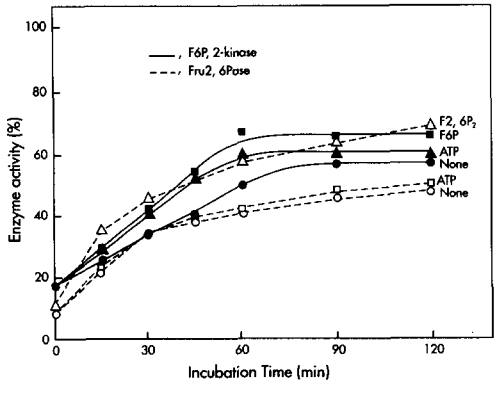

Fig. 5. Effect of ligands on refolding of Fru 6-P,2-kinase:Fru 2,6-Pase. The refolding reaction mixtures and the conditions were the same as described in Figure 4 except for the addition of 0.5 mM Fru 6-P (F6P), 5 mM ATP, and 4 μM Fru 2,6-P_2 (F2,6P2).

Additional information was gained by plotting the above data using the modified Stern-Volmer equation:

$$F_o/\Delta F = 1/f_a K_{SV}[Q] + 1/f_a, \quad (1)$$

where F_o is the fluorescence in the absence of quencher, ΔF is the difference in the fluorescence in the absence and the presence of a quencher, f_a is the fraction of accessible tryptophan, and K_{SV} and [Q] are same as above (Lehrer, 1971). The results in Figure 6A show that the f_a value of WT enzyme was approximately 0.1, implying that nearly all the Trps, including that of the N-terminus, were inaccessible to iodide. However, the fa of the deletion mutant enzyme was 1, implying that all the Trps are accessible to quencher.

Acrylamide quenching

Acrylamide is a polar nonionic quencher that generally has access to all but the most buried tryptophans (Eftink & Ghiron, 1976). Acrylamide quenching of tryptophan fluorescence of WT and Del 24 enzymes demonstrated that, in contrast to iodide, the quenching curves of the enzymes were both linear (Fig. 6B) and the fa values were 1, implying that all Trps are accessible to acrylamide. In addition, acrylamide quenching of WT enzyme was determined under 2 different denaturing conditions, namely in 0.5 M GdnHCl, under which Fru 2,6-Pase was fully active (and the enzyme was presumably dimeric) and in 3 M GdnHCl, in which the enzyme was unfolded. The Stern-Volmer (Fig. 7 inset) and the modified Stern-Volmer plots (Fig. 7) show that they are linear in both concentrations of GdnHCl and that these slopes increased in higher GdnHCl concentration, which could be due to increased solvent exposure of Trp residues. The difference in the slopes of the lines in the absence and presence of 0.5 M GdnHCl was small, indicating only a slight change in the Trp accessibility.

Effect of ligands on iodide and acrylamide quenching

At saturating concentrations of Fru 6-P, ATP, and Fru 2,6-P_2, only Fru 6-P markedly reduced quenching by iodide of WT enzyme (Fig. 8A). All these ligands increased the acrylamide quenching by approximately 25% (Fig. 8B) and the modified Stern-Volmer plots indicated that 1 or more Trp became accessible to the quencher in the presence of the ligands (Fig. 8B, inset). The Stern-Volmer constants in the absence of any ligand

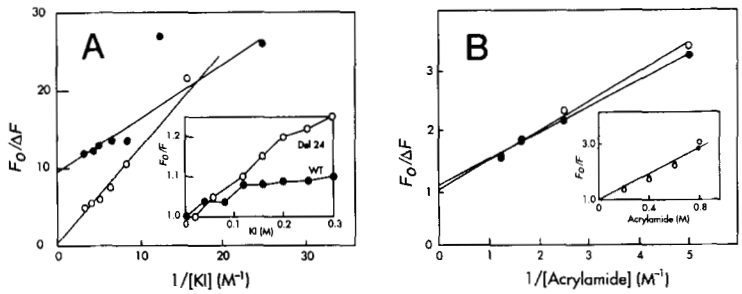

Fig. 6. Modified Stern–Volmer plots of iodide (**A**) and acrylamide (**B**) quenching of Fru 6-p,2-kinase:Fru 2,6-Pase (WT) (●) and the N-terminal deletion mutant enzyme (Del 24) (○). The tryptophan fluorescence of the enzyme (1.0 μM) was quenched with increasing concentrations of KI or acrylamide as described in the Materials and methods. The **insets** were plotted from the same data.

and in the presence of Fru 6-P, ATP, and Fru 2,6-P_2 were 1.6 M^{-1}, 2.2 M^{-1}, 2.3 M^{-1}, and 2.3 M^{-1}, respectively. These results demonstrated that Fru 6-P was able to shield 1 or more surface-exposed Trps from the solvent, but these ligands increased the number of Trps accessible to acrylamide.

Discussion

The kinetic and spectroscopic results presented for Fru 6-P,2-kinase:Fru 2,6-Pase in GdnHCl suggest that dissociation of the dimer is preceded by an initial partial unfolding of the subunits. The evidence in support of this partial unfolding is: (1) Fru 6-P,2-kinase activity decreased by GdnHCl in the range of 0–0.4 M and (2) the polarization of Trps increased in the same guanidine range. This increase in polarization is most likely due to a decrease in the local mobility of 1 or both of the tryptophans in the kinase (N-terminal) domain, a decrease in the average lifetime of these Trps, or a combination of both effects. In contrast to the kinase, Fru 2,6-Pase activity was stable in 0–0.4 GdnHCl, and the polarization of AEDANS (located primarily in the Fru 2,6-Pase domain) was unchanged in this range of GdnHCl concentration, suggesting increased stability of the phosphatase domain relative to the kinase domain. Apparently then the kinase and phosphatase domains of the bifunctional enzymes unfold independently. These observations on the conformational changes of the enzyme in GdnHCl are consistent with the mechanism:

$$D \rightarrow D' \rightarrow 2M \rightarrow 2U, \qquad (2)$$

where D, D′, M, and U are native dimer, the conformationally altered dimer intermediate, monomer, and unfolded monomer, respectively. This mechanism is also in agreement with our previous scheme for the denaturation of the enzyme in urea (Tominaga et al., 1993). The important difference between these 2 mechanisms, however, is that in low concentrations of urea, kinase activity remains essentially the same while phosphatase activity is increased significantly, whereas in GdnHCl, no such activation of phosphatase and kinase were observed. This difference suggests that 2 conformational states of D′ were formed, depending upon the types of bonds broken during the partial unfolding reactions. These intermediate states of the bifunctional enzyme probably possess the same secondary structure as the native state but weakened tertiary structure, especially in the kinase domain. In the case of D′, the quaternary structure may be weakened. In support of this idea is the observation that the N-terminal deletion mutants of the bifunctional enzymes having the same characteristics as D′ (high phosphatase/kinase ratio) have weaker quaternary structure than the WT, i.e., weaker subunit–subunit interaction (Tominaga et al., 1993). The partial unfolding step was followed with disruption of the quaternary structure, resulting in dissociation of D′ to monomers. The principle evidence for dissociation of the dimer in the range of ~0.5–2.0 M GdnHCl comes from the concentration dependence of the AEDANS polarization data[1] (Fig. 2). In the 2.0–4 M regime, the change in AEDANS polarization was independent of the enzyme concentration, indicative of a first-order process such as monomer unfolding.

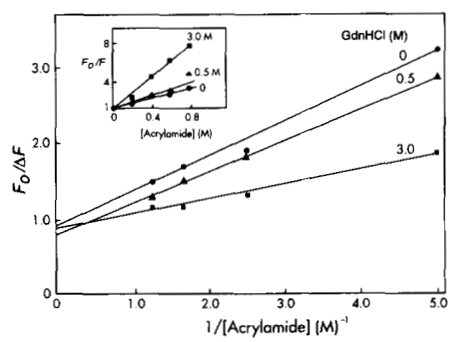

Fig. 7. Modified Stern–Volmer plots of Fru 6-P,2-kinase:Fru 2,6-Pase in GdnHCl. The enzyme (1.0 μM) in the indicated concentrations of GdnHCl was incubated for 1 h at 0 °C and the tryptophan fluorescence was determined as in Figure 6B. The **inset** was plotted from the same data.

[1] The polarization in the absence of GdnHCl was 0.282 and given the average lifetime of 18.8 ns (determined using multifrequency phase fluorometry; data not shown) one could as a first approximation calculate a rotational relaxation time of ~80 ns for the AEDANS-labeled enzyme (assuming a limited polarization of 0.45 for AEDANS at 364 nm excitation). This calculation is based on the Perrin equation: $(1/P - 1/3) = (1/P_o - 1/3)(1 + 3\tau/\rho)$ (Weber, 1966), where P is the measured polarization, P_o the intrinsic or limiting polarization in the absence of rotation, τ the fluorescence life time, and ρ the Debye rotational relaxation time. In fact, time-resolved anisotropy measurements (data not shown) indicate that the AEDANS probe experiences both "global" and "local" mobility; the "global" rotational relaxation time determined was ~180 ns, which suggests a nonspherical protein (a spherical protein of ~110 kDa would have a rotational relaxation time in the range of 120–140 ns depending upon the partial specific volume and extent of hydration). Hence, both the steady-state and time-resolved data suggested that the AEDANS moiety has local mobility. We noted that the AEDANS lifetime decreased and became more heterogenous with increasing GdnHCl concentration (~12 ns average lifetime in 2.5 M GdnHCl). Hence the actual decrease in average rotational relaxation time with increasing GdnHCl concentration was more pronounced than the decrease in polarization values indicate.

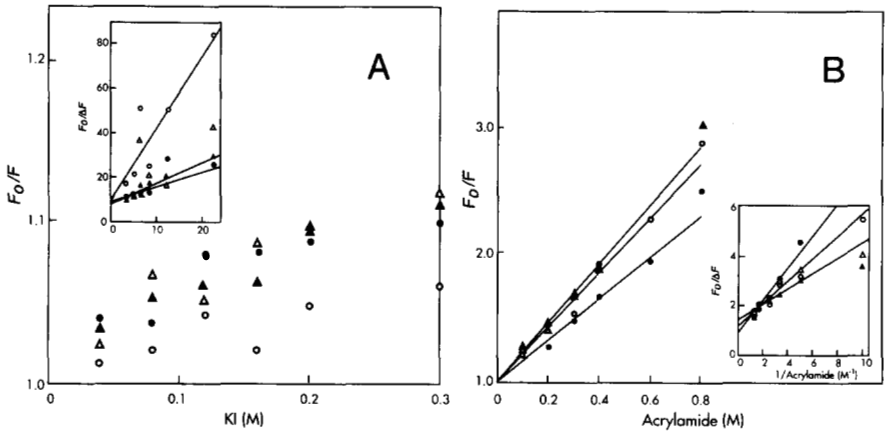

Fig. 8. The effect of ligands on the fluorescence quenching of WT. Experimental conditions were the same as in Figure 6 except for the addition of none (●), 0.5 mM Fru 6-P (F6P) (○), 1 mM ATP (▲), and 1 μM Fru 2,6-P_2 (F26P2) (△) to WT. The **inset** is a modified Stern–Volmer plot of the same data. **A:** KI. **B:** acrylamide.

Renaturation follows the reversal of the denaturation pathway (Equation 2) except for a formation of an intermediate different from D′. This intermediate (we term D″) could be a dimer or folded monomer that formed rapidly upon removal of GdnHCl and that is devoid of enzyme activity. At present we cannot distinguish between these 2 possibilities, and this point requires further experimentation. However, this intermediate is slowly converted to the native enzyme with approximately the same kinase and phosphatase activities; the rates of the recovery of both activities were essentially the same. During the conversion of the inactive intermediate (D″) to the fully active enzyme, there was no evidence of a significant concentration of an additional intermediate such as D′ with high phosphatase and low kinase. The reason for the absence of D′ during this sequence of refolding is not clear. This intermediate may represent a conformational state similar to that of the native enzyme except for a lack of final detailed interactions such as adjustment between the kinase and the phosphatase domains as well as between subunits. Based on these results, we propose the following minimum steps for the unfolding and refolding of rat testis Fru 6-P,2-kinase:Fru 2,6-Pase:

$$\mathrm{D} \begin{matrix} \nearrow \mathrm{D'} \searrow \\ \nwarrow \quad \swarrow \\ \mathrm{D''} \end{matrix} 2\mathrm{M} \leftrightarrows 2\mathrm{U} \; . \qquad (3)$$

Conversion of the unfolded monomers (U) to the folded monomer (unimolecular reaction) followed by an association (bimolecular reaction) step to form the inactive dimer was extremely rapid. It is well known that folding of many globular proteins as well as association of the subunits occurs in times ranging from a few microseconds to less than a second (reviewed by Kuwajima, 1989; Creighton, 1990).

During the renaturation, an additional folding intermediate was produced which had a propensity to aggregate, contributing to lower recovery (60%) of the native enzyme. The formation of this inactive aggregate was promoted at higher protein concentration, as indicated by appearance of turbidity, and in the absence of certain ligands such as Fru 6-P or ATP and stabilizing agents such as glycerol. Obviously, the unfolding and refolding processes of the bifunctional enzyme are very complex, involving many intermediate conformations, and thus far we have uncovered only a few of them.

A question remains whether a monomeric form has both kinase and phosphatase activities as does the dimer. Previously it was demonstrated that deletion mutants of human (Algaier & Uyeda, 1988) and rat (Tauler et al., 1988) liver enzymes, which lacked 145 amino acids and 226 amino acids, respectively, from their N-termini, occur only as monomer and contain only the phosphatase activity. The deletion mutant of the human enzyme shows less than 1% of the native phosphatase activity (Algaier & Uyeda, 1988), although that of rat is reported to have the comparable phosphatase activity as the native enzyme (Tauler et al., 1988). However, whether or not the monomeric form of the native enzyme has both activities is unknown. A comparison of the inactivation of the enzyme activities and the dissociation in GdnHCl (Fig. 1) and in urea (Tominaga et al., 1993) strongly suggests that Fru 6-P,2-kinase activity resides only in the dimeric form of the enzyme, but Fru 2,6-Pase occurs in both dimer and monomeric forms. However, the monomer has much lower (perhaps less than 10%, Fig. 1) phosphatase activity than the dimer.

The results of the iodide quenching studies presented here (Fig. 6A) show that the environment around the Trp residues is heterogeneous and most of the residues, including Trp-15 in the N-terminal peptide, appear to be buried in the native enzyme. However, the observation that the quenching of the N-terminal deletion mutant enzyme, Del 24, was homogeneous indicates that those Trps are more exposed to solvent than WT. These results imply that the heterogeneous quenching part of the Stern–Volmer plot (above 0.1 M KI) was due to either Trp-15 or to interaction of the N-terminus with other Trps. Preparation of additional constructs by mutagenesis is underway to distinguish these possibilities. Nevertheless, these iodide-quenching data demonstrated that the N-terminal peptide blocked Trps from solvent and thus suggest that it plays an important role in the dimer formation. This idea is further supported by our previous observations (Tominaga et al., 1993) that the peptide is important in stabilizing the native dimeric structure. The quenching studies revealed also the differences in the conformational states of the native and Fru 6-P-bound enzyme. Although the Trps are buried in the free enzyme, those residues in the Fru 6-P-bound enzyme are even more shielded to iodide. It is not possible at present to decide whether Trps in the kinase or phosphatase domains become shielded by Fru 6-P because this hexose-P binds to both domains as the substrate for the kinase

and the inhibitor for the phosphatase. To answer this question, specific mutations or chemical modifications of each Trp will provide information regarding the various conformational states of the enzyme.

Materials and methods

Homogeneous recombinant rat testis Fru 6-P,2-kinase:Fru 2,6-P_2-bisphosphatase was prepared from *Escherichia coli* BL21 carrying the RT2K/pT7-7 plasmid as described previously (Tominaga et al., 1993). Muscle phosphofructokinase was prepared as described (Uyeda et al., 1978). The labeling compound, IAEDANS, was a product of Molecular Probes (Eugene, Oregon). GdnHCl was purchased from Sigma Chemical Co. (St. Louis, Missouri). Electrophoretic grade acrylamide was obtained from BioRad (Richmond, California). All the solutions used for fluorescence studies were passed through a 0.22-μm filter. All other chemicals were reagent grade and obtained from commercial sources.

Preparation of labeled enzyme

Fru 6-P,2-kinase:Fru 2,6-Pase was labeled with IAEDANS as follows. The reaction mixture contained in a final volume of 0.5 mL, 50 mM Tris/phosphate (pH 7.5), 0.2 mM EDTA, 40 μM Fru 2,6-P_2, 5% glycerol, and 0.5 mg of the bifunctional enzyme. The reaction was initiated with addition of 1.2 μmol of IAEDANS dissolved in dimethylformamide and the reaction mixture was incubated for 1 h at 25 °C. At the end of the incubation, 50 μL of 1 M dithiothreitol was added to terminate the reaction, and the labeled enzyme was dialyzed extensively against 50 mM Tris/phosphate (pH 7.5), 5 mM dithiothreitol, 0.2 mM EDTA, and 5% glycerol.

To establish the location of AEDANS-labeled cysteine(s), tryptic peptides containing the fluorescent residues were isolated and sequenced. The results indicated that the AEDANS was carried primarily by Cys-332 in the Fru 2,6-Pase domain.

GdnHCl treatment

Fru 6-P,2-kinase:Fru 2,6-Pase (0.1 mg/mL) in 50 mM Tris-phosphate (pH 7.5), 0.5 mM EDTA, 2 mM dithiothreitol (buffer A), and varying concentrations of GdnHCl was incubated at 0 °C for 1 h. Aliquots were removed for the enzyme activity assays and also for fluorescence measurement. Stock solution of GdnHCl solution (5 M) was neutralized to pH 7.5 with KOH.

Fluorescence spectra

Fluorescence spectra were determined at 25 °C using an Aminco-Bowman series 2 spectrofluorometer (SLM-Aminco, Champaign, Illinois). Excitation and emission slits were set to 4 nm. For intrinsic tryptophan fluorescence, 295-nm excitation was utilized. Emission spectra were collected from 300 to 450 nm. Spectra were corrected for the buffer background but not for the instrument-response functions.

Fluorescence quenching studies

The reaction mixture contained, in a final volume of 0.1 mL, 50 mM Tris/phosphate (pH 7.5), 0.5 mM EDTA, 2 mM dithiothreitol (buffer A), varying concentrations of potassium iodide or acrylamide, the selected concentration of GdnHCl, and the enzyme at 0.1 mg/mL (0.9 μM). Additionally appropriate amounts of ammonium sulfate were added to maintain constant ionic strength. The emission spectra of the mixtures were then obtained. To prevent I_3^- formation, 0.1 mM sodium thiosulfate was included in the stock potassium iodide solution (4 M), and this mixture was prepared fresh each time. The reaction mixture was incubated at 20 °C for 5 min.

Fluorescence polarization

Polarizations were obtained using an SLM-8000 C spectrofluorometer equipped with calate prism polarizers. For the AEDANS probe, the excitation wavelength was 365 nm and emission at wavelengths greater than 380 nm was viewed through Schott KV399 cut-on filters. The standard deviation of the polarization values was generally ±0.001. The initial volume of the labeled enzyme solution (0.9 μM) was 120 μL; 5 μL of stock (5 M) GdnHCl was added up to a final volume of 240 μL solution; the sample was allowed to incubate for 5 min after each GdnHCl addition before the polarization was determined. As a control, additions of buffer alone to the labeled enzyme solution were made to reach the same enzyme dilution as in the GdnHCl experiment. The polarization of the intrinsic protein emission was also determined as a function of GdnHCl concentration (Fig. 3). The WT enzyme (0.2 mg/mL, 1.8 μM) was excited at 280 nm and emission at wavelengths >300 nm were viewed through a Schott KV320 cut-on filter; the excitation slit width was 4 nm.

Renaturation

Fru 6-P,2-kinase:Fru 2,6-Pase (0.5 mg/mL; 0.45 μM) was first denatured in 3 M GdnHCl in buffer A and incubated for 1 h at 0 °C. The denatured enzyme mixture was diluted in 0.38 M GdnHCl in buffer A containing 10% glycerol and, if stated, selected concentrations of ligands. The mixture was incubated at 20 °C. At indicated times, aliquots were removed for enzyme activity assays and for fluorescence measurements.

Assay method for Fru 6-P,2-kinase

The reaction mixture contained in a final volume of 0.1 mL 100 mM Tris/HCl (pH 7.5), 0.1 mM EDTA, 5 mM ATP, 1 mM Fru 6-P, and 10 mM $MgCl_2$. The reaction was started with the addition of Fru 6-P,2-kinase and incubated at 30 °C for 5 and 10 min. Aliquots (10 μL) were transferred to 90 μL of 0.1 N NaOH, and the diluted solution was heated at 80 °C for 1 min to stop the reaction. Appropriate aliquots of the heated reaction mixture were then assayed for Fru 2,6-P_2 as described (Uyeda et al., 1981). One unit of the enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru 2,6-P_2/min under these conditions.

Assay method for Fru 2,6-bisphosphatase

The fluorometric coupled assay was described previously (Tominaga et al., 1993). The reaction mixture contained in a final volume of 0.6 mL, 100 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 50 μM NADP, 17 μM Fru 2,6-P_2, 0.4 unit of desalted

glucose 6-P dehydrogenase, and 1 unit of phosphoglucose isomerase. The reaction was initiated with the addition of Fru 2,6-Pase and was followed at room temperature fluorometrically at excitation and emission wavelengths of 354 nm and 452 nm, respectively. One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru 6-P (as NADPH production)/min under these conditions.

Protein concentration assay

Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Acknowledgments

We thank Cu Nguyen for his excellent technical assistance and Dr. Sarah McIntire for her critical review of this manuscript. This research was supported by grants from the Department of Veterans Affairs and NIDDK (DK16194).

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