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Renaturation of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* after denaturation in 4 M guanidine hydrochloride: kinetics of aggregation and reactivation

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In 4 M guanidine hydrochloride (GdnHCl), the dimeric enzyme glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6PD) dissociated to subunits and was extensively unfolded. Rapid dilution of this high GdnHCl concentration allowed G6PD to partially renature, as measured by enzyme reactivation, to a level which depended on the conditions employed. The fraction of the enzyme which did not renature aggregated and precipitated out of solution, a process which could not be substantially prevented by stabilizing additives. Based on the enzyme concentration dependence of the reactivation yield and on a comparison of the aggregation and reactivation rates, it was determined that aggregation and reactivation compete kinetically for a partially-folded intermediate only very early in the process, during the rapid GdnHCl-dilution step. The kinetics of G6PD reactivation were sigmoidal, indicating that this process involves more than one rate-limiting reaction. The kinetics depended on enzyme concentration in a higher than first-order manner, indicating that association of subunits is one of the rate-limiting reactions. A renaturation mechanism compatible with these observations is described, which involves a bi-unimolecular (subunit association-folding) reaction sequence, with rate constants equal to 2.19 $\mu M^{-1} min^{-1}$ and 0.140 min^{-1}, respectively. This mechanism involves an inactive, dimeric, G6PD-folding intermediate, a species whose existance has recently been established by equilibrium denaturation experiments (Plomer, J.J. and Gafni, A. (1992) Biochim. Biophys. Acta 1122, 234–242).

Introduction

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6PD) has been extensively studied, resulting in the elucidation of its catalytic mechanism and the determination of the kinetic and binding constants for its substrate and coenzymes [1–7]. G6PD is a homodimeric enzyme with a subunit molecular mass of 54316 Da [8]. Although this enzyme has been crystallized, its detailed three-dimensional structure has not been reported yet [9], hence the shape and number of structural domains in each subunit as well as the geometrical arrangement of the subunits forming the native dimer are unknown.

The relationship between G6PD's function and structure was previously probed by equilibrium denaturation experiments with increasing concentrations of the denaturant guanidine hydrochloride (GdnHCl) in an effort to elucidate the pattern of denaturation and to correlate the loss of enzymatic activity with unfolding and dissociation to subunits [10]. These studies demonstrated that unlike many oligomeric enzymes [11–15], inactivation and partial unfolding of G6PD distinctly precede its dissociation to subunits, indicating that the domain containing the active site unfolds independently of structure which is essential for maintaining intersubunit contact. A number of inactive, partially unfolded, dimeric G6PD denaturation intermediates were revealed in these studies [10]. These were highly susceptible to aggregation into high-molecular-mass structures, and were unable to reactivate significantly upon reduction of the GdnHCl concentration to levels expected to support renaturation to native G6PD.

When G6PD was extensively denatured in 4.0 M GdnHCl, its ability to reactivate upon GdnHCl dilution was better than G6PD which was only partially dena-

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Abbreviations: ANS, 8-anilino-1-naphthalene sulfonate; CMC, critical micelle concentration; G6PD, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*; GdnHCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; Xaa, any amino acid.

tured using lower GdnHCl concentrations [10]. Under these renaturing conditions, the percentage of enzymatic activity recovered was 55%. Moreover, the specific activity of the renatured fraction approached 100% while the remaining 45% of the enzyme aggregated and precipitated out of solution. A reactivation yield less than 100% is common for oligomeric proteins, where refolding is often accompanied by formation of inactive aggregates [16–18]. Haghighi and Levy [19] reported that when exposed to 8 M urea, *Leuconostoc mesenteroides* G6PD denatures, with complete loss of enzymatic activity, extensive unfolding, and subunit dissociation. Upon dilution of urea, they observed a 45–70% reactivation yield, a value which is similar to that observed using GdnHCl [10]. In contrast, though, Haghighi and Levy [19] concluded that formation of inactive aggregates did not occur since no decrease in reactivation yield was observed at high G6PD concentrations; however, it appears that no attempt was made to detect aggregates directly.

The first objective of this study was to determine the dependence of G6PD reactivation yield, following denaturation in 4 M GdnHCl, on enzyme concentration in order to assess to what extent aggregation competes with reactivation. The kinetics of aggregation were also directly measured by light scattering and compared to those of reactivation. Furthermore, the effects of other renaturation conditions (i.e., residual GdnHCl concentration, temperature, presence of additives, and the length of denaturation prior to renaturation) on reactivation yield were examined. Results presented here support the conclusion that aggregation competes with reactivation only very early in the renaturation process, reflecting the transient presence of a partially-folded monomeric intermediate with a high tendency to aggregate. Competing with this aggregation is a conformational transition which converts this intermediate to a form that reactivates with high yield. The second objective of this study was to establish the mechanism of reactivation, following GdnHCl denaturation, by measuring G6PD reactivation kinetics as a function of the concentration of reactivateable subunits. The results support the hypothesis, formerly derived from equilibrium denaturation experiments [10], that renaturation of G6PD proceeds through an inactive, dimeric intermediate.

Materials and Methods

Materials

Lyophilized G6PD from *L. mesenteroides*, glucose 6-phosphate, $NADP^+$, ammonium sulfate, sucrose, dodecyl maltoside, 8-anilino-1-naphthalene sulfonate (ANS) and trypsin were all purchased from Sigma. Ultrapure GdnHCl was purchased from Calbiochem. All other chemicals were of reagent grade.

Methods

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%. Concentration of G6PD was determined from the absorbance at 280 nm using the extinction coefficient $E^{280}_{0.1\%} = 1.15\ cm^{-1}$ and monomeric molecular mass = 54 316 Da [8]. The ratio of the absorbance at 280 nm to that at 260 nm was found to be 1.95, indicating that the G6PD was in the apo form [20]. Also, when glucose 6-phosphate was added to a sample of the enzyme, 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 initial rate of NADPH production (absorbance at 340 nm) at 25°C as described by Olive et al. [5] using a Milton-Roy Spectronic 1201 spectrophotometer. 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 [21].

The critical micelle concentration of dodecyl maltoside, an additive used in a renaturation experiment described below, in 0.250 M GdnHCl, 50 mM Tris-HCl (pH 7.8) was determined at 25°C by the fluorescence method of De Vendittis et al. [22] using the dye 8-anilino-1-naphthalene sulfonate (ANS) at 10 μM. ANS fluorescence (excitation and emission wavelengths set at 370 nm and 490 nm, respectively) as a function of dodecyl maltoside concentration was measured using a Spex Fluorolog II fluorometer with 0.25-m single grating excitation monochromator and a 0.25-m double grating emission monochromator (data not shown). The bandwidth for excitation was 1.8 nm, while that for emission was 7.2 nm. From the inflection point in the fluorescence curve, the critical micelle concentration was found to be 170 μM, a value similar to that previously reported (157–166 μM) in the literature [23,24].

Denaturation of G6PD was performed in the following manner. Stock solutions of G6PD (typically 15 mg/ml) and GdnHCl (8 M) were mixed with 50 mM Tris-HCl (pH 7.8), with G6PD being added last, to give 4.0 M GdnHCl and the desired concentration of enzyme (see figures for details). 4.0 M GdnHCl was previously shown to cause complete inactivation and very extensive unfolding of G6PD [10]. Each mixture was allowed to equilibrate by incubation at 25°C for 1 h, unless otherwise noted, before initiating renaturation.

Renaturation was initiated by rapidly diluting each denatured G6PD sample into renaturation buffer (50 mM Tris-HCl (pH 7.8)) to give a residual GdnHCl concentration of 0.25 M (unless otherwise noted) and the desired enzyme concentration. The residual GdnHCl concentration was carefully adjusted to the

desired concentration by adding a proper amount of GdnHCl to the renaturation buffer, if needed. To determine reactivation yield, each mixture was incubated at 25°C (in one case at 5°C) for at least 24 h before enzymatic activity was measured and compared to control G6PD which had not been denatured in 4.0 M GdnHCl, but that was incubated under identical renaturation conditions (i.e., enzyme concentration, residual GdnHCl concentration, temperature, or presence of additive). Enzyme reactivation was used as the criterion for correct renaturation, an assumption supported by the absence of partially active enzyme species during either denaturation or reactivation of denatured G6PD [10]. A reactivation yield less than 100% was shown to be due to the formation of a mixture of fully active enzyme and completely inactive aggregates of G6PD [10]. Incubation at 25°C, or in one case at 5°C, of reactivated and control enzyme was continued from 24 h for time periods up to 96 h, and the enzymatic activity of both remained constant throughout under all renaturation conditions employed.

The reactivation yield was found to critically depend on the mixing efficiency during the dilution step used to initiate renaturation, and decreased as the rate of mixing decreased. To ensure reproducibly rapid mixing, the dilution step was accomplished by adding the volume of 4 M GdnHCl-denatured G6PD directly into the renaturation buffer, which was undergoing continuous agitation with a vortex mixer. Mixing was complete in less than 1 s.

The kinetics of reactivation at 25°C were determined by measuring enzymatic activity as a function of time (0–120 min) following the mixing of denatured G6PD with renaturation buffer. In order to prevent reactivation during the activity assay, which would result in a non-linear NADPH production course, 50 μg/ml trypsin was included in the assay mixture [25]. This concentration of trypsin was found to quench reactivation, ostensibly by proteolytic cleavage of denatured G6PD, without affecting the activity of already active enzyme. Under all conditions and enzyme concentrations used, the reactivation yield reached a plateau by 120 min or less.

The kinetics of aggregation during renaturation were determined by monitoring the intensity of light scattering at 450 nm vs. time, using the Spex Fluorolog II fluorometer described above, with the light scattering intensity determined at 90° to the incident beam and with both incident and scattered beams vertically polarized. The bandwidths were 1.8 nm for the incident beam and 14.4 nm for the scattered beam.

Results

Experiments in which a fixed concentration of denatured G6PD was reactivated by dilution to yield different GdnHCl concentrations, but identical concentrations of reactivating enzyme, showed the reactivation yield to be independent of the residual GdnHCl concentration between 0.06–0.30 M. When the latter concentration was greater than 0.30 M, reactivation was increasingly prevented and became negligible above 0.50 M denaturant (data not shown).

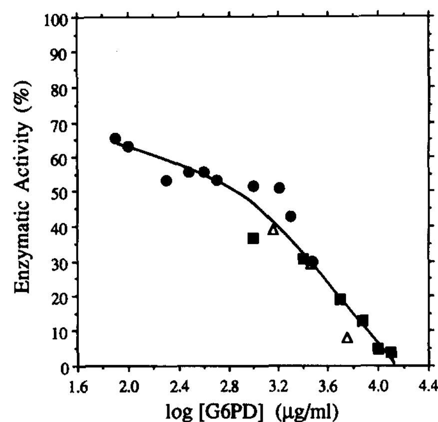


Fig. 1. Dependence of G6PD reactivation yield on the concentration of denatured enzyme. The concentrations of enzyme and residual GdnHCl in the renaturation buffer were, respectively: (●), 5 μg/ml, 0.250 M; (■), 37 μg/ml, 0.148 M; (△), 125 μg/ml, 0.350 M.

Dependence of reactivation yield on G6PD concentration

It has been previously reported that the yield of enzyme reactivation frequently dramatically improves, with corresponding decrease in aggregation, as the protein concentration is decreased [26–30]. This effect is thought to reflect a higher reaction order for aggregation than for reactivation, the two processes occurring from a common refolding intermediate.

Fig. 1 shows that the yield of reactivation indeed steadily decreased as the concentration of denatured enzyme was increased, with the concentrations of enzyme and GdnHCl in the renaturation buffer being held constant. This decrease was most precipitous at denatured enzyme concentrations greater than 1.0 mg/ml. This result may indicate that a fraction of a folding intermediate generated very rapidly during denaturant dilution forms inactive aggregates, i.e., that early in G6PD folding aggregation competes with reactivation.

To test the competition between reactivation and aggregation, the dependence of the reactivation yield on the concentration of enzyme in the renaturation buffer was measured with denatured enzyme and residual GdnHCl concentrations being held constant at 1.6 mg/ml and 0.250 M, respectively. These two concentrations allow the reactivation yield to be maximized. Fig. 2 shows that the reactivation yield did not depend on the concentration of enzyme in the renaturation buffer, indicating that once the rapid dilution of GdnHCl was complete, reactivation could not be sub-

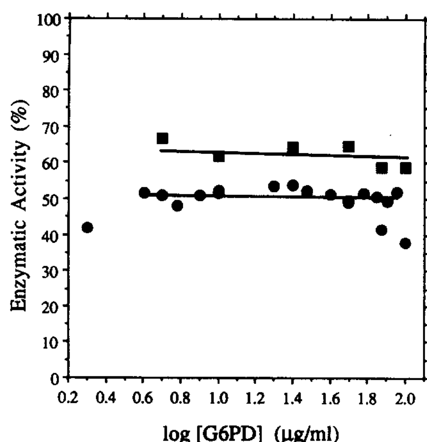


Fig. 2. Dependence of G6PD reactivation yield on the concentration of enzyme in the renaturation buffer. The concentration of denatured enzyme was 1.6 mg/ml. The renaturation temperature was either 25°C (●) or 5°C (■).

stantially improved by lowering the enzyme concentration. Evidently, aggregation does not compete with reactivation once mixing/dilution is complete.

Fig. 2 also shows that lowering the temperature to 5°C improved the reactivation yield by about 10% at all enzyme concentrations but had no effect on the shape of the curve. This result suggests a different temperature-dependence of the reactions governing aggregation and reactivation [31].

Dependence of the reactivation yield on denaturation time

Experiments in which G6PD was allowed to incubate in 4.0 M GdnHCl between 3 s and 60 min prior to dilution of the denaturant are presented in Fig. 3. It is clear that the reactivation yield did not depend on the incubation time and remained relatively constant at about 50%.

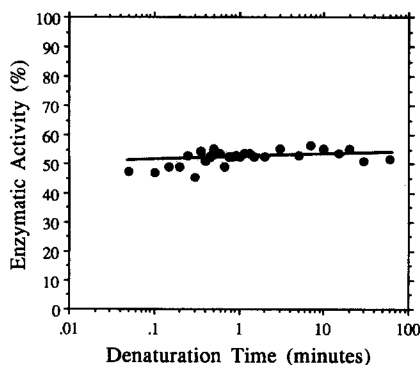


Fig. 3. Dependence of G6PD reactivation yield on denaturation time. The concentrations of denatured enzyme and enzyme in the renaturation buffer were 1.6 mg/ml and 50 μg/ml, respectively.

TABLE I

Dependence of the reactivation yield on additives to the renaturation buffer

First, 1.6 mg/ml G6PD was denatured in 4.0 M GdnHCl, 50 mM Tris-HCl (pH 7.8) for 1 h at 25°C. Subsequently, this mixture was rapidly diluted 32-fold into 50 mM Tris-HCl (pH 7.8) containing 0.129 M GdnHCl and an additive, to give 50 μg/ml G6PD, 0.250 M residual GdnHCl, and one of the additive concentrations listed below. Following incubation at 25°C for 24 h, enzymatic activity was measured and expressed as a % of control, which was not denatured in 4.0 M GdnHCl but was incubated under identical renaturation conditions.

Additive	Enzymatic activity (%)
None	47
Sucrose (30%, w/v)	64
Glucose 6-phosphate (10 mM)	44
$NADP^+$	
60 μM	46
600 μM	52
Dodecyl maltoside [a]	
100 μM	46
125 μM	40
150 μM	18
175 μM	11
200 μM	0
Ammonium sulfate (50% saturation)	0

[a] These concentrations of dodecyl maltoside did not affect the enzymatic activity of native G6PD.

Dependence of the reactivation yield on the presence of additives

Table I summarizes these experiments and shows that inclusion of 30% (w/v) sucrose in the renaturation buffer improved the reactivation yield by about 16% with respect to that which was obtained without additive. However, neither the presence of glucose 6-phosphate or $NADP^+$, each at a concentration capable of saturating native G6PD, improved the reactivation yield over that which was obtained without ligand. Addition of dodecyl maltoside to the renaturation buffer increasingly prevented reactivation as its concentration was increased from 100 to 200 μM. This concentration range includes the critical micelle concentration which was determined to be 170 μM under the conditions of renaturation (see Materials and Methods for more details). Addition of ammonium sulfate at 50% saturation completely prevented reactivation of G6PD.

Kinetics of G6PD reactivation and aggregation

The time-course of reactivation of G6PD denatured in 4.0 M GdnHCl was followed and compared to the time-course of enzyme aggregation under the same conditions (Fig. 4). Aggregation, monitored through scattered light intensity, increased very rapidly upon dilution of the GdnHCl, while enzymatic activity recov-

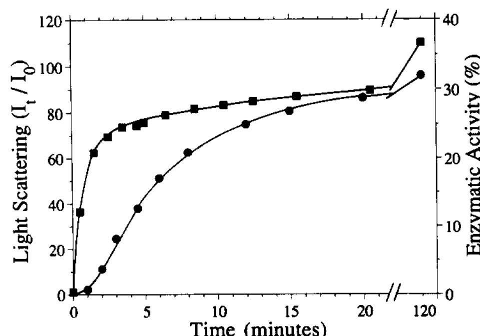


Fig. 4. Kinetics of G6PD reactivation (●) compared to aggregation as measured by light scattering (■). The scattering intensity at time zero (I_0) was that of denatured (monomeric) G6PD. The concentrations of denatured enzyme and enzyme in the renaturation buffer were 1.6 mg/ml and 100 μg/ml, respectively.

ered much slower. Thus, within one minute scattered light intensity increased about 36-fold while reactivation reached less than 1%, indicating little formation of native enzyme. The light scattering intensity continued to slowly increase over longer time, indicating a gradual increase in the size of aggregates. These results further support the idea that aggregation occurs during mixing in the dilution process.

To characterize the reactivation mechanism of G6PD, the time-course of this reaction as a function of the initial concentration of reactivatable protein was followed. This concentration ($[M_2]_0$ in Scheme I) was obtained by subtracting the fraction of enzyme that rapidly aggregates from the total concentration of enzyme in the reactivation buffer. Fig. 5 shows the time-course of relative reactivation for various values of $[M_2]_0$. In these plots, the maximal activity obtainable at each concentration (activity at 120 min) was normalized to 100%. At low concentrations, these kinetics were clearly sigmoidal, indicating that G6PD reactivation does not obey simple first- or second-order kinetics. As $[M_2]_0$ was increased, the reactivation became faster, less sigmoidal, and less dependent on enzyme concentration. In fact, at $[M_2]_0 = 0.589$ μM, the reactivation obeyed a linear semi-log plot (Fig. 5, inset), indicating that first-order kinetics are approached at high enzyme concentrations.

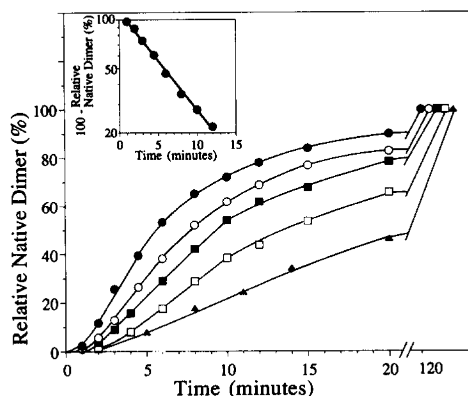
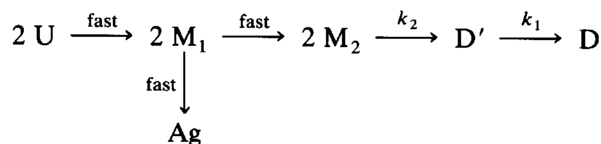


Fig. 5. Relative reactivation kinetics: G6PD concentration dependence. The concentration of denatured enzyme was 1.6 mg/ml. The initial concentrations of renaturing enzyme ($[M_2]_0$, μM) were: 0.589 (●); 0.217 (○); 0.101 (■); 0.0361 (□) and 0.0188 (▲). The enzymatic activity present at 120 min was set to 100%. Inset: Semi-log plot of the reactivation of 0.589 μM monomer.

Reactivation kinetics data analysis

The minimal renaturation scheme of dimeric G6PD involves the following sequential steps: subunit folding providing the surface required for correct subunit recognition, subunit association, and, possibly, dimer conformational adjustment [32]. The apparent sigmoidicity of the reactivation profiles indicates that their rate cannot be limited by only one reaction. That subunit association must be involved is demonstrated by the fact that the reactivation kinetics (Fig. 5) highly depend on subunit concentration. This behavior, frequently observed in oligomeric enzymes [33,34], reflects the significant fact that monomeric G6PD cannot be active. Also, aggregation of G6PD is evident as a side-reaction which competes with reactivation from a partially folded intermediate which is formed very early in the pathway. The following model was used to analyze the reactivation data of Fig. 5:



Scheme I

where U is extensively unfolded G6PD subunit, M_1 and M_2 are inactive, monomeric folding intermediates, Ag is aggregated enzyme, D′ is an inactive, dimeric folding intermediate, and D is native G6PD. k_2 and k_1 are second-order and first-order rate constants respectively. This combination of folding and association reactions is initiated when the denaturant is diluted from a high concentration, which supports existence of U, to a concentration low enough to support the formation of native enzyme (D).

The reactivation mechanism presented in Scheme I is based on the results shown in Figs. 1, 2 and 4 demonstrating that the degree of enzyme aggregation increases with the concentration of denatured enzyme (U), but does not depend on the concentration of enzyme in the renaturation buffer (when the various concentrations of this species are generated from the same sample of denatured enzyme). It is thus clear that aggregation and reactivation compete only during mixing/dilution of the denaturant, and that once M_2 has formed it is committed for reactivation. Moreover, the increased yield of aggregation with G6PD concentra-

tion is in line with the high reaction order of this process. The initial aggregates continue to grow steadily by self-association, not depleting the fraction of unaggregated monomeric folding intermediate (M_2). Our analysis of the kinetics of reactivation was therefore not complicated by the aggregation side-reaction, which was subtracted out.

The second-order and first-order rate constants k_2 and k_1, respectively, were obtained by analyzing the data in Fig. 5 in the following way. For each reactivation plot, an inflection point was identified and the slope around this point, which represents the maximal rate of native dimer formation, V_{max}, was measured.

$$V_{max} = d[D]_{max}/dt = k_1[D']_{max} \quad (1)$$

The subscript 'max' is used to refer to the parameters (concentrations, reaction rates, etc.) at this inflection point. From Scheme I, it is clear that at this portion of the curve

$$d[D']_{max}/dt = 0 \quad (2)$$

and since

$$d[D']_{max}/dt = k_2[M_2]^2_{max} - k_1[D']_{max} \quad (3)$$

it follows that

$$k_1[D']_{max} = k_2[M_2]^2_{max} \quad (4)$$

Therefore, from Eqn. 1

$$V_{max} = k_2[M_2]^2_{max} \quad (5)$$

$$\log V_{max} = \log k_2 + 2\log[M_2]_{max} \quad (6)$$

As an approximation, it was assumed that

$$[M_2]_{max} = [M_2]_0 - 2[D']_{max} \quad (7)$$

Since $[D']_{max} = V_{max}/k_1$,

$$\log V_{max} = \log k_2 + 2\log\{[M_2]_0 - (2V_{max}/k_1)\} \quad (8)$$

Fig. 5, inset, shows that, as expected, $D' \rightarrow D$ becomes rate-limiting for reactivation at high concentration of $[M_2]_0$ and the reactivation kinetics become first-order. k_1 was calculated from the data to be 0.140 min^{-1} and this value, which was consistent from experiment to experiment to ± 0.004 min^{-1}, was used in Eqn. 8. A plot of the data according to Eqn. 8 is shown in Fig. 6, and the second-order rate constant (k_2) was calculated from the y-intercept to be 2.19 $\mu M^{-1} min^{-1}$ ($3.65 \cdot 10^4$ $M^{-1} s^{-1}$). The observed reaction order, calculated from the slope, was 2.13, which is close to the expected value of 2.00 for the reaction $2M_2 \rightarrow D'$.

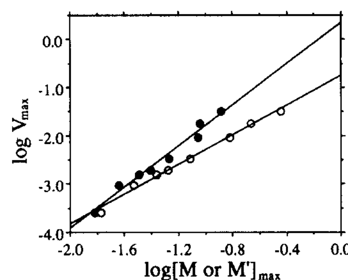
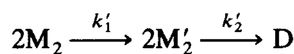


Fig. 6. Log-log plot of G6PD reactivation kinetics, according to Eqns. 9 (●) and 11 (○).

An alternative model for reactivation, comprising a uni-bimolecular reaction sequence, also warrants consideration:



$$2M_2 \xrightarrow{k_1'} 2M_2' \xrightarrow{k_2'} D$$

Scheme II

where M_2' is another inactive, monomeric folding intermediate, which replaces D' in Scheme I. The unimolecular and bimolecular rate constants are k_1' and k_2', respectively. Using reasoning analagous to that presented above, the following relationship was derived from Scheme II, relating the measured maximal rate of native dimer formation (V_{max}) to $[M_2]_0$:

$$\log V_{max} = \log k_2' + 2\log\{[M_2]_0 - (V_{max}/k_1')\} \quad (9)$$

where $[M_2]_0 - (V_{max}/k_1') = [M_2']_{max}$ and $k_1' = k_1 =$ 0.140 min^{-1}. A plot of the data according to Eqn. 9 is shown in Fig. 6, and from the y-intercept, the second-order rate constant (k_2') was calculated to be 0.170 $\mu M^{-1} min^{-1}$ ($0.283 \cdot 10^4$ $M^{-1} s^{-1}$). The observed reaction order, calculated from the slope, was 1.53.

Discussion

It was previously demonstrated that renaturation of G6PD denatured with either urea [19] or GdnHCl [10] yields about 55% reactivation, a value which could not be substantially improved by decreasing the concentration of the renaturing enzyme [19]. The present study confirms this observation and makes the new observation that G6PD reactivation yield depends greatly on the concentration of denatured protein (Figs. 1 and 2). This dependence provides convincing evidence that aggregation and reactivation compete kinetically only very early in the folding, i.e., during the GdnHCl dilution step. Further evidence for this conclusion is provided by the observation that aggregation occurs much more rapidly than reactivation (Fig. 4). A likely interpretation for these observations is that high local protein concentrations which exist transiently during dilution facilitate rapid aggregation of 'sticky' early

folding intermediates. This is consistent with the often encountered strong dependence of the reactivation yield on the manner used to dilute a denatured protein into the renaturation buffer. In general, kinetic competition between aggregation and reactivation is common, especially among oligomeric proteins [18,27,28,30,32, 35].

Another possible explanation for the observation that reactivation and aggregation compete only very early in the folding process is that two populations of extensively unfolded G6PD exist, one 'earmarked' for aggregation and another for reactivation upon denaturant dilution. Such two, noncompeting populations might be generated by isomerization of Xaa-proline peptide bonds in extensively unfolded G6PD [36], where molecules which contain a non-native Xaa-proline peptide bond conformation are destined for misfolding and aggregating under renaturing conditions. This, however, appears unlikely, since the ratio of these two species would not be expected to show the observed strong dependence on denatured enzyme concentration. Moreover, Brandts et al. [37] found that proline isomerization in small model dipeptides occurs with a relaxation time of 1–7 min at room temperature and one would therefore expect the degree of G6PD reactivation to depend on the length of incubation of the enzyme in 4 M GdnHCl if proline isomerization was involved. Fig. 3 clearly shows that G6PD reactivation yield was independent of the length of denaturation in 4 M GdnHCl. The ratio of G6PD aggregation and reactivation is thus not likely to be determined by Xaa-proline isomerization.

30% sucrose was found to improve the reactivation yield of G6PD by 16% (Table I). Similar protective effects of sucrose against protein aggregation have been demonstrated before [38] and may reflect a decrease in the rate of aggregation, a diffusion-dependent process, due to increased solution viscosity [39]. Also, sucrose may stabilize the native structure of proteins by causing preferential hydration of the polypeptide by increasing the surface tension of water [40].

The lack of an improvement in G6PD reactivation yield by excess $NADP^+$ or glucose 6-phosphate (Table I) clearly supports the notion that the division into aggregating and reactivating enzyme occurs before the latter is able to bind either ligand. In contrast, Teipel and Koshland demonstrated that the presence of substrate or cofactor increased both the rate and yield of reactivation of six different enzymes, including three dehydrogenases [41].

Table I also shows that dodecyl maltoside did not improve reactivation and even prevented it as the concentration was increased to above the critical micelle concentration. Horowitz et al. [42,43] showed dodecyl maltoside to improve the renaturation of several enzymes, perhaps by masking exposed hydrophobic surfaces present in renaturation intermediates, thus preventing aggregation. In view of this evidence for the beneficial effect of dodecyl maltoside on protein renaturation, the reason for lack of a positive effect on G6PD reactivation is unclear.

Ammonium sulfate completely prevented reactivation in favor of aggregation. This was not unexpected since sulfate is a kosmotropic (water structure maker) ion [44], and high concentrations of ammonium sulfate lead to protein precipitation by competing for water molecules [39]. It thus apparently promotes self-association of the already vulnerable renaturation intermediate M_1 during the dilution step.

Of the two renaturation models presented in Schemes I and II in Results the first, which involves a bi-unimolecular reaction sequence, is clearly more compatible with the data. This conclusion is based on the fact that the value of 2.1 derived for the reaction order of subunits dimerization using Scheme I was closer to the predicted value of 2.0 than the value of 1.5 obtained using Scheme II. Moreover, the bimolecular rate constant evaluated based on Scheme I ($k_2 = 3.65 \cdot 10^4$ $M^{-1} s^{-1}$) is in good agreement with the values reported for other oligomeric proteins [32], while the corresponding value derived from Scheme II ($0.283 \cdot 10^4$ $M^{-1} s^{-1}$) is not. For example, reconstitution of GdnHCl-denatured pig skeletal muscle lactic dehydrogenase and pig mitochondrial malic dehydrogenase both involve a bimolecular rate constant of $3 \cdot 10^4$ $M^{-1} s^{-1}$. [33,45]. It should be noted that all the values quoted above are much larger than the bimolecular rate constant of $4.9 \cdot 10^{-3}$ $\mu M^{-1} min^{-1}$ (82 $M^{-1} s^{-1}$) reported by Haghighi and Levy [19] for the reassociation of *L. mesenteroides* G6PD from urea-denatured subunits. The reason for this discrepancy is not clear.

Finally, it is interesting to note that the presence of an inactive, dimeric form of G6PD (D′) inherent in Scheme I is fully consistent with our previous finding that such species exist during equilibrium denaturation of G6PD [10]. Equilibrium denaturation and the kinetics of reactivation thus portray a coherent picture of G6PD folding.

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