Denaturation of glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides by guanidine hydrochloride; identification of inactive, partially unfolded, dimeric intermediates

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(Received 15 January 1992)

Key words: Glucose-6-phosphate dehydrogenase; Denaturation; Guanidine hydrochloride; Denaturation intermediate; Enzyme conformation

The denaturation of the dimeric enzyme glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides by guanidine hydrochloride has been studied using enzymatic activity, intrinsic fluorescence, circular dichroism, and light scattering measurements. Equilibrium experiments at 25°C revealed that between 0.9 and 1.2 M denaturant the enzyme underwent a conformational change, exposing tryptophan residues to solvent, with some loss of secondary structure and a complete loss of enzymatic activity but without dimer dissociation to subunits. This inactive, partially unfolded, dimeric intermediate was susceptible to slow aggregation, perhaps due to exposure of 'sticky' hydrophobic stretches of the polypeptide chain. A second equilibrium transition, reflecting extensive unfolding and dimer dissociation, occurred only at denaturant concentrations above 1.4 M. Kinetics experiments demonstrated that in the denaturant concentration range of 1.7-1.9 M the fluorescence change occurred in two distinct steps. The first step involved a large, very rapid drop in fluorescence whose rate was strongly dependent on the denaturant concentration. This was followed by a small, relatively slow rise in the emission intensity, the rate of which was independent of denaturant concentration. Enzymatic activity was lost with a denaturant-concentration-dependent rate, which was approx. 3-times slower than the rate of the first step in fluorescence change. A denaturation mechanism incorporating several unfolding intermediates and which accounts for all the above results is presented and discussed. While the fully unfolded enzyme regained up to 55% of its original activity upon dilution of denaturant to a concentration that would be expected to support native enzyme, denaturation intermediates were able to reactivate only minimally and in fact were found to aggregate and precipitate out of solution.

Introduction

In spite of extensive research over the last two decades, the way in which a protein's sequence of amino acids codes for its three-dimensional structure is not well understood [1-4]. For example, does the amino-acid sequence code for the final (native) three-dimensional structure or for the correct folding of an intermediate species which, in turn, guides the folding process in the right direction [5]? This so called "protein folding problem" is particularly challenging when the protein is oligomeric. Not only must the individual polypeptide chains fold properly, but the subunits must associate correctly since their specific associations are usually vital to catalytic activity. Failure of many oligomeric enzymes to regain activity when refolded from denatured subunits has been well documented [6]. The correct folding and assembly of oligomeric proteins is, therefore, currently a problem of great importance.

Glucose-6-phosphate dehydrogenase (G6PD) from Leuconostoc mesenteroides is a dimeric protein which catalyzes the oxidation of glucose 6-phosphate to 6-phosphoglucono-6-lactone by either NADP⁺ or NAD⁺ [7]. The dimeric molecular weight is 103 700 [8]. Interestingly, NAD⁺ binds to the enzyme more weakly than NADP⁺, but causes a larger conformational change than the latter coenzyme [9,10]. Although this protein has been crystallized, the detailed three-dimensional
structure has not been reported yet [11]. Several studies have shown that the association of the two identical subunits of *L. mesenteroides* G6PD is vital for enzymatic activity [12,13]. Haghhighi and Levy [12] measured the kinetics of renaturation of G6PD from urea-denatured subunits, and proposed a model in which the unfolded subunits rapidly refold to an inactive structure that can dimerize slowly to generate native enzyme. Renatured and native enzymes were indistinguishable based on physicochemical and enzymological criteria.

The purpose of the present study was to explore the denaturation of G6PD under equilibrium conditions using increasing concentrations of the denaturant GdnHCl in an effort to determine the relationship between enzymatic activity, folding and association of G6PD subunits. Several experimental properties which reflect different aspects of the enzyme’s structural integrity were monitored throughout the course of the equilibrium denaturation. Thus, enzymatic activity was monitored to determine when active site integrity is lost, while intrinsic protein fluorescence was utilized to detect changes in the local environment of tryptophan residues, as exposure of such residues to solvent is known to affect their fluorescence. Circular dichroism (CD) was used to monitor changes in the secondary structure of the G6PD molecule, while light scattering was utilized to follow the enzyme’s dissociation pattern. Denaturation curves were obtained for each of these properties that represent equilibrium structural rearrangements of G6PD. Also, the rate of reaching equilibrium was determined by measuring the kinetics of inactivation, unfolding and dissociation. The results demonstrate that complete inactivation as well as a substantial amount of unfolding occur at GdnHCl concentrations well below those needed to induce the dissociation of the enzyme into monomeric subunits. Unlike many oligomeric enzymes, inactivation of G6PD thus distinctly precedes its dissociation to subunits. The inactive, partially unfolded, dimeric intermediates which form are highly susceptible to aggregation and reactivate only minimally upon dilution of GdnHCl. In fact, dilution of the denaturant enhanced aggregation and precipitation of the enzyme possibly indicating that in the partially unfolded dimers some hydrophobic stretches are exposed which have the tendency to adhere to one another.

Materials and Methods

G6PD in lyophilized form as well as glucose-6-phosphate and NADP⁺ were purchased from Sigma. Ultrapure GdnHCl was purchased from Calbiochem. Reagents for the Bradford protein assay [14] were purchased from Bio-Rad. All other chemicals were of reagent grade.

Stock solutions of G6PD were made by reconstituting the lyophilized enzyme with 50 mM Tris-HCl (pH 7.8). Enzyme homogeneity was verified by SDS-PAGE to be better than 99%. Stock solutions of G6PD to be used in light scattering experiments (see below) were passed through a Sephadex G-200 column to remove a small amount (3%) of high-molecular-weight protein, likely aggregated G6PD. Concentration of G6PD was determined from the absorbance at 280 nm using the extinction coefficient $E_{280} = 1.15$ cm⁻¹ [8]. Alternatively, protein concentration was determined by the method of Bradford [14]. The ratio of the absorbance of G6PD at 280 nm to that at 260 nm was found to be 1.95, indicating that G6PD was in the apo form [8]. Also, glucose 6-phosphate was added to a sample of the enzyme, and no change in absorbance at 340 nm was detected, confirming that G6PD was in the apo form. The activity of the enzyme was determined spectrophotometrically by measuring the rate of NADPH production (absorbance at 340 nm) as described by Olive et al. [15], using a Milton-Roy Spectronic 1201 spectrophotometer, with one unit of activity being defined as the production of 1.0 μmol NADPH per min (initial velocity). The reaction mixture consisted of 2.50 mM glucose 6-phosphate and 0.1 mM NADP⁺ in 50 mM Tris-HCl (pH 7.8), maintained at 25°C. Manageable NADPH production rates were obtained using G6PD concentrations in the range of 0.05–0.20 μg/ml. The specific activity of G6PD was 238 ± 16 units/mg using the above reaction conditions. Stock solutions of GdnHCl were made in 50 mM Tris-HCl (pH 7.8), and the concentration of denaturant determined from the refractive index as described by Nozaki [16].

Denaturation of G6PD was performed in the following manner. Stock solutions of G6PD (typically 5 mg/ml) and GdnHCl (8 M) were mixed with 50 mM Tris-HCl (pH 7.8) with G6PD being added last, to give the desired concentration of protein and denaturant (see Figs. for details). Each mixture was allowed to equilibrate by incubation at 25°C for 24 h, a sufficient time to reach the limiting values of all enzyme properties listed below at each GdnHCl concentration used, before either enzymatic activity, protein fluorescence, or CD was measured as described in more detail below. Enzymatic activity was measured in the presence of very low concentration of GdnHCl (typically <3 mM). While it is possible that an undetected, instantaneous, partial reactivation could have occurred upon addition of the enzyme to the assay mixture, no reactivation of the enzyme was observed during the assay as evidenced by a completely linear NADPH production curve. Light scattering (described in more detail below) was used in order to identify the GdnHCl concentration needed to dissociate G6PD to subunits. Protein-GdnHCl mixtures on which light scattering measurements were done were incubated at 25°C for
only 2 h in order to minimize the amount of G6PD aggregation, a slow process which by 24 h at certain GdnHCl concentrations results in a large increase in light scattering intensity, which would obscure the expected 50% decrease in light scattering when the dimeric G6PD dissociates. A 2 h incubation time was chosen since, in the presence of 1.4 M GdnHCl, it is sufficient to completely inactivate G6PD, an event whose possible relation to dissociation of enzyme sub-units was tested. The kinetics of G6PD denaturation were determined by measuring either enzymatic activity, protein fluorescence, or light scattering at various times after mixing (see Fig. 2–4 and 6 for details).

Renaturation of G6PD was performed in the following manner. Firstly, the enzyme was denatured by mixing stock solutions of G6PD and GdnHCl with 50 mM Tris-HCl (pH 7.8), with G6PD being added last, to give the desired molar concentration of GdnHCl ([GdnHCl]) (see Fig. 5) and X μg/ml protein, where X = ([GdnHCl]) / (125) / (0.35). Each mixture was allowed to incubate at 25°C for 2 h. Secondly, renaturation was facilitated by rapidly diluting each solution into 50 mM Tris-HCl (pH 7.8) to give the low residual GdnHCl concentration of 0.35 M and 125 μg/ml G6PD and allowing the sample to equilibrate by incubation at 25°C for 24 h. Subsequently, each solution was filtered through a Gelman Sciences 0.2 μm disposable syringe filter and 14.4 nm for the scattered beam. In the denaturation experiments, the concentration of G6PD was 500 μg/ml in a 0.4 × 1.0 cm quartz cuvette. In order to remove dust particles before measurement, solutions were passed through a 0.2 μm Gelman Sciences syringe filter directly into the quartz cuvette for immediate measurement. The ratio of apparent molecular weights in the presence, $M_i$, and the absence, $M_o$, of GdnHCl was determined using the following equation [17,18]:

$$M_i = \frac{(n_i^2 \frac{dn}{dc_i})^2 (C_i)(I_{0i})}{(n_o^2 \frac{dn}{dc_o})^2 (C_o)(I_{0o})}$$

where $n$ represents the refractive index of the medium, $dn/dc$ is the refractive index increment due to the increase of protein concentration ($C$) at constant chemical potential, and $I$ is the scattered light intensity at 90° after subtraction of the scattering of the solvent. Subscripts i and o indicate the system with and without GdnHCl, respectively. The refractive index increment was assumed to decrease linearly from 0.177 to 0.130 over the GdnHCl concentration range of 0 to 5.7 M as described by Liang et al. [17]. The refractive index of the medium for the same range of GdnHCl concentrations was measured with an Abbé type refractometer.

The validity of using light scattering to detect changes in protein molecular weight was tested empirically by measuring light scattering intensity as a function of protein concentration (10–500 μg/ml) for native G6PD as well as for the native form of several standard proteins of known molecular weight (30–150 kDa). In each case it was found that light scattering intensity increased linearly with protein concentration, and the slope of this increase was proportional to the molecular weight of the protein being measured in all cases. Therefore, it is clear that at any given protein concentration the light scattering intensity is proportional to the protein’s molecular weight.

CD measurements were made at 25°C using a Jasco J-40 Recording Spectropolarimeter equipped with a CD attachment. The change in CD signal was monitored at 220 nm. The concentration of G6PD was 500 μg/ml in 0.1 cm path-length quartz cuvette.
Results

Denaturation of G6PD

Fig. 1 shows the dependence of enzymatic activity, intrinsic fluorescence, and CD on the concentration of GdnHCl under equilibrium conditions. Enzymatic activity was lost in a single, sharp, transition centered at 1.1 M GdnHCl, and the enzyme was completely inactivated by 1.2 M GdnHCl. The position of this transition was found not to depend on the concentration of G6PD in the range of 500–0.5 μg/ml (data not shown). The intrinsic fluorescence of G6PD at 340 nm was reduced with increasing concentrations of GdnHCl, to a limiting value (reached above 3.0 M denaturant) of 58% of the fluorescence without denaturant. However, in contrast to the inactivation, the fluorescence decline occurred in two, well resolved, steps of approximately the same size. The first, sharp, step coincided with the loss of enzymatic activity, since it was centered at 1.1 M GdnHCl. The second phase was less steep and was complete by 3.0 M GdnHCl. The reduction in fluorescence intensity was due in part to a shift in the emission wavelength maximum from 340 nm in the absence of GdnHCl to 363 nm at GdnHCl concentrations greater than 3.0 M. Moreover, this shift occurred in a two-step manner paralleling the decrease in intensity. The change in CD at 220 nm, a measure of protein secondary structure, with GdnHCl concentration also showed a two step transition similar to that displayed by the fluorescence. These results show that G6PD undergoes a conformational change, exposing tryptophan residues to solvent, with some loss of secondary structure and a complete loss of active site integrity between 0.9 and 1.2 M GdnHCl while a second transition, reflecting more extensive unfolding of the enzyme, begins only at GdnHCl concentrations above 1.4 M. The denaturation transitions of 50 μg/ml G6PD, incubated at 25°C for 24 h, as monitored through enzymatic activity and fluorescence in the presence of a saturating concentration of glucose-6-phosphate or NADP⁺ were also measured (data not shown). The concentrations used were ten fold the dissociation constants for each of these ligands [9], and the denaturation transitions were unaffected by the presence of these ligands.

Insight into the molecular events involved in the two transitions described above was provided by the results of the light scattering experiments, presented in Fig. 2, which showed no decrease in G6PD molecular weight throughout the GdnHCl concentration range of 0–1.9 M, indicating that no dissociation of G6PD to subunits occurs. In fact, the light scattering intensity of samples incubated with 1.0–1.4 M GdnHCl began to increase slowly after about 4 h, indicating the gradual formation of high molecular weight aggregates. This aggregation, depicted in Fig. 2 (inset), occurred without a loss of protein from solution as precipitate. To avoid interference of this slow aggregation in the light scattering experiments all the data presented in Fig. 2 were recorded following a 2 h incubation of the enzyme in GdnHCl. While complete equilibration was possibly
not reached during this shortened incubation time, it is important to note that at 1.4 M GdnHCl G6PD was completely inactive but no dissociation of the enzyme to subunits occurred. A dissociation of the G6PD dimer to monomeric subunits only occurred at concentrations of GdnHCl where the second transition in fluorescence and CD take place. This was indicated by the decrease in light scattering intensity by about a half in the range of 2.0–2.2 M GdnHCl. The kinetics of this decrease was too fast (< 20 s) to be measured with our methods (data not shown). This observation demonstrates that an inactive, partially unfolded, dimeric species of G6PD forms during denaturation between 0.9 and 1.2 M GdnHCl at 25°C. This denaturation intermediate is susceptible to slow aggregation. Unfolding of G6PD subunits appears to be complete at GdnHCl concentration between 3.0–4.0 M, as indicated by the lack of further substantial changes in any of the spectroscopic parameters followed.

The kinetics of G6PD denaturation upon mixing of the enzyme with 1.4 M GdnHCl as described in Materials and Methods were followed through both the loss of enzymatic activity and the reduction in tryptophan fluorescence as shown in Fig. 3. Both followed first-order kinetics with rate constants, evaluated from the data, of 0.052 min⁻¹ and 0.165 min⁻¹ for the loss of enzymatic activity and reduction in fluorescence, respectively. The approximately 3-fold difference between these rate constants implies that the two parameters reflect different events in G6PD unfolding with fluorescence being lost in a parallel transition which does not affect the enzymatic activity of G6PD. Enzymatic activity at the earliest recorded time (time zero in Fig. 3 but approx. 60 s following mixing) was 87% of that of native enzyme. This rapid initial slight loss of enzymatic activity might be due to the high ionic strength imparted by GdnHCl to the mixture, which could have an immediate, but small, inhibitory effect on G6PD’s activity independent of unfolding.

That the unfolding of G6PD involves distinct kinetic intermediates is demonstrated by the data in Fig. 4, which describes the change in fluorescence intensity with time upon mixing the enzyme with various concentrations of GdnHCl. At all concentrations of the denaturant, an initial first-order decline in fluorescence was observed, whose rate rapidly increased with increasing GdnHCl concentrations. The first-order rate constants for this initial decline in fluorescence were 0.165 min⁻¹, 1.14 min⁻¹, and 3.29 min⁻¹ when G6PD was mixed with 1.4, 1.6 and 1.7 M GdnHCl, respectively (Fig. 4, inset A). At GdnHCl concentrations of 1.7 M and above, the kinetics of fluorescence change became biphasic. For example, mixing G6PD with 1.8 M GdnHCl resulted in a rapid initial drop in fluorescence (< 30 s) followed by a relatively slow first-order recovery (Fig. 4, inset B) to the final levels observed in equilibrium measurements. The rate constant for this recovery was 0.37 min⁻¹. It is important to note that the rapid initial drop in fluorescence was accompanied by inactivation of G6PD, which lost 90% of its enzy-
enzyme was allowed to denature at 25°C (Fig. 6). The percent reactivation dropped from approx. 28 to 6% as the denaturation time increased from 20 s to more than 9 min. This denaturation time period corresponded to the time-frame in which the enzyme's fluorescence increased, as is shown in the superimposed fluorescence intensity time-course. It is important to note again that this fluorescence recovery was preceded by a rapid drop in fluorescence and by inactivation of G6PD.

G6PD denatured with GdnHCl concentrations greater than 2.0 M allowed a higher fraction of enzymatic activity to be regained, concomitant with less precipitation (Fig. 5). Furthermore, the specific enzymatic activity (i.e., total enzymatic activity/[protein] in solution) of the fraction of G6PD which remained in solution steadily increased, approaching 100%, as higher concentrations of GdnHCl were used in the denaturation step. Even under these conditions, however, approx. 45% of the protein precipitated from solution.

When G6PD which had precipitated during renaturation was isolated by centrifugation and mixed with 4.0 M GdnHCl, 50 mM Tris-HCl (pH 7.8), the protein completely dissolved. In order to facilitate reactivation, enzymatic activity within 30 s when mixed with 1.8 M GdnHCl (data not shown). In contrast to the strong dependence of the initial drop in fluorescence on the GdnHCl concentration, the first-order increase which followed was found to be virtually identical when 1.8 and 1.9 M GdnHCl were used (Fig. 4, inset B). The rate constant in the latter case being 0.33 min⁻¹.

**Renaturation of G6PD**

The data presented in Fig. 5 show that the fraction of denatured G6PD that can be reactivated upon dilution of the denaturant critically depended on the concentration of GdnHCl used for denaturation. Practically no enzymatic activity was regained when G6PD inactivated in 1.4–1.8 M GdnHCl was diluted. Approx. 80% of the protein precipitated out of solution upon dilution of denaturant from initial values in the above range, and the diluted solutions were visibly cloudy. The G6PD remaining in solution, approx. 20%, was practically inactive and highly aggregated as shown by the high light scattering values.

Interestingly, the percent reactivation which was obtained from dilution of G6PD denatured in 1.8 M GdnHCl critically depended on the length of time the enzyme was allowed to denature at 25°C (Fig. 6). The percent reactivation dropped from approx. 28 to 6% as the denaturation time increased from 20 s to more than 9 min. This denaturation time period corresponded to the time-frame in which the enzyme's fluorescence increased, as is shown in the superimposed fluorescence intensity time-course. It is important to note again that this fluorescence recovery was preceded by a rapid drop in fluorescence and by inactivation of G6PD.

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When G6PD which had precipitated during renaturation was isolated by centrifugation and mixed with 4.0 M GdnHCl, 50 mM Tris-HCl (pH 7.8), the protein completely dissolved. In order to facilitate reactivation,
this solution was subsequently diluted with 50 mM Tris-HCl (pH 7.8), bringing the GdnHCl concentration to 0.35 M and giving a protein concentration equal to 125 μg/ml. After 2 h incubation at 25°C, this mixture was filtered through a Gelman Sciences 0.2 μm syringe filter to remove precipitated protein, which amounted to approx. 75% of the protein. The 25% protein remaining in solution had a specific activity 60% that of fully active G6PD. That the reactivated precipitate possessed only 60% specific activity is probably due to the presence of soluble, inactive aggregates not removed by filtration. However, this experiment does demonstrate that precipitated G6PD is reactivatable.

Discussion

The dissociation of oligomeric enzymes to subunits upon exposure to appropriate concentrations of a denaturant is often the first step in denaturation and usually results in the loss of enzymatic activity [19–22]. This observation reflects the fact that the intersubunit interactions are weak relative to the interactions that stabilize the secondary and tertiary structure of each subunit. In contrast, the experimental evidence presented here strongly supports the conclusion that the first step in G6PD denaturation, in which enzymatic activity is completely lost and the intrinsic fluorescence is partially lost, occurs while the enzyme is in its dimeric form, and hence does not involve its dissociation. The simplest mechanism for the denaturation of G6PD which is compatible with this observation is:

\[ K \frac{D}{D^*} = \frac{K_u}{2U} \]  

where the equilibrium constants \( K \) and \( K_u \) depend on denaturant concentration, \( D \) is native dimer, \( D^* \) is inactive, partially unfolded and fluorescently modified dimer and \( U \) is monomeric, extensively unfolded subunit. The experiments presented in Figs. 1 and 2 show conclusively that between 0.9–1.2 M GdnHCl, \( D^* \) forms under equilibrium conditions. The equilibrium constant for the formation of \( D^* \) in the absence of GdnHCl (\( K_u \)) was evaluated by extrapolating a plot of ln \( K \) vs. [GdnHCl] according to Pace [23], \( K \) being determined from the inactivation transition in Fig. 1, and was found to be \( 2.6 \cdot 10^{-9} \). Rinas et al. [24] recently made a similar observation that dimeric blood coagulation factor XIIIa undergoes a conformational change and inactivation without dissociation to subunits as the first step in denaturation in GdnHCl. They also observed complete dimer dissociation to subunits only in the presence of high concentrations of GdnHCl. Liang et al. [17] also reported rapid inactivation of lobster muscle D-glyceraldehyde-3-phosphate dehydrogenase before dissociation and unfolding of the enzyme.

The conclusion that at intermediate concentrations of GdnHCl G6PD loses enzymatic activity without dimer dissociation to subunits is also supported by the observation that the position of the inactivation transition does not depend on protein concentration. For a mechanism involving inactivation with dissociation:

\[ K_d \frac{D}{D^*2U} \]  

where the equilibrium constant \( K_d \) depends on GdnHCl concentration, one would expect lower concentrations of G6PD to have inactivated at lower GdnHCl concentrations, since monomer-dimer equilibria depend on protein concentration. This was not observed over the wide range of G6PD concentrations employed (0.5–500 μg/ml).

The loss of active site integrity which characterizes the intermediate \( D^* \) results from a conformational change (partial unfolding) which apparently exposes to solvent previously buried tryptophan residues causing a decline and red-shift in the fluorescence and a disruption in secondary structure, probably α-helix, as evidenced from the change in CD. This change affects a domain which contains the active site and that unfolds independently of structure which is essential for maintaining intersubunit contact [25]. Moreover, the data presented in Fig. 2 (inset) show that in this conformationally modified dimer hydrophobic domains, originally buried, become exposed leading to slow aggregation. Similar aggregation of denaturation intermediates has been previously reported for a wide variety of proteins, usually oligomeric ones [17,19,20,22,24,26,27]. Above about 1.4 M GdnHCl, \( D^* \) undergoes further unfolding as shown by the second fluorescence and CD steps and the dimer dissociates to monomers (2 U) in the range of 2.0–2.2 M GdnHCl, a transition which is evident from the decline in light scattering to a value which reflects a molecular weight of approximately one half of native G6PD (Fig. 2).

The observation that the denaturation transition as followed both by enzymatic activity and by intrinsic fluorescence was not shifted to higher denaturant concentration in the presence of saturating concentrations of either glucose 6-phosphate or NADP⁺ was unexpected, since ligand binding to the native form of a protein tends to stabilize it [28]. It is possible, but unlikely, that each of these two ligands remains bound to G6PD throughout the denaturation transition so that the preferential stabilization of the native enzyme is lost. Alternatively, if low concentrations of GdnHCl cause the dissociation of the ligand prior to, and independent of, G6PD denaturation, then the ligand would also lose its ability to preferentially stabilize the native enzyme. Direct studies of glucose 6-phosphate and NADP⁺ binding to G6PD in the presence of GdnHCl are needed to clarify this phenomenon.
The kinetics of G6PD denaturation with 1.4 M GdnHCl shown in Fig. 3 clearly demonstrate that the fluorescence is lost in a transition which does not affect enzymatic activity, since fluorescence is lost 3-times faster than the latter and both processes are independently first-order. Thus, it appears that a partially unfolded but active species of G6PD (Df) also forms transiently during the approach to equilibrium. Thus the denaturation mechanism becomes:

\[ D \xrightarrow{D_f} D_i \xrightarrow{2U} D_{ni} \] 

where the modifications that produce Df (the species with modified fluorescence) and Di (inactive G6PD) occur independent of each other and possibly involve different domains of the protein, so that D and Df inactivate (to Df and Di) at the same rate. The data presented in Fig. 4 clearly demonstrate the strong dependence of the rate of decline in G6PD fluorescence on the concentration of denaturant. More interestingly, when GdnHCl is 1.7 M or above, the reaction becomes biphasic with a fast drop in fluorescence to below the equilibrium value being followed by a slower increase. The latter was found to be first-order with a rate of about 0.33–0.37 min⁻¹, which was largely independent of GdnHCl concentration. Since in the relevant GdnHCl concentration range, 1.7–1.9 M, the enzyme dimer does not dissociate (Fig. 2), we conclude that this second phase represents a conformational transition in the dimer, forming other partially unfolded, dimeric intermediates (Df* and Di*), which develop subsequent to the initial transition induced by denaturant (and whose rate depends on denaturant concentration). A denaturation mechanism compatible with all the above mentioned observations is:

\[ D \xrightarrow{D_f} \xrightarrow{D_i} \xrightarrow{2U} D_{ni} \] 

The relative concentration of dissociated subunits becomes predominant only above 2.0 M GdnHCl (see Fig. 2). The numerical value of the rate constant for the second fluorescence transition, and the observation that this rate is not sensitive to GdnHCl concentration are compatible with the possibility that this transition represents isomerization of Xaa-proline peptide bonds. The latter process is known to occur on the time scale of 1–7 min during the unfolding of small model peptides [29]. This second transition probably also occurs at GdnHCl concentrations less than 1.7 M but is masked by the initial transition (which generates Df and Df*), which at these concentrations becomes slower.

Fig. 5 clearly demonstrates that when GdnHCl in the range of 1–2 M is diluted to a concentration that would be expected to support native G6PD, the inactive dimeric species are able to reactivate only minimally and most of the fraction of inactive protein aggregates and precipitates out of solution. The inability to reactivate develops with the time of incubation with denaturant parallel to the formation of Df* as shown in Fig. 6. This behavior again reflects that Di and Di* are in a conformational state characterized by exposed hydrophobic stretches of the polypeptide chain. A similar effect has been observed for pig muscle lactic dehydrogenase [30]. Removal of GdnHCl by dilution leads to rapid, incorrect, intermolecular interactions between these hydrophobic stretches resulting in aggregation and precipitation rather than allowing the correct hydrophobic core of the native protein to reform [6]. When G6PD is denatured more extensively with higher concentrations of GdnHCl, its ability to reactivate upon denaturant dilution improves since Dn and Dn* are increasingly avoided by more complete unfolding. Similarly, it was shown that an associated GdnHCl denaturation intermediate of bovine growth hormone is relatively insoluble in the absence of GdnHCl [31,32]. This intermediate precipitates rather than renatures when GdnHCl is removed. The possibility that covalent modifications develop in G6PD during denaturation producing enzyme forms which aggregate during renaturation is unlikely since higher denaturing concentrations of GdnHCl would be expected to enhance such damage and to progressively decrease reactivation rather than increase it as observed. Furthermore, the finding that precipitated G6PD is reactivatable is also evidence against the possibility that covalent damage to G6PD is responsible for aggregation.

While equilibrium studies of G6PD reactivation, as here reported, can yield a wealth of information about this process, it is clear that for a more complete resolution between potential refolding and reassociation pathways, including aggregation, complementary kinetics data are indispensable. Such studies are currently underway.

Acknowledgements

This work was supported by a Presidential Initiative Fund award to the University of Michigan from the W.K. Kellogg Foundation and by a grant from the Office of Naval Research. J.J.P. was supported by a training grant from the National Institute on Aging (T32AG00114).

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