

REASSOCIATION AND REACTIVATION OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM *STREPTOMYCES AUREOFACIENS* AFTER DENATURATION BY 6 M UREA

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Abstract

Glucose 6-phosphate dehydrogenase (G6PD) from *Streptomyces aureofaciens* was purified and denatured in 6 M urea. Denaturation led to complete dissociation of the enzyme into its inactive monomers, 98% loss of the enzyme activity, about 30% decrease in the protein fluorescence and a 10 nm red shift in the emission maximum. Dilution of urea-denatured enzyme resulted in regaining of the enzyme activity and the native protein fluorescence. The renatured enzyme was indistinguishable from the native enzyme based on a number of enzymological and physicochemical criteria. Regaining of the protein fluorescence occurred immediately after diluting the denatured enzyme and before reactivation started. The reactivation process was also monitored by measuring the accessibility of histidine residues toward diethylpyrocarbonate modification. As the reactivation proceeded, less histidine residues were able to be modified. Nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate (NADP^+) and glucose 6-phosphate stimulated the reactivation rate at different degrees. It seemed likely that specific ligands stimulated reactivation by binding to an inactive form of the enzyme leading to a different pathway of refolding. The data are consistent with a model for enzyme renaturation and reactivation in which NAD^+ and NADP^+ pull the enzyme toward different conformational structures.

Keywords: Glucose 6-phosphate Dehydrogenase; Urea-denaturation

Introduction

Glucose 6-phosphate dehydrogenase (G6PD, EC.

1.1.1.49) in most organisms is either NAD^+ or NADP^+ linked. In eukaryotes and many prokaryotes G6PDs are NADP^+ specific or NADP^+ preferring [1]. An important

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function of this coenzyme is to generate NADPH used for various biosynthetic reactions. In some prokaryotes G6PD participates in two or more metabolic pathways. Such organism possesses either two distinct G6PDs, NAD⁺ and NADP⁺ preferring, respectively, or a single enzyme that can catalyze both reactions [1]. Among the later, G6PD from *Locunostoc mesenteroides* has been studied extensively [1-3]. Another dual nucleotide specific enzyme is G6PD from *Streptomyces aureofaciens* [4]. Primary kinetic and inhibition studies have shown two different forms of *S. aureofaciens* G6PD for NAD⁺- and NADP⁺-linked activities [4,5]. The mechanism by which this enzyme selects coenzyme, however, is not yet understood. Denaturation and renaturation kinetic studies have been utilized to investigate conformational changes of proteins induced upon ligand binding or other experimental conditions. We have previously reported that kinetics of reassociation and reactivation of *L. mesenteroides* G6PD from urea-induced inactive subunits revealed different conformational transitions for NAD⁺ or NADP⁺ binding [6]. Others also studied the kinetics of denaturation and renaturation of this enzyme in guanidine hydrochloride [7]. Refolding pathway and association of other dehydrogenases such as glutamate dehydrogenase [8], estradiol 17- β -dehydrogenase [9] and D-glyceraldehyde 3-phosphate dehydrogenase [10] have been studied.

In this study *S. aureofaciens* G6PD was inactivated in 6 M urea and then allowed to reactivate under various conditions. The kinetics of several stages in this process was monitored by measuring changes in the activity, intrinsic protein fluorescence and reactivity of histidine residues. The effect of adding coenzyme during reactivation was also examined and suggested different enzyme conformation induced by the coenzymes.

Experimental Procedures

Materials

Streptomyces aureofaciens (#1119) was obtained from Iranian Scientific Research Organization (Tehran). Glucose-6-phosphate, NAD⁺, NADP⁺, DEAE-Cellulose, Sephadex G-100, urea and Tris were purchased from Sigma Co. (U.S.A.). All other chemicals were of the best grade commercially available.

Enzyme Assay

Standard assays of G6PD for NAD⁺- and NADP⁺-linked reactions were performed in a Perkin Elmer spectrophotometer model 551S at 25°C as described by Neuzil *et al.* [4]. The assay mixtures were brought to

25°C prior to each assay.

Fluorescence studies were done in a Perkin Elmer spectrophotofluorometer model Ls-3B at 25°C. Protein concentration was determined by the method of Lowry *et al.* [11].

Enzyme Purification

Streptomyces aureofaciens was grown according to Behal *et al.* [12]. The cells (20 g/100ml) were suspended in 0.2 M Tris-HCl buffer pH 7.4 containing 2 mM EDTA, 5% (v/v) glycerol and 1 mM β -mercaptoethanol and sonicated at 22 kHz for 30 sec. Sonication repeated 13 cycles to disrupt the cells (99%). The disrupted cell solution was diluted (1:3) in the above buffer and centrifuged at 20,000 g for 30 min. The supernatant (crude extract) was kept at -20°C for the enzyme purification. G6PD from the crude extract was precipitated with 60% ammonium sulfate. The precipitated enzyme was dissolved in 10 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA, 0.025 mM sucrose and 0.05% (v/v) β -mercaptoethanol and dialyzed against the same buffer. The enzyme was then applied on a DEAE-Cellulose column (2×20 cm) equilibrated with 50 mM Tris-HCl buffer pH 7.5 and eluted with a linear gradient of 0-0.5 M NaCl in 200 ml of the starting buffer. Fractions (4 ml) containing the enzyme activity (31-43) were combined, precipitated with saturated ammonium sulfate, redissolved in small volume of the starting buffer and dialyzed against the same buffer. Finally, the enzyme was further purified on a Sephadex G-100 column (2×91cm) equilibrated with 50 mM Tris-HCl buffer pH 7.5 and the only sharp peak of the enzyme was collected and concentrated with saturated ammonium sulfate as above.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of the native and renatured enzyme and SDS-PAGE for the determination of enzyme subunits M.W. was performed as essentially described by Harris *et al.* [13]. Activity staining was done in 0.1 M Tris-HCl buffer pH 7.4 containing 2 mM NADP⁺, 5 mM glucose 6-phosphate, 0.07 mM phenazine methosulfate and 0.5 mM tetra nitro blue tetrazolium [5].

Molecular Weight Determination

Subunit molecular weight of the denatured enzyme was determined on PAGE by the method of Hedrick and Smith [14] except that 6 M urea was included in the cathodic buffer as well as in the gel. A standard curve

Table 1. Purification of G6PD from *Streptomyces aureofaciens*

Steps	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield %
Crude extract	3189	45	0.014	100
Ammonium sulfate precipitate (60%)	1000	44.2	0.044	98.3
DEAE-Cellulose	83.2	31.9	0.384	71
Gel filtration (Sephadex G-100)	11.2	22.9	2.1	51

The whole purification performed twice and similar results obtained

was generated with bovine serum albumin, egg albumin, pepsin trypsinogen, β -lactoglobulin and lysozyme. Molecular weights of the native and renatured enzyme was estimated by gel filtration on a Sephadex G-100 (2 \times 91 cm) column equilibrated with 50 mM Tris-HCl buffer pH 7.4. The column was calibrated with immunoglobulin, β -galactosidase, transferrine and bovine serum albumin.

Denaturation

Denaturation of G6PD was performed by incubating the enzyme in 40 mM Tris-HCl buffer pH 7.4 containing urea concentration ranging from 2 M to 8 M. The mixture was gently stirred for 2 h at room temperature. Complete denaturation was achieved with 6 M urea.

Renaturation

Completely denatured enzyme with 6 M urea was reactivated by diluting rapidly at least 100-fold in 40 mM Tris-HCl buffer pH 7.4 and incubating at 25°C. Aliquots were removed at different time intervals and added to the assay mixture. The mixture was rapidly mixed and the change in the absorbance at 340 nm was measured. A short assay time (*ca.* 1 min) was employed to minimize the possible change in initial velocity resulting from reactivation during the assay period.

The fluorescence change during reactivation was monitored at room temperature. The urea-denatured enzyme was directly diluted into the cuvette followed by a rapid mixing (*ca.* 10 sec). The fluorescence was measured in the above Tris-HCl buffer containing 6 M urea and corrected for the fluorescence of the urea. The fluorescence of the native enzyme was also determined in the buffer lacking urea. The excitation wavelength was 280 nm and the emission measured at 320-420 nm.

Kinetic Studies

Kinetic constants for the native and renatured enzymes were determined in 40 mM Tris-HCl buffer pH 7.4 for NADP⁺-linked and in 50 mM Imidiazol-HCl buffer pH 6.6 for NAD⁺-linked reactions. Initial velocities were measured at four concentrations of each of coenzymes and substrate. The range of concentrations was 0.125-1 mM NADP⁺, 0.25-2 mM NAD⁺ and 1-5 mM glucose 6-phosphate. The lines for double-reciprocal plots and replots were drawn using linear regression analysis. The kinetic constants were evaluated from the replots.

Modification of Histidine Residues during Reactivation

The accessibility of histidine residues during the period of reactivation was demonstrated with diethylpyrocarbonate (DEPC). The number of modified histidine residues was measured using a molar extinction coefficient for *N*-carbethoxyimidazole of $\epsilon = 3.2 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 240 nm [15]. Urea-denatured enzyme was rapidly diluted (1 to 60) to start reactivation. At each time point 0.4 ml was taken and 10 μM of DEPC (0.138 M diluted in ethanol) was added and mixed. The reaction was allowed to proceed at 25°C for 10 min, and the increase in absorbance at 240 nm resulting from formation of *N*-carbethoxyimidazole residues was recorded. Control experiments were performed by omitting protein or adding ethanol instead of DEPC. In a similar experiment the activity of the enzyme during the renaturation period was measured.

Results

Enzyme Purification

Purification steps are summarized in Table 1. More than 98% of the enzyme activity appeared in 60% ammonium sulfate precipitate. In DEAE-Cellulose

chromatography enzyme activity appeared in only one peak (fraction 31-43) (Fig. 1). In the last step, on a gel filtration sephadex G-100 column the enzyme was eluted as a single peak (Fig. 2) and another 150-fold purification was obtained having specific activity of 2.1 U/mg protein. The purified enzyme showed only a single band on SDS-PAGE with a M.W. of about 50,000 Da (data not shown). Activity staining of the enzyme on polyacrylamide gel electrophoresis showed one major and two minor bands (data not shown). It is probable that in addition to the active dimer, other forms such as trimer or tetramer also exhibit minor catalytic activity.

Enzyme Inactivation and Dissociation

Inactivation was performed by incubation of enzyme with various concentrations of urea in 40 mM Tris-HCl buffer pH 7.4 (Fig. 3). Complete inactivation was achieved by 6 M urea after about 70 min incubation at 25°C. The denatured enzyme displayed a decrease of about 30% in intrinsic protein fluorescence and a red shift of about 10 nm in the maximum of fluorescence emission spectrum. The corrected fluorescence emission spectra of the denatured, renatured and native enzymes are shown in Figure 4. The molecular weight of urea-denatured enzyme, estimated by polyacrylamide gel electrophoresis in the presence of 6 M urea (see Methods), was $49,000 \pm 1,500$ Da corresponding to that of the single subunit obtained from SDS-PAGE experiment of the native enzyme. No catalytic activity remained in the denatured enzyme since, after direct dilution into a cuvette containing assay mixture, no catalytic reaction took place for about 4 min. These experiments provide evidence that complete denaturation and dissociation of this enzyme is achieved in 6 M urea.

Renaturation and Reactivation

Immediately after urea-denatured enzyme was diluted in renaturation buffer, the regain of the intrinsic protein fluorescence took place and no more fluorescence change was detected (measured for 2 h). Regain of the enzyme activity after about 2 h was about 50% of the original native enzyme (Fig. 5).

The reactivation process was also monitored by measuring the accessibility of histidine residues toward DEPC modification (Fig. 5). Immediately after the denatured enzyme was diluted to start reactivation, a total of 8 M of histidine residue/mol of enzyme dimer was accessible to this modification. As the reactivation proceeded, less histidine residues were able to react with DEPC. After about 140 min of incubation, when

53% of the enzymatic activity was recovered, only 2 M of histidine/mol of enzyme could be modified with DEPC. Figure 5 illustrates that burying of histidine residues parallels reactivation.

Since both native and reactivated enzymes are dimers (see below), it is suggested that reactivation step involves both dimerization and a conformational change not detected by a fluorescence change

Effects of Substrates on Reactivation

The influence of substrate on the rate of reconstitution was examined by inclusion of the substrates in the renaturation mixture either at zero time or after initiation of reactivation. Addition of NAD^+ and NADP^+ at 2 mM (~ 10 Km) and 1 mM (~ 12 Km), respectively, to the reactivation mixture did not affect the rate of reactivation. NAD^+ at 10 mM (~ 50 Km) stimulated reactivation when either one was present at zero time and also when it was added after initiation of reactivation (Fig. 6). NADP^+ , however, at concentration of 4 mM (~ 50 Km) did not affect the reactivation rate, but at much higher concentration (~ 100 Km) sharply stimulated the reactivation rate (Fig. 7). Glucose 6-phosphate also at 10 mM (5 Km) concentration stimulated the reactivation rate (data not shown). Increase in the buffer concentration did not influence the reactivation rate, indicating that the rate is not affected by ionic strength.

Characterization of Reactivated and Renatured Enzymes

Kinetic and physicochemical characteristics of the enzyme in its native, denatured and renatured states were compared (Table 2). Renatured and native enzymes both eluted with an identical elution volume from a gel filtration column. Calibration of this column revealed a molecular weight of 107,000 Da for both renatured and native enzymes. A molecular weight of 49,000 Da was obtained for the denatured enzyme using polyacrylamide electrophoresis in the presence of 6-M urea. Renatured enzyme exhibited essentially the same Km values for NAD^+ , NADP^+ and glucose 6-phosphate as those of the native enzyme. The same ratio of maximum velocities of the NAD^+ - and NADP^+ -linked reactions was also obtained for both enzymes.

The mobility of renatured enzyme on polyacrylamide gel electrophoresis was the same of that of the native enzyme. Recovery of intrinsic fluorescence upon renaturation was 93% with a maximum emission fluorescence at 345 nm. The data, therefore, indicate that the renatured enzyme is indistinguishable from the fully active enzyme in its native state.

Discussion

Studies concerning structure-function relationship of enzymes require purified protein. G6PD from *Streptomyces aureofaciens* was purified 150-fold and showed a single band on polyacrylamide gel. Other workers have reported a 10-fold purification for this enzyme [4]. Determination of molecular weight of the enzyme using SDS-PAGE and by gel filtration chromatography revealed that *S. aureofaciens* G6PD is a dimer of identical subunits (Table 2). Several other dehydrogenases such as *L. mesenteroides* G6PD [21] and *Pseudomonas c.* G6PD [22] have also similar structure.

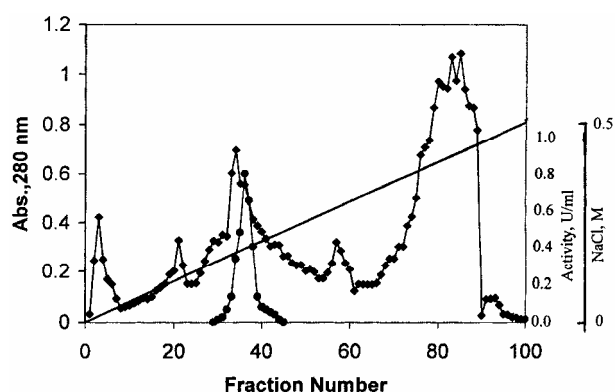


Figure 1. Elution profile of ion exchange chromatography of *S. aureofaciens* G6PD on DEAE-cellulose column. Enzyme activity (●) and protein as detected by A_{280} (◆) were eluted with a linear gradient of NaCl from 0 to 0.5 M in the equilibrating buffer. For details see text.

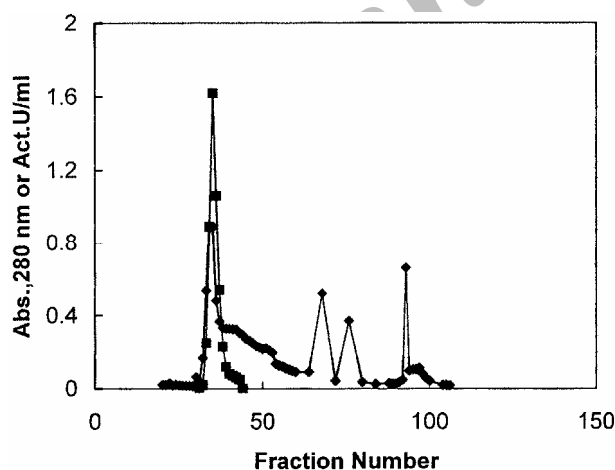


Figure 2. Elution profile of gel filtration chromatography of *S. aureofaciens* G6PD on Sephadex G-100 column. The enzyme activity (■) and protein as detected by A_{280} (◆) were eluted as described in the methods.

Appearance of 3 active bands of *S. aureofaciens* G6PD on polyacrylamide gel, one major and 2 minor bands, suggested that either other polymeric forms exhibit slight activity or isomers of dimeric enzyme are formed on the gel. Rat mammary gland G6PD shows 3 isomers of the dimeric form on polyacrylamide gel [23].

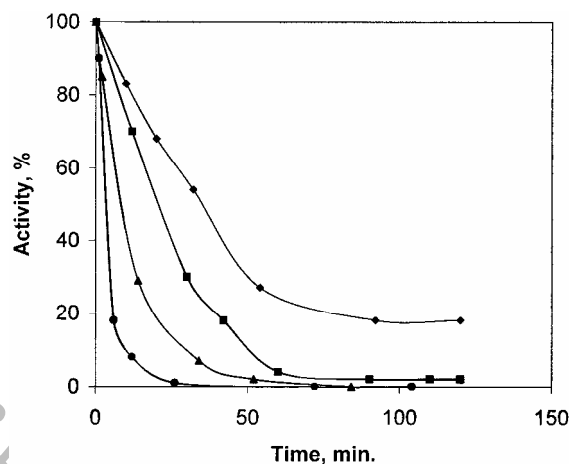


Figure 3. Denaturation of G6PD in different concentrations of urea. Enzyme (0.6 mg/ml) was incubated at 2 M (◆), 4 M (■), 6 M (▲) and 8 M (●) urea at room temperature and the enzyme activity measured at the indicated time intervals. Values represent the average of two independent experiments.

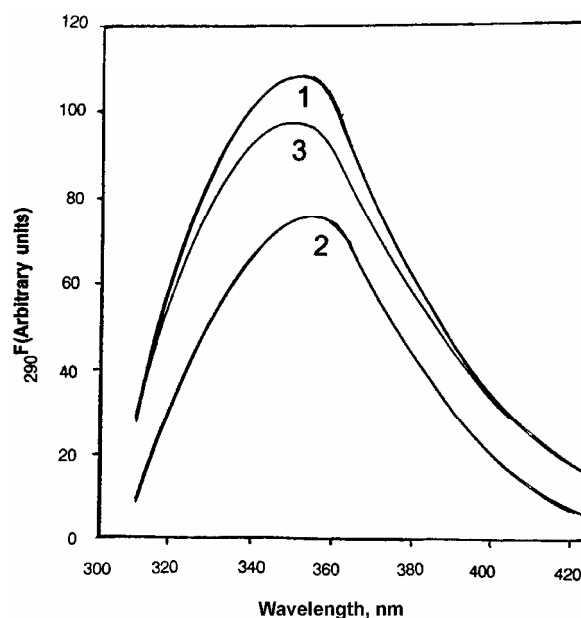


Figure 4. Protein fluorescence emission spectra of native (1), denatured (2) and renatured G6PD (3). The spectra of 2 and 3 were corrected for fluorescence of the corresponding urea concentrations. For detail see text. Similar spectra were obtained from several independent experiments.

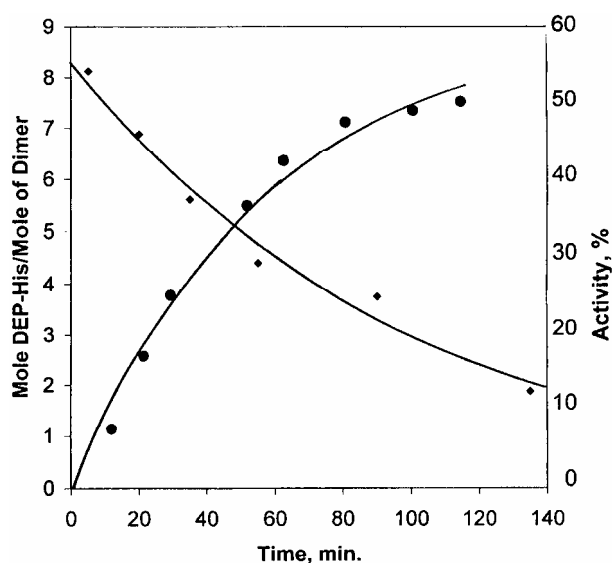


Figure 5. Accessibility of histidine residues to DEPC modification during reactivation of urea-denatured G6PD. (■), histidine residues modified as a function of time; (●), percent reactivation. Protein concentration was 500 nM. Values represent the average of two independent experiments.

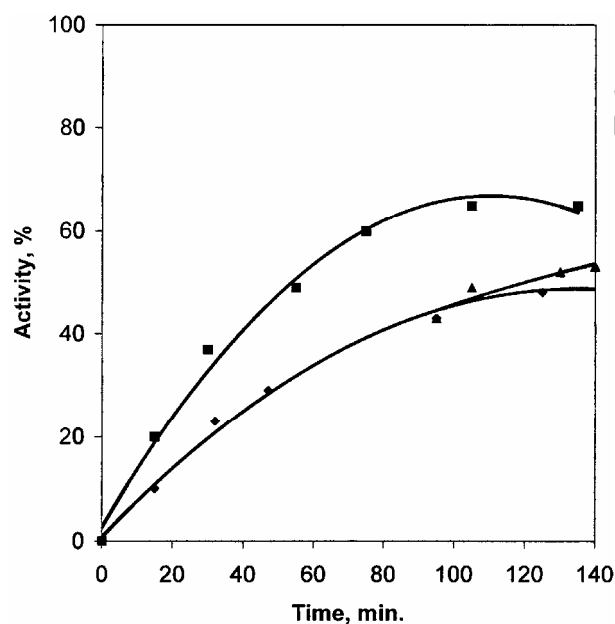


Figure 6. Reactivation of urea-denatured G6PD in the presence and absence of NAD^+ . NAD^+ was included in the renaturation buffer as indicated. (♦), No NAD^+ present; (■), NAD^+ was present at the zero time; (▲), NAD^+ was added at the time shown by arrow. Enzyme concentration was 15 $\mu\text{g}/\text{ml}$ and NAD^+ concentration was 10 mM. Values represent the average of two independent experiments.

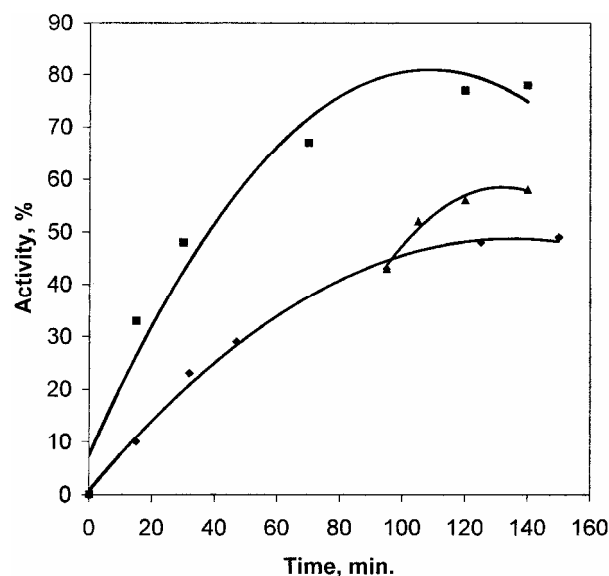


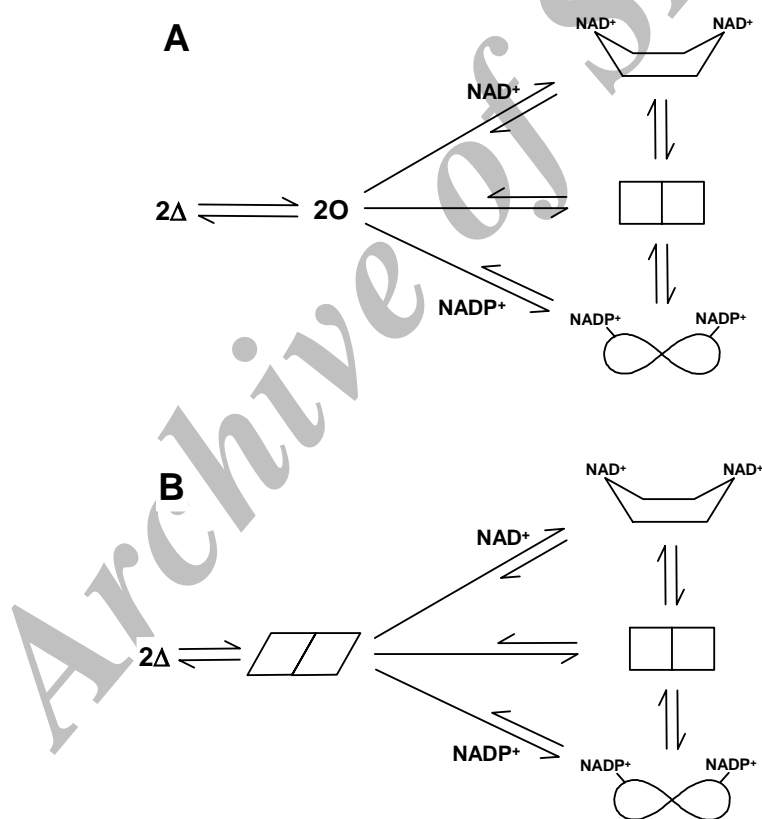
Figure 7. Reactivation of urea-denatured G6PD in the presence and absence of NAD^+ . Symbols and concentrations are the same as in Figure 4. Values represent the average of two independent experiments.

Kinetic studies of the *S. aureofaciens* G6PD showed K_m values of 0.19 mM and 0.08 mM for NAD^+ and NADP^+ , respectively (Table 2). Neuzil *et al.* [4] have also reported a K_m value for NAD^+ which was 3 times greater than that of NADP^+ using partially purified enzyme. For other dual nucleotide specific G6PDs such as those of *L. mesenteroides* [21] and *Pseudomonas c.* [22] the $K_m^{\text{NAD}^+}/K_m^{\text{NADP}^+}$ ratios of 18 and 13 have been reported, respectively. If NAD^+ and NADP^+ bind the same site on *S. aureofaciens* G6PD, as it occurs in *L. mesenteroides* enzyme [6], the difference observed in K_m values of the coenzymes suggests two different conformational forms of the enzyme for coenzyme binding. The successful application of kinetic methods for denaturation and reactivation studies requires to demonstrate that the native and reconstituted proteins are identical and that denaturation is reversible [16]. G6PD from *S. aureofaciens* was completely dissociated in 6 M urea (Fig. 3). The decrease in intrinsic fluorescence and the red shift in fluorescence maximum of the dissociated enzyme showed that a gross conformational change had occurred (Fig. 4). The final state was described by the homogenous, inactive and probably completely unfolded monomers. We have also previously shown that G6PD from *L. mesenteroides* displayed a shift in the fluorescence maximum from 330 to 350 nm and a large decrease in fluorescence intensity upon denaturation in 8 M urea [6]. It seems likely that tryptophan-containing enzymes share these features in

Table 2. Characteristics of G6PD at its native, denatured and renatured states

	Native	Renatured	Denatured
Molecular weight (Da)	107,100	107,600	49,000
K _m for coenzyme:			
<i>NAD</i> ⁺ (mM)	0.188	0.139	
<i>NADP</i> ⁺ (mM)	0.076	0.070	
K _m for glucose 6-phosphate:			
<i>NAD</i> ⁺ -Linked (mM)	0.84	1.08	
<i>NADP</i> ⁺ -Linked (mM)	0.77	0.95	
<i>V</i> _{NAD⁺} / <i>V</i> _{NADP⁺}	1.66	1.70	
<i>R</i> _m on polyacrylamide gel:			
Stained for activity	0.23	0.23	
Stained for protein	0.35	0.34	
Intrinsic fluorescence:			
<i>F</i> _{rel} 330 nm (%)	100	93	71
<i>λ</i> _{max} (nm)	345	345	355

The values represent the average of two independent experiments

**Figure 8.** Two possible models (A and B) for reconstitution of urea-denatured G6PD. The completely unfolded urea-denatured monomers are shown at the extreme left.

common. Teipel & Koshland [17] have shown that the fluorescence spectrum of a mixture of the *N*-acetyl

esters of *L*-tryptophanamide, *L*-tyrosinamide and *L*-phenylalaninamide resembles that of the guanidine

hydrochloride denatured forms of five oligomeric enzymes, with an emission maximum around 350 nm. Other studies have shown similar results [18-19]. The fluorescence spectra of the model compounds showed a blue shift when the dielectric constant of the solvent was decreased [20]. This finding suggests that upon enzyme refolding, tryptophan residues are transferred from a hydrophilic to a hydrophobic environment. Our previous work revealed that a solution of glycytryptophan exhibits spectral characteristics of those of urea-denatured *L. mesenteroides* G6PD, under the same conditions with an emission maximum of 355 nm [6]. It is, therefore, conceivable that the aromatic residues of *Streptomyces aureofaciens* G6PD in 6 M urea are also fully exposed to the solvent, indicating that the enzyme is extensively unfolded.

Kinetic and physical characteristics of the native and reassociated enzymes show that they are indistinguishable from each other (Table 2). The transition from denatured to native enzyme was accompanied by an increase in maximum fluorescence intensity and a blue shift in the emission spectrum, both in the direction of the native enzyme (Fig. 3). Since the regain of the native-fluorescence occurs within a few seconds at 25°C before reactivation started, it is probable that reactivation takes place in at least two steps. First, fast refolding or dimerization of the subunits and then, slow refolding of the dimeric form or dimerization of partially folded monomers and regaining of the native structure which parallels appearance of the enzyme activity. The latter was monitored by the test for accessibility of histidine residues to DEPC modification (Fig. 5). This experiment proved that the initial fast refolding and/or dimerization generate a partially organized structure that results in burying most of the histidine residues. Further structure formation then occurs as judged by decreased accessibility of histidine residues with incubation time. These results differ from the results with mitochondrial malate dehydrogenase, which showed that fluorescence changes paralleled reactivation but the subunit reassociation preceded these changes [25,26].

The yield of reactivation in the present study was 85%, in the presence of NADP⁺, probably due to instability of denatured and dissociated enzyme.

Reactivation of the denatured enzyme was stimulated by the presence of coenzymes (Figs. 6-7). Reactivation of several enzymes is affected by the presence of specific ligands [6,27]. Such ligands may stimulate the reactivation rate either by affecting the rate limiting step in the overall process or by stabilizing the end or intermediate products in the correct folding pathway. Addition of 10 mM NAD⁺ (50 Km) and glucose 6-

phosphate (6 Km) to the renaturation mixture significantly stimulated the reactivation rate (Fig. 6), whereas NADP⁺ at the same 50 Km (1 mM) did not. At 10 mM NADP⁺, however, the reactivation rate was sharply increased (Fig. 7). Therefore, these compounds do not exert the effects by their relative affinities to the native enzyme, and they do not pull the equilibrium along the reactivation pathway toward the native structure. Rather, it appears likely that these compounds bind to an inactive form of the enzyme such as incompletely refolded monomer or dimer leading to a different pathway of refolding. Since the presence of each coenzyme in the reactivation mixture at zero time stimulated the reactivation rate greater than its presence 1.5 h after initiation of reactivation, it is likely that the ligands bind preferably to the unfolded structures.

The differential effects of NAD⁺ and NADP⁺ on the rate of inactivation suggest different conformations for the binary complex of *S. aureofaciens* G6PD with NAD⁺ and NADP⁺ bind to two different conformation of the native enzyme [28]. It is, therefore, postulated that the unfolded monomers first refold to either folded monomers or to inactive partially structured dimers. Subsequently dimerization of the folded monomer or transition of the inactive dimer to the active dimer occurs. Alternative pathways for reactivation from either folded monomer or inactive dimer may be proposed, depending upon the kind of ligand present in the reactivation mixture. A plausible model for subunit reassociation based on the present findings is presented in Figure 8. Further studies are needed to obtain the kinetic constants of the individual steps and the rate-limiting step in the reconstitution pathway. The data also are consistent with the idea that NAD⁺ and NADP⁺ bind to different conformational isomers of dual nucleotide dehydrogenases as has been found for *L. mesenteroides* G6PD [1-3,6].

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