Glucose-6-phosphate Dehydrogenase from *Leuconostoc mesenteroides*. Kinetics of Reassociation and Reactivation from Inactive Subunits†

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ABSTRACT: Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* is denatured in 8 M urea and dissociated into its two inactive subunits. Denaturation leads to an approximately 80% decrease in protein fluorescence and a 20-nm red shift in the emission maximum. Upon dilution, the urea-treated enzyme regains catalytic activity (approximately 70%). The reactivated enzyme is indistinguishable from the native enzyme based on a number of physicochemical and enzymological criteria. The kinetics of renaturation and reactivation were monitored by measuring the rates of regain of native fluorescence and appearance of activity and the accessibility of histidine residues toward diethyl pyrocarbonate modification. Regain of native fluorescence was too rapid to measure at 25 °C; at 5 °C the initial phase was also too rapid, but a slower phase was monitored and shown to obey first-order kinetics with $k = (5.9 \pm 1.3) \times 10^{-3}$ s^{-1}. Reappearance of activity was measured at several protein concentrations; reactivation followed second-order kinetics with $k = (4.85 \pm 0.47) \times 10^{-3}$ M^{-1} min^{-1}. Reactivation was stimulated to different degrees by either the initial or delayed addition of NAD$^+$, NADP$^+$, or glucose 6-phosphate. During the initial, rapid phase of renaturation, approximately 3 of the enzyme's 12 histidine residues become unreactive toward diethyl pyrocarbonate; concomitant with the subsequent reactivation, approximately 7 more histidines become inaccessible to diethyl pyrocarbonate. The data are consistent with a model for enzyme renaturation and reactivation in which the unfolded subunits rapidly refold to an inactive structure that can dimerize slowly to generate native enzyme. Specific ligands stimulate reactivation by binding to refolded subunits or incompletely folded dimers.

Many oligomeric proteins that have been denatured into their constituent subunits can reassemble spontaneously to their native state when the denaturant is removed (Jaenicke, 1978). Reactivation involves changes in the tertiary and quaternary structures of these proteins, and a variety of studies have been employed to elucidate kinetic and thermodynamic aspects of the overall process (Wetlaufer & Ristow, 1973; Creighton, 1978). The effects of the addition of substrates or coenzymes on the kinetics or reactivation have suggested that the correct folding of some oligomeric enzymes may be influenced in vivo by the presence of these ligands (Teipel & Koshland, 1971a,b).

Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* is a dimeric protein with a molecular weight of 103 700 (Olive & Levy, 1971) containing two apparently identical subunits (Haghighi et al., 1982) of molecular weight 54 800 (Ishaque et al., 1974; Kawaguchi & Bloch, 1974). The enzyme shows little or no tendency to form larger aggregates (Olive & Levy, 1971; Milhausen, 1974). Reactivity of *L. mesenteroides* glucose-6-phosphate dehydrogenase with NAD$^+$ and NADP$^+$ appears to involve two distinct conformational forms of the enzyme (Haghighi & Levy, 1982). Little is known about subunit interactions in this enzyme, except that these must differ from those in glucose-6-phosphate dehydrogenase of *Candida utilis* and *Saccharomces cerevisiae*, based on the resistance of the *L. mesenteroides* enzyme to inhibition and dissociation by palmitoyl-CoA (Kawaguchi & Bloch, 1974).

We describe here experiments in which *L. mesenteroides* glucose-6-phosphate dehydrogenase is inactivated in 8 M urea and then allowed to reactivate under various conditions. The kinetics of several stages in this process were monitored by measuring changes in activity, intrinsic fluorescence, and reactivity of histidine residues. The effects of adding substrates and coenzymes during reactivation were examined and are consistent with differences in enzyme conformation produced by these ligands as suggested by fluorescence studies (Haghighi & Levy, 1982).

Experimental Procedures

Materials

Glucose-6-phosphate dehydrogenase was purified to homogeneity from *L. mesenteroides* (ATCC 12291, American Type Culture Collection) as described previously (Haghighi et al., 1982). Glucose 6-phosphate, NAD$^+$, NADP$^+$, *N*-(2,4-dinitrophenyl)-L-phenylalanine, bovine serum albumin (BSA),[1] ovalbumin, yeast alcohol dehydrogenase, and catalase were obtained from Sigma Chemical Co.; ultrapure urea was a product of Schwarz/Mann, Inc.; chymotrypsinogen A came from Pharmacia Fine Chemicals and ADP-ribose from P-L Biochemicals Inc. All other chemicals were reagent grade. Most reagent solutions were prepared daily and brought to the appropriate pH before use. All solutions used for fluorescence measurements, except for the enzyme, were filtered through a 0.45-μm membrane filter (Millipore) before use.

Methods

Standard assays of glucose-6-phosphate dehydrogenase were performed in a Gilford 240 spectrophotometer with a thermostated cell compartment maintained at 25 °C, attached to a Gilford 6051 recorder as described previously (Haghighi et al., 1982). The assay mixtures were brought to 25 °C prior

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[1] Abbreviations: DEP, diethyl pyrocarbonate; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane. For designation of fluorescence excitation and emission wavelengths, see Haghighi & Levy (1982).

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to each assay. Fluorescence measurements were carried out with a Perkin-Elmer MPF-3L fluorescence spectrophotometer attached to a Leeds & Northup Speedomax XL 620 series flatbed recorder. The temperature of the cell compartment was controlled with a Forma constant temperature bath and circulator and remained at 25 °C, unless otherwise noted. Protein concentration was determined by a modification of the biuret method outlined by Zamenhof (1957).

Denaturation. Denaturation was performed by incubating the enzyme in 33 mM Tris-HCl, pH 7.8, containing 8 M urea. The mixture was gently stirred for 2 h at room temperature.

Reactivation. The urea-denatured enzyme was reactivated by diluting rapidly at least 100-fold in 33 mM Tris-HCl, pH 7.8, and incubated at 25 °C, unless otherwise specified. Aliquots were removed at different time intervals and added to the assay mixture. After rapid mixing, the rate of change in absorbance at 340 nm was measured. A short assay time (ca. 1 min) was employed to minimize any possible change in initial velocity resulting from reactivation during the assay period.

The fluorescence change during the fast refolding step was measured at 5 °C. The urea-denatured enzyme was directly diluted into the cuvette followed by a mixing period lasting 8–10 s. Immediately afterward the fluorescence change was recorded. The fluorescence of denatured enzyme was determined in the buffer containing 8 M urea and corrected for fluorescence of the urea. The excitation wavelength was 290 nm, and the fluorescence was measured at either 330 or 350 nm. Both excitation and emission sites were set at 8 nm.

Modification of Histidine Residues during Reactivation. The accessiblity of histidine residues to modification within the period of reactivation was demonstrated by reaction with DEP. The extent of reactivation was quantitated, using a molar extinction coefficient for *N*-carbethoxyimidazole of $\epsilon = 3.2 \times 10^3\ M^{-1}\ cm^{-1}$ at 240 nm (Muhlrad et al., 1969). Urea-denatured enzyme was rapidly diluted (1 to 60) to start renaturation. At each time point, 0.8 mL was taken, and 8 μL of DEP (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 DEP. In a similar experiment the appearance of enzyme activity was monitored with time.

Kinetics. Kinetic constants for the renatured enzyme were determined in Tris-HCl, pH 7.8. Initial velocities were measured at five concentrations each of coenzyme and substrate in the Gilford spectrophotometer by using cells of 1- and 5-cm light path for the NAD- and NADP-linked reactions, respectively. The range of concentrations was 47.8–478 μM NAD^+, 2.67–18.7 μM $NADP^+$, and 25–200 μM glucose 6-phosphate. The lines for double-reciprocal plots were drawn by using a computer program based on the direct linear plot (Eisenthal & Cornish-Bowden, 1974; Cornish-Bowden & Eisenthal, 1978) and the kinetic constants evaluated from replots by using linear regression analysis.

Gel Electrophoresis and Molecular Weight Determination. Disc gel electrophoresis was performed according to the method of Gabriel (1971). The molecular weight of urea-denatured enzyme was determined by the procedure of Hedrick & Smith (1968) except that 8 M urea was included in the cathodic buffer as well as in the gels. A standard curve was generated with ovalbumin, chymotrypsinogen A, myoglobin, and BSA. The molecular weights of native and renatured enzyme were determined by gel filtration on a column of Bio-Gel A-0.5m (1.5 × 70 cm) equilibrated with 66 mM Tris-HCl, pH 7.8. The column was calibrated with ovalbumin, BSA, yeast alcohol dehydrogenase, catalase, and native glucose-6-phosphate dehydrogenase from *L. mesenteroides*.

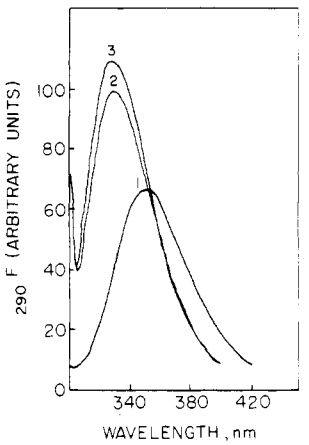

FIGURE 1: Protein fluorescence emission spectra of native (3), denatured (1), and renatured (2) glucose-6-phosphate dehydrogenase. The spectrum of denatured enzyme is not corrected for the fluorescence due to 8 M urea (see Experimental Procedures). The enzyme concentration was 295 nM. For explanation of fluorescence symbols in this figure and Figure 3, see footnote 1.

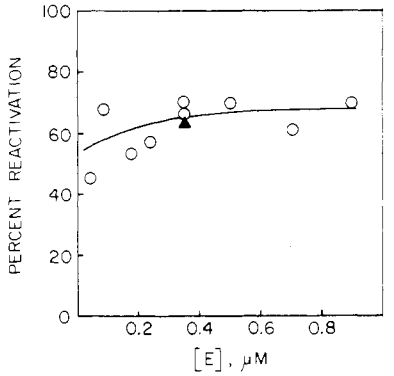

FIGURE 2: Effect of enzyme concentration (E) on the extent of reactivation of glucose-6-phosphate dehydrogenase after denaturation with 8 M urea. Reactivation time was extended until no more change in the activity was observed. (○) Reactivation in absence of ligands; (▲) reactivation in the presence of NAD^+, $NADP^+$, or glucose 6-phosphate.

Results

Enzyme Inactivation and Dissociation. Inactivation was achieved by incubation of enzyme with 8 M urea in 33 mM Tris-HCl, pH 7.8, as described under Experimental Procedures. The denatured enzyme displayed a decrease of about 80% in intrinsic protein fluorescence and a red shift of 20 nm in the maximum of the fluorescence emission spectrum. The uncorrected fluorescence emisson spectra of the denatured, renatured, and native enzymes are shown in Figure 1. The molecular weight of urea-denatured enzyme, estimated by the method of Hedrick & Smith (1968) in the presence of 8 M urea (see Methods), was 50000 ± 2000, corresponding to that of the single subunit reported by Ishaque et al. (1974). 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 3 min. These experiments provide evidence that the enzyme is denatured and completely dissociated to its subunits in 8 M urea.

Yield of Reactivation. As shown in Figure 2, in the concentration range 0.35–0.9 μM, the yield of reactivation has a constant value of around 70%. At concentrations less than

Table I: Comparison of *L. mesenteroides* Glucose-6-phosphate Dehydrogenase in Its Native, Renatured, and Denatured States

	native	renatured	denatured
molecular weight	103 700[a]	104 000	50 000[b]
K_m for coenzyme			
NAD^+ (μM)	106[a]	102	
$NADP^+$ (μM)	5.7[a]	4.7	
K_m for glucose 6-phosphate			
NAD linked (μM)	52.7[a]	78	
NADP linked (μM)	81[a]	89.9	
V_{NAD^+}/V_{NADP^+}	1.82[a]	1.71	
R_m on polyacrylamide gel			
stained for activity	0.437	0.433	
stained for protein	0.443	0.432	
intrinsic fluorescence			
F_{rel} 330 nm (%)	100	95	22[c]
λ_{max} (nm)	330	330	350

[a] From Olive & Levy (1971). [b] Measured in 8 M urea. [c] Calculated from data in Figure 1, corrected for urea fluorescence.

0.3 μM, the yield decreased. Similar results have been reported for lactate dehydrogenase (Rudolph & Jaenicke, 1976). Reactivation was allowed to proceed until no more change in the activity appeared (usually 2–3 days), although the major regain of activity occurred much faster at high enzyme concentration.

Characterization of Reactivated and Renatured Enzyme. A comparison of *L. mesenteroides* glucose-6-phosphate dehydrogenase in its native, denatured and renatured states using kinetic and physicochemical criteria is shown in Table I. The renatured enzyme was eluted from a gel filtration column with an elution volume identical with that of the native form. Calibration of this column revealed a molecular weight of 104 000 for both renatured and native enzymes. The mobility of renatured enzyme on polyacrylamide gels was in excellent agreement with that of native enzyme. Recovery of intrinsic protein fluorescence was 95% with a maximum emission fluorescence at 330 nm. Renatured enzyme also exhibited essentially the same ratio of maximum velocities of the NAD- and NADP-linked reactions and K_m values for NAD^+, $NADP^+$, and glucose 6-phosphate as those of the native enzyme. These observations demonstrate that the final product of renaturation is indistinguishable from the fully active enzyme in its native state. Therefore, the reactivation kinetics can be used to study the correlation between refolding, association, and catalytic function.

Renaturation. Regain of the intrinsic protein fluorescence took place within a few seconds after addition of urea-denatured enzyme to the renaturation buffer at 25 °C, and no more fluorescence change was detected (measured for 3 h). This fluorescence change was monitored at 5 °C where refolding was relatively slow. Immediately after addition of the urea-denatured enzyme to renaturation buffer, a very fast increase in protein fluorescence occurs within about 20 s, followed by yet another rapid phase lasting for 3–4 min. No further change in the fluorescence was detected (measured for 3–4 h). Here again, the analysis of the very fast phase was not possible, but the slower phase was analyzed according to the first-order equation (Figure 3). The rate of this phase of fluorescence change was independent of protein concentration. A first-order rate constant of $(5.9 \pm 1.3) \times 10^{-3}$ s^{-1} was calculated from the data. Similar first-order rate constants for the fast refolding step were reported for creatine kinase (Grossman et al., 1981) and several dehydrogenases (Jaenicke, 1978). Since the rate-limiting reactivation step was shown to be a bimolecular reaction (see below), both refolding steps described here must be unimolecular processes.

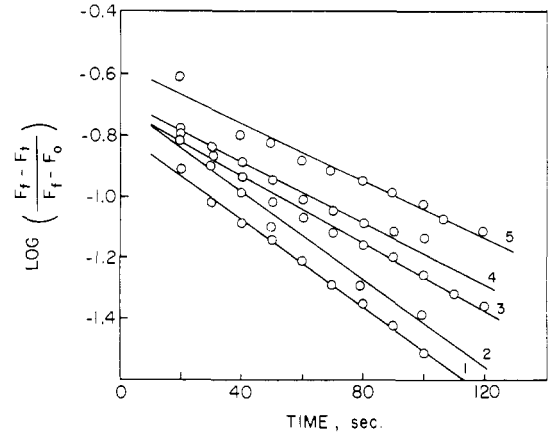

FIGURE 3: First-order kinetics of increase in protein fluorescence during renaturation of urea-denatured glucose-6-phosphate dehydrogenase. Enzyme concentrations were 500 (1), 59 (2), 900 (3), 700 (4), and 175 (5) nM. F_0, F_t, and F_f represent fluoresence at times zero, t, and final, respectively.

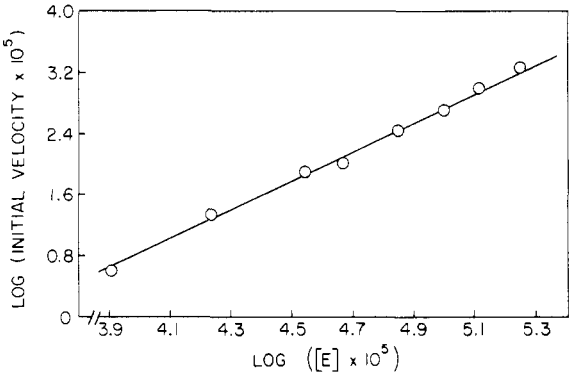

FIGURE 4: Second-order kinetics of reactivation of glucose-6-phosphate dehydrogenase after inactivation in 8 M urea. Inactivation and reactivation were done at 25 °C at the following enzyme concentrations (nM): 40.4, 88, 177, 235, 354, 500, 707, and 900.

Reactivation. Urea-denatured enzyme wad diluted in renaturation buffer and incubated at 25 °C. The appearance of enzymic activity, followed by sampling aliquots as defined time intervals, showed no sigmoidicity, indicating that the reaction can be described by a single, rate-limiting step. The order of reactivation was calculated from a double logarithmic plot of initial velocities vs. enzyme concentration (Figure 4). The initial velocities were obtained from the tangents to reactivation profiles expressed as concentration of active dimer formed vs. time. A reaction order of $n = 1.92$ was determined, indicating the rate-limiting step to be a second-order reaction. The second-order rate constant, calculated from second-order plots, was $k = (4.85 \pm 0.47) \times 10^{-3}$ μM^{-1} min^{-1}.

The reactivation process was also monitored by measuring the accessibility of histidine residues toward DEP modification (Figure 5). A total of 9 mol of histidine residue/mol of enzyme dimer was accessible to this modification immediately after the denatured enzyme was diluted to start reactivation. As the reactivation proceeded, less histidine residues were able to react with DEP. After 137 min of incubation, when 65% of the enzymatic activity was recovered, only 2.4 mol of histidine/mol of enzyme could be modified with DEP. Figure 5 also illustrates that burying of histidine residues parallels dimerization, suggesting that the reactivation step involves both dimerization and probably a conformational change not detected by a fluorescence change.

Influence of Substrates on Refolding and Reassociation. For demonstration of whether coenzymes (NAD^+, $NADP^+$)

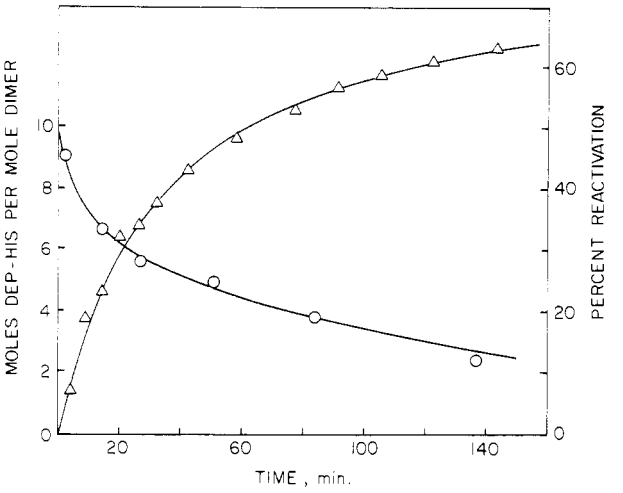

FIGURE 5: Accessibility of histidine residues to DEP modification during reactivation of urea-denatured glucose-6-phosphate dehydrogenase. (○) Histidine residues modified as a function of time; (△) percent reactivation. Protein concentration was 900 nM.

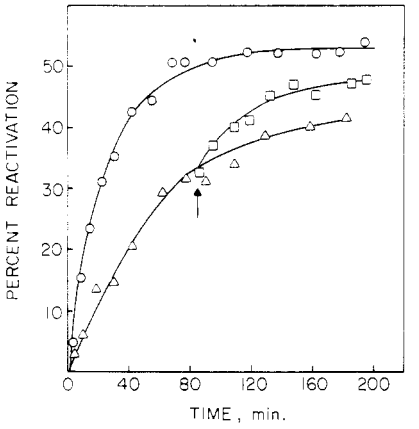

FIGURE 6: Comparison of the reactivation rate of urea-denatured glucose-6-phosphate dehydrogenase in the presence and absence of NAD^+. NAD^+ was included in the renaturation buffer as indicated. (○) NAD^+ was present at zero time; (□) NAD^+ was added at the time shown by the arrow; (△) no NAD^+ present. NAD^+ concentration was 21.4 mM, and enzyme concentration was 0.35 μM.

or glucose 6-phosphate might influence the rate of reconstitution, experiments were conducted in which each compound was added to the renaturation mixture either at zero time or after reactivation was initiated. Although there was some variability in the rate and extent of reactivation in these experiments, all the ligand effects were reproducible. The results of one such experiment are shown in Figure 6. Both 21.4 mM NAD^+ and 6.3 mM glucose 6-phosphate stimulated the rate of reactivation. Stimulation was observed when NAD^+ or glucose 6-phosphate was present at zero time and also when they were added after reactivation was initiated. $NADP^+$, at a concentration of 30 μM, did not influence the reactivation rate significantly, whereas at a concentration corresponding to that of NAD^+ (21.4 mM), a rapid and large rate enhancement was observed. The second-order rate constants calculated from these data were greater than that obtained in the absence of substrate or coenzyme. Galactose 6-phosphate, a substrate analogue, and ADP-ribose plus nicotinamide, coenzyme analogues, had little or no effect on the reactivation rate. Potassium phosphate did not change the rate, indicating that the reactivation rate is not influenced by ionic strength. A summary of all rate constants is presented in Table II.

Table II: Kinetic Constants of Reconstitution of *L. mesenteroides* Glucose-6-phosphate Dehydrogenase from Inactive Subunits

ligand present	ligand concn (mM)	kinetic constants[a] $k_2 \times 10^3$ (s^{-1})[b]	k_3[d] $\times 10^3$ (μM^{-1} min^{-1})
none	–	5.9 ± 1.3	4.9 ± 0.5
NAD^+	21.4	–[c]	13 ± 0.2
$NADP^+$	0.03	–	4.9
$NADP^+$	21.4	–	23.0
glucose 6-phosphate	6.3	–	8.9 ± 2.6
ADP-ribose + nicotinamide	21.4 (each)	–	4.1
galactose 6-phosphate	6.3	–	6.5

[a] For designation of rate constants see Figure 7. [b] Measured at 5 °C. [c] Not measured. [d] Includes values for k_3' and k_3''.

Stability of Renatured Enzyme. Enzyme renatured in the presence or absence of glucose 6-phosphate or coenzymes was allowed to incubate at room temperature, and enzymic activity was monitored. Enzyme renatured in the absence of any substrate or in the presence of 30 μM $NADP^+$ retained its activity over 100–120 h. The presence of 21.4 mM $NADP^+$ caused a slight decline in the activity. When NAD^+ (21.4 mM) or glucose 6-phosphate (6.3 mM) was present, the enzyme was less stable, and after 60–100 h it lost essentially all its activity. The loss in activity appeared to result from aggregate formation since the solution finally became turbid. These aggregates were removed by centrifugation, and the supernatant was concentrated by ultrafiltration using an Amicon PM10 membrane. Each sample was then subjected to polyacrylamide gel electrophoresis. Both protein and activity stains showed that there was essentially no protein or enzymic activity in samples incubated with NAD^+ or glucose 6-phosphate. However, the samples incubated with $NADP^+$ (high and low concentration) showed catalytic activity with R_m values corresponding to that of native enzyme.

Discussion

The successful application of kinetic methods for investigating the reactivation of denatured proteins necessitates the demonstration that the reconstituted and native proteins are identical and that the denaturation is reversible (Jaenicke, 1978). Complete dissociation of *L. mesenteroides* glucose-6-phosphate dehydrogenase was achieved in 8 M urea. The intrinsic fluorescence of the dissociated enzyme showed that a gross conformational change had occurred (Figure 1). The final state was fully described by the homogeneous, inactive, and probably completely unfolded monomers. The enzyme displays a shift in the fluorescence maximum from 330 to 350 nm and a large decrease in fluorescence intensity upon denaturation. It seems likely that tryptophan-containing enzymes share these features in common. Teipel & Koshland (1971b) showed 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. The fluorescence intensities of these enzymes were significantly diminished upon denaturation. A blue shift has been demonstrated for the spectra of model compounds when the dielectric constant of the solvent is decreased (Bell, 1981), suggesting that upon refolding, tryptophan residues are transferred from a hydrophilic to a hydrophobic environment. A solution of glycyltryptophan exhibits spectral characteristics similar to those

of urea-denatured glucose-6-phosphate dehydrogenase, under the same conditions with an emission maximum of 355 nm; the presence of 8 M urea does not alter these properties (data not shown). These observations suggest that the aromatic residues of *L. mesenteroides* glucose-6-phosphate dehydrogenase in 8 M urea are fully exposed to the solvent, indicating that the enzyme is extensively unfolded. Determination of the molecular weight of the denatured enzyme in 8 M urea revealed complete dissociation of enzyme dimer to its monomeric subunits (Table I).

According to several criteria, the reconstituted enzyme is indistinguishable from the native enzyme (Table I). The transition from the denatured to renatured state 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 (Figure 1). The regain of the native fluorescence occurs within a few seconds at 25 °C and within 2–3 min at 5 °C. This change appeared to take place in at least two stages. Analysis of the slower phase showed it to be a first-order process. The very fast fluorescence change could not be monitored but is likely to be first order as well, since the subsequent association of the subunits is the slow, rate-limiting step. This finding differs from those seen with other enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Krebs et al., 1979) and lactate dehydrogenase (Rudolph & Jaenicke, 1976) where a gross fluorescence change parallels the association reaction. It also differs from the results with mitochondrial malate dehydrogenase, which showed that fluorescence changes paralleled reactivation but the subunit reassociation preceded these changes (Wood et al., 1981). Reconstitution of creatine kinase revealed that the dimerization occurs after renaturation and reactivation are complete (Grossman et al., 1981).

The kinetic data show that reactivation follows a second-order reaction (Figure 4), suggesting that the active dimers formed in the rate-determining association reaction are responsible for the regain of enzymatic activity. This feature has also been reported for other dehydrogenases (Jaenicke, 1978). Obviously, under these circumstances the monomeric form of the enzyme cannot be catalytically active. Although, in the present case, dimerization was not accompanied by any detectable fluorescence change, the test for accessibility of histidine residues to DEP modification permitted monitoring structural changes associated with regain of the enzymatic activity (Figure 5). This experiment proved that the initial, fast refolding of subunits generates a partially organized structure that results in burying about 3 out of the 12 histidine residues originally present per mol of dimer (Ishaque et al., 1974). Presumably, most of the tryptophan residues are also buried in this rapid phase, as the original fluorescence was rapidly restored. Further structure formation then occurs upon dimerization of the subunits as judged by decreased accessibility of histidine residues with incubation time. It was also found that treatment of the native enzyme with DEP results in loss of enzymatic activity, suggesting the possible necessity of histidine residue(s) for activity (data not shown). A preliminary experiment showed that reactivation at pH 9.0 takes place much more slowly than at pH 7.6, suggesting that protonation of one or more ionizable groups promotes reactivation. Such a group(s) might be histidine; the enzyme lacks cysteine residue (Ishaque et al., 1974). Firm conclusions from these observations require further investigation.

The yield of reactivation appeared to be independent of the enzyme concentration, within a range of 0.35–0.9 μM. A slight decrease in yield at low enzyme concentration (below 0.3 μM) can be attributed to concentration-dependent inactivation and/or insufficient time for reactivation. The fact that the reactivation yield does not exceed 70% could be due to instability of denatured and dissociated enzyme. Formation of inactive aggregates did not occur since no decrease in yield at high enzyme concentration was observed.

Reconstitution of several oligomeric enzymes is affected by the presence of specific ligands (Rudolph & Jaenicke, 1976; Jaenicke et al., 1979; Krebs et al., 1979; Teipel & Koshland, 1971a,b). Such ligands may stimulate reactivation by accelerating the rate-limiting step in the overall process or by stabilizing the end product or intermediates in the correct folding pathway. Furthermore, refolding of some denatured proteins occurs only in the presence of a specific ligand. Liver alcohol dehydrogenase folds only in the presence of an optimum concentration of Zn^{2+} (Rudolph et al., 1978), and Deal (1969) reported an absolute requirement for NAD^+ for reactivation of glyceraldehyde-3-phosphate dehydrogenase. Although no such absolute requirement was found for *L. mesenteroides* glucose-6-phosphate dehydrogenase, addition of NAD^+, $NADP^+$, or glucose-6-phosphate, either at zero time or later in the refolding process, does stimulate the reactivation rate (e.g., Figure 6). These observations show that at least the rate-limiting dimerization is affected by these ligands but do not establish whether they also affect the fast steps in the refolding of monomers. Furthermore, the effects are specific, as compounds structurally similar to the substrate or coenzymes had little or no effect (Table II).

In seeking to explain the mechanism whereby substrate and coenzymes stimulate reactivation, one should note that, whereas 21.4 mM ($3K_D$) NAD^+ and 6.3 mM ($10K_D$) glucose 6-phosphate have a significant effect, 0.03 mM $NADP^+$ ($4.6K_D$) does not, although 21.4 mM $NADP^+$ does (Table II). Therefore, the effectiveness of these compounds is not dictated by their relative affinities to the native enzyme, and they do not stimulate primarily by pulling the equilibrium along the reactivation pathway toward the native structure. Rather, it appears more likely that they bind to an inactive form of the enzyme—either the refolded monomer with restored protein fluorescence or an incompletely folded dimer—leading to different pathways of refolding. Bartholmes & Jaenicke (1978) reported that refolded monomers of yeast glyceraldehyde-3-phosphate dehydrogenase are capable of binding NAD^+.

The differential effects of NAD^+, glucose 6-phosphate, and $NADP^+$ on the rate of enzyme reactivation as well as their differential effects on stability of renatured enzyme are consistent with the postulated differences in the conformations of the binary complexes of *L. mesenteroides* glucose-6-phosphate dehydrogenase with NAD^+, glucose 6-phosphate, and $NADP^+$ (Haghighi & Levy, 1982). A plausible model for subunit reassociation that takes these findings into account is presented in Figure 7. For a dimeric enzyme, the pathway for reconstitution requires one or more first-order isomerizations and one second-order dimerization step. In the present case, the data suggest that the unfolded monomers first refold to inactive monomers with fluorescence characteristics indistinguishable from those of the native dimer. A subsequent, slow dimerization then allows the formation of active dimer, indistinguishable from the native enzyme form. Alternative pathways for dimerization are proposed, depending upon the kind of ligand present in the reactivation buffer. For subunit refolding it was possible to monitor only the slower phase of the fluorescence change at 5 °C. Obviously, the rate constant obtained for this step at 5 °C will be different from that at

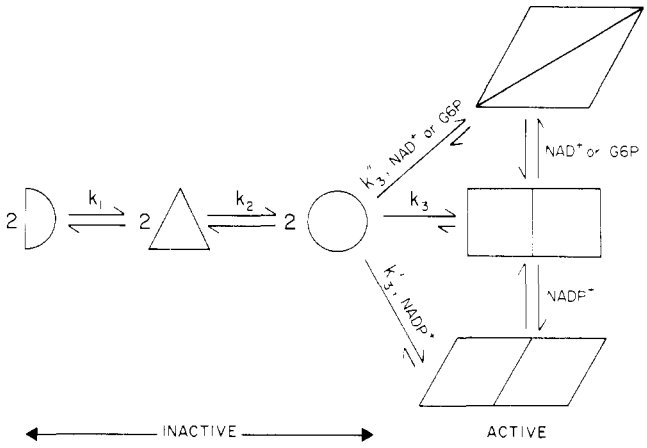

FIGURE 7: Model for reconstitution of urea-denatured glucose-6-phosphate dehydrogenase. The completely unfolded, urea-denatured monomers are shown at the extreme left. For rate constants see Table II.

25 °C. A comparison between the rate constants shows $k_2 > k_3' > k_3'' > k_3$.

Our results imply that in vivo assembly of *L. mesenteroides* glucose-6-phosphate dehydrogenase is not dependent on the presence of specific ligands but that the concentrations of glucose 6-phosphate, NAD^+, and $NADP^+$ in the cell may influence the rate of this process.

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Reversibility of Coated Vesicle Dissociation†

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ABSTRACT: The dissociation of the coated vesicles to clathrin and uncoated vesicles and their reassociation have been studied under various conditions. The extent of reassociation is pH dependent and increases slightly with increasing concentrations of the components. Unlike the self-association of clathrin which is strongly salt dependent, the reassociation of clathrin and uncoated vesicles is practically independent of salt concentration. The coated vesicle gradually loses its coat with increasing pH, and the dissociation process is not an all or none reaction. Ca^{2+} inhibits dissociation of the coated vesicles and enhances the reassociation of clathrin and uncoated vesicles. Our results show that, although many conditions result in reassociation of protein and lipid vesicle, few conditions result in vesicles of both the same size and composition as native coated vesicles.

Coated pits and vesicles are involved in receptor-mediated endocytosis (Goldstein et al., 1979; Ockleford & Whyte, 1977; Pearse, 1980), secretion of glycoproteins (Rothman et al., 1980), and membrane exchange (Heuser & Reese, 1973). Clathrin is readily dissociated from coated vesicles (CV)[1] by various methods which are mild enough not to cause denaturation, i.e., elevated pH, 2 M urea, and 0.50 M Tris (Pearse, 1975; Shook et al., 1979; Keen et al., 1979; Woodward &

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[1] Abbreviations: CV, coated vesicle(s); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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