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# Kinetic Aspects of Conformational Changes in Proteins. I. Rate of Regain of Enzyme Activity from Denatured Proteins*

John W. Teipel and D. E. Koshland, Jr.

ABSTRACT: The enzymes fumarase, enolase, aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase, denatured in 6 M guanidine hydrochloride, were renatured *in vitro* and the effects of environmental conditions on the kinetics of reactivation examined. The presence of substrate or cofactor was found to increase both the rate and final extent of reactivation for all six enzymes. Changes in the ionic strength and protein concentration of the renaturation medium also influenced the regain of biological activity, however, both the direction and magnitude of these effects varied from enzyme to enzyme. The rate of equilibration between the different conformational species formed during renaturation was relatively rapid for the enzymes fumarase, enolase, and aldolase, but very slow for glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase.

This result suggests that for the former group of enzymes the denatured protein refolds to final conformational state(s) which are determined solely by thermodynamic considerations, whereas for the latter group of enzymes the denatured protein refolds to metastable conformational state(s) specified by kinetic factors.

Sela, White, and Anfinsen (1957) and Anfinsen and Haber (1961) first demonstrated that reduced and denatured ribonuclease could be refolded to a conformational state possessing full biological activity. Since these pioneering studies, many other proteins have been shown to refold spontaneously from a denatured to the native state upon removal of the denaturing conditions (*cf.* review by Sund and Weber, 1966). These experiments provide convincing evidence that the information needed to specify the three-dimensional structure of a protein resides within its primary amino acid sequence.

Further study of this important phenomenon has inevitably raised questions about other facets of the folding problem. A recent investigation of the renaturation of a large number of enzymes, for example, revealed that the per cent regain of biological activity varied dramatically from enzyme to enzyme even when they were renatured under identical conditions (Cook and Koshland, 1969). Also, some enzymes when refolded in the presence of their substrates or cofactors yield a significantly higher level of enzymatic activity than that found when renaturation occurs in the absence of metabolite (Hill and Kanarek, 1964; Chilson *et al.*, 1965; Deal, 1969). These facts raise questions concerning the role of the environment in the refolding process and the extent to which refolding might be affected by specific metabolites in the media.

In addition several enzymes possessing the same amino acid sequence have been found to refold into different conformational forms. Thus, it has been suggested that different conformers of malic dehydrogenase have been found *in vivo* even though their primary covalent structures appear to be identical (Kitto *et al.*, 1966, 1970). Also lactic dehydrogenase (Chilson *et al.*, 1966), ribonuclease (Pflumm and Beychok, 1969), and creatine kinase (Dawson *et al.*, 1965), refolded *in vitro*, have been reported to regain biological activity but may possess different structural properties than their original native conformation. These observations have been contested by others (Schechter and Epstein, 1968; Mann and Vestling, 1968) but they raise questions as to whether a single conformational species is predetermined by a single sequence and, further, if such a species is so predetermined, whether kinetic or thermodynamic factors are more important in specifying the final conformational state.

An investigation of some of the kinetic aspects of protein renaturation was therefore undertaken in an effort to throw

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received June 5, 1970.* The authors acknowledge the support of the U. S. Public Health Service (Grant AM-09765) and the American Cancer Society.

light on the questions raised above and to gain further insight into the kinetic processes associated with conformational changes in general. The renaturation of six enzymes was examined—glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:DPN oxidoreductase (phosphorylating), EC 1.2.1.12), lactic dehydrogenase (L-lactate:DPN oxidoreductase, EC 1.1.1.2), malic dehydrogenase (L-malate:DPN oxidoreductase, EC 1.1.1.37), fumarase (fumarate hydratase, EC 4.2.1.2), enolase (phosphopyruvate hydratase, EC 4.2.1.11), and aldolase (fructose 1,6-diphosphate:D-glyceraldehyde 3-phosphate lyase, EC 4.1.2.). In each case the protein was denatured under conditions which produced loss of three-dimensional structure. The kinetics of the refolding process were then examined in relation to the regain of activity and the regain of three-dimensional structure. Moreover, the influence of environment on this process was examined. The results of the reactivation studies are discussed in this paper and the structural studies presented in the following paper (Teipel and Koshland, 1971).

## Experimental Section

*Enzymes.* All enzymes were obtained from commercial sources as crystalline suspensions in ammonium sulfate solution. Fumarase, lactic dehydrogenase, and malic dehydrogenase from pig heart, and enolase from rabbit muscle were purchased from Boehringer Mannheim Corp. Aldolase and glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle were obtained from Calbiochem. All were used without further purification.

*Reagents.* Oxalacetic acid, L-malic acid, and dithiothreitol were obtained from Calbiochem. Sodium pyruvate, fructose 1,6-diphosphate, and 2-phosphoglycerate were purchased from Boehringer Mannheim Corp. DPN and DPNH were obtained from Sigma Chemical Co. and guanidine hydrochloride (Ultra Pure) from Mann Research Laboratories. DL-Glyceraldehyde 3-phosphate was prepared from the barium salt of the diethyl acetal (Sigma) as described by Racker *et al.* (1959). All other chemicals were of the highest purity commercially available.

*Analytical Methods.* Absorbance measurements were made on a Gilford Model 2000 recording spectrophotometer, equipped with a constant-temperature circulating bath. The pH of all solutions was measured with a Radiometer type TTTlC titrator.

*Enzyme Solutions.* Solutions of native enzyme were routinely prepared by dissolving a pellet of the crystalline enzyme in 0.05 M Tris-acetate–$1 \times 10^{-3}$ M EDTA (pH 7.5). The solution was then centrifuged at 30,000*g* for 15 min to remove any undissolved material.

The concentration of enzyme solutions was determined spectrophotometrically at 280 mμ. The extincton coefficients ($E^{1\%}_{280\ m\mu}$) employed for these determinations were as follows: fumarase, 5.1 (Kanarek and Hill, 1964); enolase, 9.0 (Holt and Wold, 1961); malic dehydrogenase, 2.8 (Thorne, 1962); lactic dehydrogenase, 11.6 (Pfleiderer and Jeckel, 1957); glyceraldehyde phosphate dehydrogenase, 10.0 (Fox and Dandliker, 1956); and aldolase, 9.1 (Kabashi *et al.*, 1966).

*Enzyme Assays.* The catalytic activity of all enzymes was measured spectrophotometrically at 25°. A small aliquot (5–50 μg) of native or reactived enzyme was added to 1 ml of the assay mixture and the change in optical density monitored with time. The specific assay conditions employed for the individual enzymes are described below.

Fumarase was assayed in 0.02 M L-malate–0.01 M sodium phosphate (pH 7.3) in a modification of the procedure described by Kanarek and Hill (1964). The conversion of L-malate into fumarate was monitored at 240 mμ.

Enolase activity was measured in a solution containing $3 \times 10^{-3}$ M 2-phosphoglycerate, $1 \times 10^{-3}$ M $MgSO_4$, $1 \times 10^{-4}$ M EDTA, and 0.05 M Tris-chloride (pH 7.0) in a modification of the procedure of Malmström (1962). The conversion of 2-phosphoglycerate into phosphoenolpyruvate was monitored at 240 mμ.

Malic dehydrogenase was assayed in $1 \times 10^{-3}$ M oxalacetate, $2.5 \times 10^{-4}$ M DPNH, $1 \times 10^{-3}$ M EDTA, and 0.05 M Tris-acetate (pH 7.3). The oxidation of DPNH was monitored at 340 mμ.

Lactic dehydrogenase activity was measured in a solution containing $3.3 \times 10^{-4}$ M pyruvate, $2.5 \times 10^{-4}$ M DPNH, and 0.05 M sodium phosphate (pH 7.4)—a slight modification of the procedure described by Pesce *et al.* (1964). The oxidation of DPNH was monitored at 340 mμ.

The assay solution for glyceraldehydephosphate dehydrogenase contained $1 \times 10^{-3}$ M DL-glyceraldehyde 3-phosphate, $1 \times 10^{-3}$ M DPN, $1 \times 10^{-3}$ M EDTA, 0.01 M sodium arsenate, and 0.05 M sodium pyrophosphate (pH 8.5). The reduction of DPN was monitored at 340 mμ.

The activity of aldolase was measured by the hydrazine method as described by Jagannathan *et al.* (1956). Into 1 ml of solution containing $2.3 \times 10^{-3}$ M hydrazine sulfate, $1 \times 10^{-3}$ M EDTA, and 0.05 M Tris-acetate (pH 7.5), was added 40 μl of 0.1 M fructose 1,6-diphosphate. The increase in absorbance was monitored at 240 mμ. Aldolase was then added to the assay mixture and the change in absorbance at 240 mμ was again followed. The difference in rate between the aldolase catalyzed and control reactions was taken as a measure of aldolase activity.

*Denaturation.* Enzymes were denatured by incubation in a solution of 6 M guanidine hydrochloride, 0.01 M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.0) for 2 hr at room temperature. Enzyme–guanidine hydrochloride solutions were prepared either by directly dissolving a pellet of the crystalline enzyme in 6.5 M guanidine hydrochloride (a slight molar excess of the denaturant was employed to compensate for the small volume of ammonium sulfate solution still associated with the pellet) or diluting 4:1 a solution of the native enzyme into 8 M guanidine hydrochloride.

*Renaturation.* Enzymes denatured in guanidine hydrochloride were renatured by lowering the concentration of the denaturant either by dialysis or dilution.

In the dialysis procedure, 1 ml of the denatured enzyme was dialyzed against 125 ml of 0.05 M Tris-acetate, $1 \times 10^{-3}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) for 4 hr at room temperature. After dialysis was completed, the enzyme was assayed for regain in activity.

In the dilution procedure, 0.1 ml of the denatured enzyme was added slowly (over a period of 30 sec), with stirring, to 10 ml of buffer at 25° (101-fold dilution). The reactivation buffer contained 0.05 M Tris-acetate, 0.01 M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) in the presence or absence of specific metabolites (see Results). Aliquots were removed with time and assayed for enzymatic activity.

## Results

*Extent of Renaturation and Effect of Substrate.* Enzymes denatured in guanidine hydrochloride were renatured by diluting the enzyme–guanidine hydrochloride solution into an excess of buffer (see Experimental Section). At the low enzyme

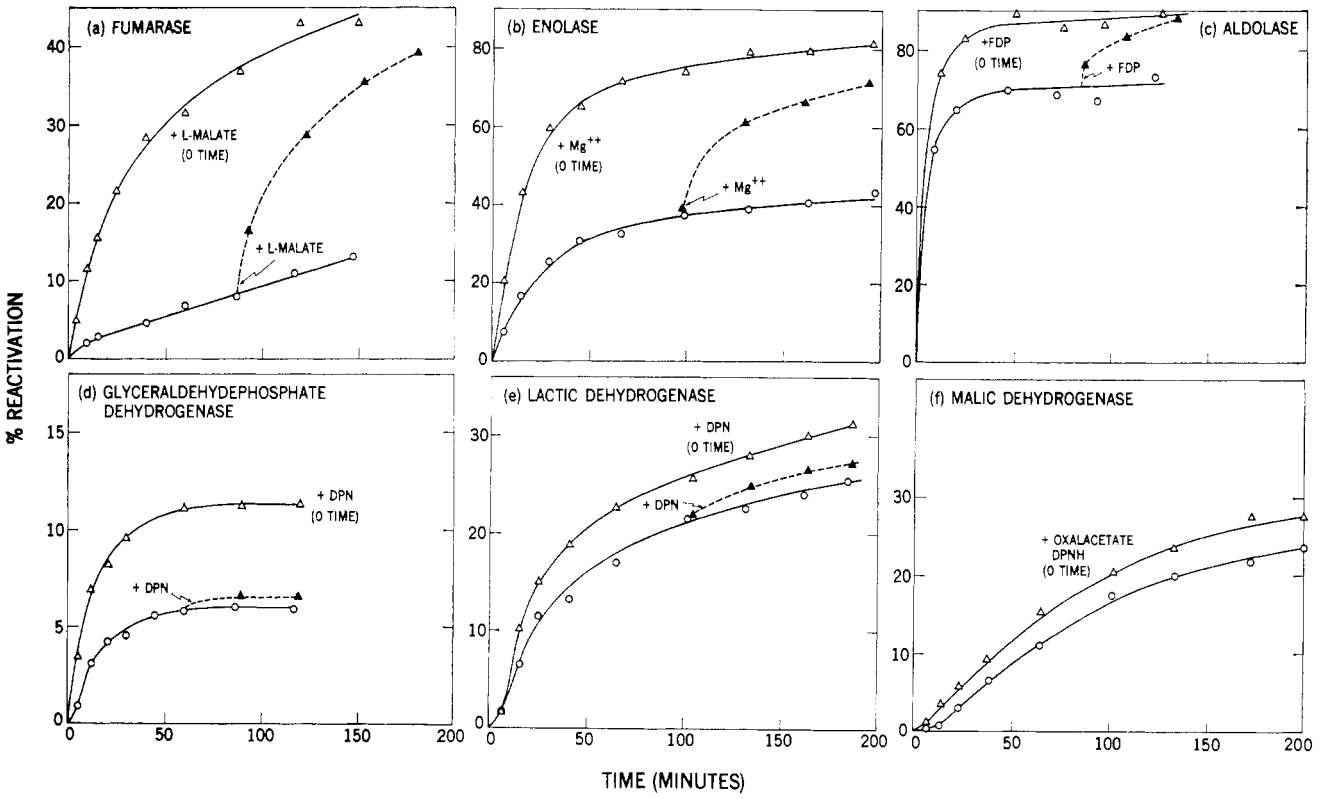


FIGURE 1: Reactivation in the presence and absence of substrate. Enzymes denatured in 6 M guanidine hydrochloride were reactivated by a 101-fold dilution into a solution of $5 \times 10^{-2}$ M Tris-acetate, $1 \times 10^{-2}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) at 25 ± 1° in the presence (△) and absence (○) of substrate or cofactor. The same concentrations of substrate or cofactor were also added to the reactivation medium initially devoid of metabolite 60–100 min after the start of renaturation (▲). The final concentrations of enzymes and metabolites in the reaction medium were as follows: (a) 0.028 mg/ml of fumarase, $5 \times 10^{-3}$ M L-malate, and $1 \times 10^{-2}$ M sodium phosphate; (b) 0.027 mg/ml of enolase and $2 \times 10^{-3}$ M $MgSO_4$; (c) 0.048 mg/ml of aldolase and $1 \times 10^{-3}$ M fructose 1,6-diphosphate (FDP); (d) 0.01 mg/ml of glyceraldehyde phosphate dehydrogenase, $1 \times 10^{-3}$ M DPN, and $1 \times 10^{-2}$ M sodium arsenate; (e) 0.01 mg/ml of lactic dehydrogenase and $1 \times 10^{-3}$ M DPN; and (f) 0.02 mg/ml of malic dehydrogenase, $1 \times 10^{-3}$ M oxalacetate, $1 \times 10^{-3}$ M DPNH, and $1 \times 10^{-2}$ M sodium phosphate.

concentrations employed no precipitation of protein was evident after dilution. Each enzyme was renatured in the presence and absence of its substrate or cofactor and the extent of renaturation, as judged by regain in enzymatic activity, monitored with time. Approximately 60–100 min after renaturation was initiated, substrate was added to the reactivation medium originally devoid of the metabolite, and any further regain in activity measured. The results of these experiments are shown in Figure 1.

Several observations may be made concerning the reactivation profiles seen in Figure 1. Firstly, in the absence of substrate, under identical renaturation conditions, both the extent of reactivation and the rate of reactivation differed significantly among the six enzymes investigated. For example, the per cent regain of native activity, after approximately 120 min, varied from 72% for aldolase to only 6% for glyceraldehydephosphate dehydrogenase. Half-times of reactivation varied from 4 min for aldolase to at least 75 min for fumarase and malic dehydrogenase.

Secondly, the presence of substrate or cofactor in the reactivation medium at zero time always stimulated higher regains in activity relative to renaturation in the absence of metabolite. The magnitude of this effect, however, once again differed with each enzyme. The presence of L-malate increased the extent of reactivation of fumarase over 300% whereas the presence of DPN and arsenate stimulated the reactivation of malic dehydrogenase only 20%. Thirdly, the addition of substrate or cofactor to the reactivation medium, 60–100 min after renaturation was initiated, had quite different effects among the enzymes investigated. In the cases of aldolase, enolase and fumarase, the delayed addition of metabolite increased the extent of reactivation to a level almost comparable to that observed when substrate was present at zero time. For glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase, however, no significant increase was seen when substrate was added after appreciable refolding had taken place. For malic dehydrogenase the difference in reactivation level caused by the addition of substrate was too small to compare the effects of the addition of metabolite at zero time and after renaturation was initiated. It should be noted that those enzymes most stimulated by the later addition of substrate, *i.e.*, aldolase, enolase, and fumarase, also displayed the highest regain of activity in the presence of substrate at zero time. Finally, the rates of reactivation were not observed to obey simple first- or second-order kinetics. In the case of glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase an initial lag period in the reactivation was observed, suggestive of a higher order process.

*Effect of Salt.* In Figure 2 is shown the effect of increased salt concentration on the reactivation of glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase in the presence and absence of DPN. The renaturation conditions in these experiments were essentially

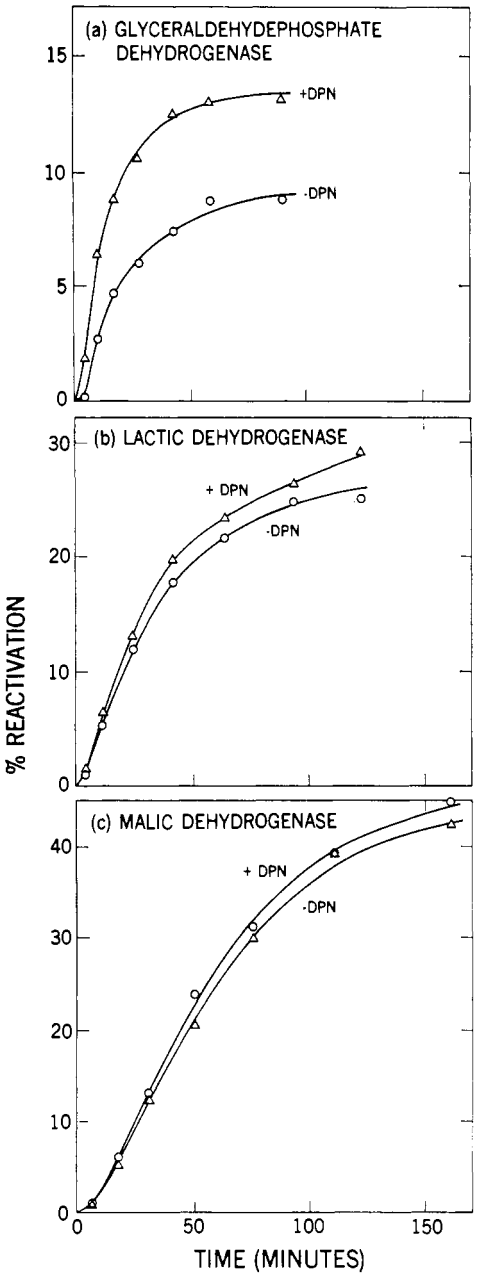


FIGURE 2: Reactivation in the presence of 0.2 M KCl. Enzymes denatured in 6 M guanidine hydrochloride were reactivated by a 101-fold dilution into a solution of 0.2 M KCl, $5 \times 10^{-2}$ M Tris-acetate, $1 \times 10^{-2}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) at 25 ± 1° in the presence (△) and absence (○) of metabolite. The final concentrations of enzymes and metabolites, respectively, in the reactive medium were as follows: (a) 0.009 mg/ml of glyceraldehyde phosphate dehydrogenase, $1 \times 10^{-4}$ M DPN, and $1 \times 10^{-2}$ M sodium arsenate; (b) 0.01 mg/ml of lactic dehydrogenase and $1 \times 10^{-3}$ M DPN; and (c) 0.02 mg/ml of malic dehydrogenase and $1 \times 10^{-3}$ M DPN.

the same as those described in Figure 1 except that the renaturation medium now also contained 0.2 M KCl.

A comparison of Figure 1 and Figure 2 reveals that the added presence on 0.2 M KCl generally increased the level of reactivation for all three enzymes. This effect of ionic strength on the regain of activity was slightly more pronounced when renaturation was conducted in the absence of DPN.

*Effect of Protein Concentration.* Enzymes denatured in guanidine hydrochloride were renatured at various protein

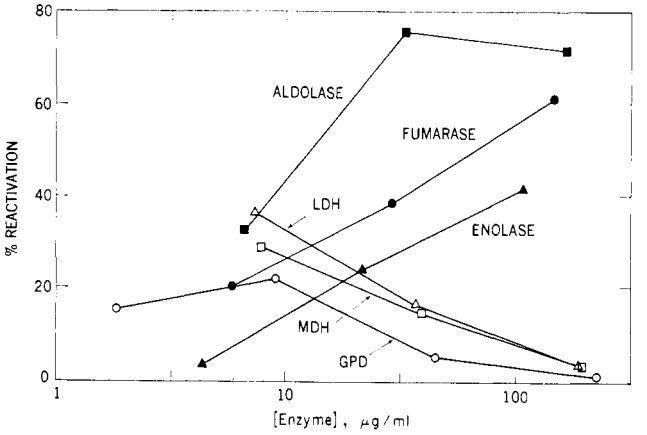


FIGURE 3: Effect of enzyme concentration on extent of reactivation. Enzymes denatured in 6 M guanidine hydrochloride were reactivated by dialysis against a 125-fold excess of 0.05 M Tris-acetate, $1 \times 10^{-3}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) for 4 hr at room temperature. Per cent reactivation after 4 hr has been plotted as a function of protein concentration for the enzymes fumarase (●), enolase (▲), aldolase (■), glyceraldehyde phosphate dehydrogenase (GPDH) (○), lactic dehydrogenase (LDH) (△), and malic dehydrogenase (MDH) (□).

concentrations by dialysis against a large excess of buffer (*cf.* Figure 3). For aldolase, fumarase, and enolase regain in enzymatic activity generally increased with protein concentration, whereas for lactic dehydrogenase, malic dehydrogenase, and glyceraldehyde phosphate dehydrogenase it decreased. The former group of enzymes also displayed higher maximum levels of reactivation. There was no evidence of precipitation of the enzyme by the dialysis procedure with the exception of the two highest concentrations of glyceraldehyde phosphate dehydrogenase and the highest concentration of aldolase. The latter observation probably accounts for the fact the extent of reactivation of aldolase decreased slightly at the highest concentration.

A comparison of the dialysis *vs.* dilution procedures for renaturation reveals that the regain of activity was generally higher by the dialysis method. The largest difference observed was for the reactivation of fumarase in the absence of substrate, where the dialysis procedure gave a threefold greater regain of activity. Such a comparison, however, must take into account the fact that the activity of the dialyzed enzyme was measured 4 hr after the initiation of dialysis, whereas the activity of enzymes renatured by dilution was generally not measured beyond 150 min. Extrapolation of the rate of reactivation of fumarase on dilution to 4 hr yielded more comparable levels of activity.

*Enolase.* As shown in Figure 1b, the presence of $Mg^{2+}$ in the reactivation medium at zero time or addition of the cation 100 min later significantly increases the regain of activity by enolase. It was therefore of interest to determine whether enhancement of this reactivation by $Mg^{2+}$ were reversible, *i.e.*, would the removal of $Mg^{2+}$ from enolase renatured in the presence of $Mg^{2+}$ decrease the activity of the enzyme. Denatured enolase was therefore reactivated in the presence of $Mg^{2+}$ and, 100 min after initiation of refolding, EDTA added to the renaturation medium. It may be seen (Figure 4) that the complexing of $Mg^{2+}$ by EDTA does in fact lower the activity of the renatured enolase. This result is particularly interesting in view of the fact that EDTA has no effect on the activity of native enolase. The upper dashed curve in Figure 4 shows the result of first adding $Mg^{2+}$, then EDTA to the

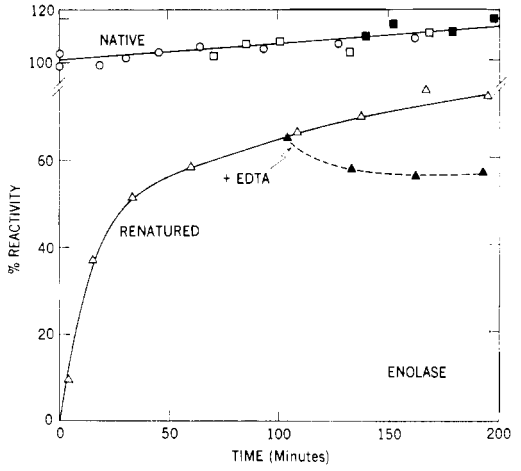


FIGURE 4: Effect of EDTA on native and renatured enolase. Enolase denatured in 6 M guanidine hydrochloride was reactivated by a 101-fold dilution into a solution of $5 \times 10^{-2}$ M Tris-acetate, $1 \times 10^{-2}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) containing $2 \times 10^{-3}$ M $MgSO_4$ (△). After approximately 100 min, $1 \times 10^{-2}$ M EDTA was added to the reactivated enzyme (▲). To a solution of native enolase in 0.05 M Tris-acetate, $1 \times 10^{-2}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) (○) were added $2 \times 10^{-3}$ M $MgSO_4$ after 70 min (□) and $1 \times 10^{-2}$ M EDTA after 130 min (■).

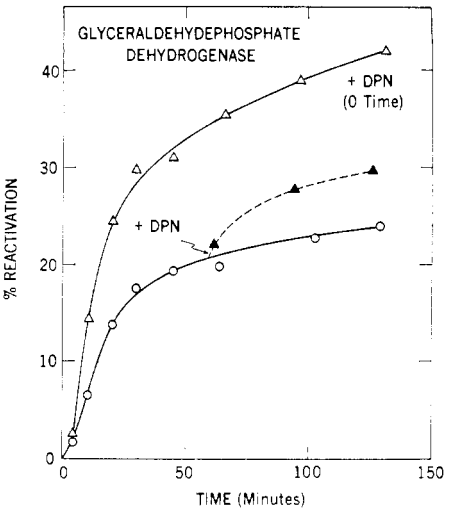


FIGURE 5: Effect of substrate on reactivation of glyceraldehyde phosphate dehydrogenase at a low enzyme concentration. Glyceraldehyde phosphate dehydrogenase, denatured in 6 M guanidine hydrochloride, was reactivated by a 101-fold dilution in 0.05 M Tris-acetate, $1 \times 10^{-2}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) at 25 ± 1° in the presence (△) and absence (○) of $1 \times 10^{-3}$ M DPN and $5 \times 10^{-3}$ M sodium arsenate. The same concentrations of DPN and arsenate were added to the reactive medium initially devoid to these metabolites 60 min after the start of renaturation (▲). The final concentration of the enzyme was 0.002 mg/ml.

native enzyme. The solution of native enolase was prepared by directly dissolving a small aliquot of the ammonium sulfate suspension of the enzyme in buffer. The gradual increase of activity with time, seen for the native enzyme, probably reflects a slight renaturation of the enzyme from its previous ammonium sulfate environment.

*Glyceraldehyde Phosphate Dehydrogenase.* The inability of an enzyme to regain an appreciable extent of its native activity after denaturation might be attributed to the fact that its primary structure is covalently modified during the denaturation and renaturation process. To test this possibility the enzyme glyceraldehyde phosphate dehydrogenase, which displayed the lowest regain of activity of the enzymes examined, was subjected to two cycles of denaturation and reactivation. After the first denaturation, 6% of the activity of the native enzyme was recovered (Figure 1d). If this low level of reactivation resulted primarily from some irreversible modification of the primary structure, then a second denaturation and reactivation might be expected to yield $6 \times 6\%$ or ~0.36% of the native activity. A second denaturation and reactivation of the enzyme in the presence of DPN, however, yielded 9% of the native activity. This discovery represents 80% of that observed after one denaturation and reactivation in the presence of DPN (Figure 1d), and indicated that the low levels of reactivation for this enzyme do not result primarily from a covalent modification of its primary structure.

Comparing the results shown in Figure 1 and Figure 3, it may be observed that glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase, which displayed the least increase in activity after the delayed addition of substrate, were also among the enzymes whose level of reactivation decreased with increasing protein concentration. It was therefore of interest to determine if there were any correlations between protein concentration and the minimal reactivation after the delayed addition of substrate. The experiment shown in Figure 1d was thus repeated for glyceraldehyde phosphate dehydrogenase except that the final protein concentration was reduced from 0.01 to 0.002 mg per ml. The results, presented in Figure 5, reveal a higher overall yield of reactivation both in the presence and absence of DPN. Moreover, there was observed at the lower glyceraldehyde phosphate dehydrogenase concentration a fourfold greater enhancement of activity by DPN, added 60 min after refolding was initiated, than seen for the delayed addition of DPN to the more concentrated glyceraldehyde phosphate dehydrogenase solution. This observation suggests that irreversible aggregation of the polypeptide chains of glyceraldehyde phosphate dehydrogenase to an inactive species might be primarily responsible for the fact that DPN does not significantly increase the extent of reactivation when added after renaturation is initiated.

*Effect of Time of Addition of Substrate on Refolding.* The reactivation of glyceraldehyde phosphate dehydrogenase in the presence and absence of DPN (Figure 1d) was repeated, except that DPN was now added to the reaction medium initially devoid of the metabolite within 30 sec after dilution of the enzyme. The results of this experiment, shown in Figure 6, reveal that the addition of DPN, less than a minute after the initiation of renaturation did not increase the degree of reactivation of the enzyme. The presence of DPN at time zero, however, as before, increased the extent of reactivation nearly twofold. This observation indicates that the refolding process which, for the most part, predetermines the final reactivation level of the enzyme occurs within 30 sec and that DPN must be present during this interval to have its maximum effect on the correct folding.

When the above experiment was conducted at slightly lower temperatures and enzyme concentrations, it was observed that DPN could be added several minutes after the initiation of renaturation and still increase the level of reactivation. The effect of DPN on the kinetics of glyceraldehyde phosphate dehydrogenase renaturation thus appears to be a sensitive function of temperature as well as enzyme concen-

tration. The effect of temperature was pursued further by renaturing the enzyme at 5° in the presence and absence of DPN. Under these ocnditions the addition of DPN 30 min after the initiation of renaturation gave the same final level of activity as that observed when DPN was present at time zero.

## Discussion

The results of the kinetic studies of the refolding process, as monitored by the regain in enzymatic activity, lead to certain simple conclusions. First, it is evident that environmental factors have a decided influence on the *in vitro* refolding of denatured polypeptide chains. For all of the enzymes investigated, the presence of substrate or cofactor increased the regain of enzymatic activity relative to renaturation in the absence of metabolite. The effect of L-malate on the reactivation of fumarase and of DPN on the reactivation of lactic and malic dehydrogenase are in agreement with earlier studies by Hill and Kanarek (1964), and Chilson *et al.* (1965). Deal has also observed that glyceraldehyde phosphate dehydrogenase from yeast is affected by DPN (Deal, 1969). The kinetic studies here, however, reveal additional facets of the nature of this substrate activation process. It is noted that in the case of fumarase, enolase, and aldolase the same amount of activity is regained whether the substrate is present initially or added at a latter stage in the refolding process. The results strongly suggest that for these enzymes there is a relatively rapid rate of reshuffling between different conformational isomers formed upon refolding. Thus, folding to an incorrect form in the absence of substrate or cofactor can be corrected by addition of this cofactor even after an appreciable amount of the protein has been folded incorrectly. On the other hand, in the case of the enzyme glyceraldehyde phosphate dehydrogenase, the later addition of substrate is less important in the recovery of protein activity. In this case folding to give the wrong conformer cannot be completely corrected by the addition of DPN. Lactic dehydrogenase appears to fall midway between these two extremes. Thus it would appear from these studies that the conformational isomers of some proteins can exist in rapid equilibrium and the initial formation of an incorrect isomer can be corrected by a reshuffling process to give the thermodynamically most stable conformer. However, in other cases the kinetics of the renaturation process can lead to incorrectly folded conformers and once in this form reshuffling to give the thermodynamically stable species does not occur within reasonable time intervals.

In addition to the presence or absence of metabolite other environmental conditions also have a considerable influence on the total activity regained and the rate of regain of activity. Changes in the ionic strength of the medium were found to influence reactivation of the enzymes glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase. When renaturation was conducted in 0.2 M KCl, levels of reactivation, both in the presence and absence of DPN, increased as much as 100%.

The regain of enzymatic activity was also found to vary with protein concentration. The extent of the activation increased with increased protein concentration for the enzymes fumarase, enolase, and aldolase, but decreased for the enzymes glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase. For the former group of enzymes it may be postulated that the association of monomers to form the quaternary structure is involved in the rate process for

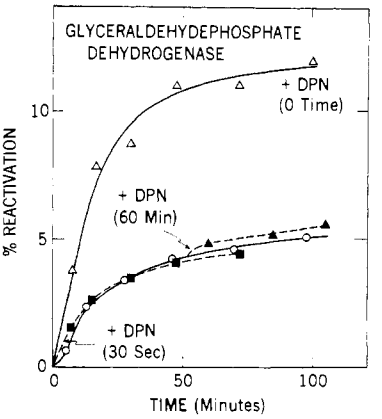


FIGURE 6: Effect of the time of addition of metabolite on the reactivation of glyceraldehyde phosphate dehydrogenase. Glyceraldehyde phosphate dehydrogenase, denatured in 6 M guanidine hydrochloride, was reactivated by a 101-fold dilution into 0.05 M Tris-acetate, $1 \times 10^{-2}$ M dithiothreitol, and $1 \times 10^{-3}$ M EDTA (pH 7.5) at 25° ± 1° in the presence (△) and absence (○) of $1 \times 10^{-3}$ M DPN and $1 \times 10^{-2}$ M sodium arsenate. The same concentrations of DPN and arsenate were added to the reactivation medium initially devoid of metabolite at both 30 sec (■) and 60 min (▲) after the start of renaturation. The final concentration of enzyme was 0.01 mg/ml.

regain of activity. In the latter group polymerization of incorrectly folded conformers to form inactive aggregates is probably responsible for the decrease in the level of reactivation with protein concentration. The existence of such inactive aggregates is supported by the fact that at the higher concentrations of glyceraldehyde phosphate dehydrogenase precipitation is observed.

The role of magnesium ion in native and renatured enolase illustrates that renaturation to a biologically active species does not demonstrate per se the formation of a conformer identical to the native enzyme. Although essentially full activity is regained in the case of enolase, the effect of EDTA is not the same in the native and renatured forms of the enzyme. Clearly, something in the nature of the tertiary structure is different even though the active catalytic site is intact. More than one conformational isomer, therefore, can give rise to biological activity.

The reactivation studies with glyceraldehyde phosphate dehydrogenase provide evidence that the refolding of the protein from an unstructured to a highly structured state involves intermediate conformational states. It was found, for example, that the initial presence of DPN in the renaturation medium increases the level of reactivation of the enzyme. However, if DPN is added to the renaturation medium seconds after dilution of glyceraldehyde 3-phosphate dehydrogenase from the guanidine hydrochloride solution, DPN has essentially no effect on reactivation. During this interval there is virtually no native enzyme formed, as judged by the regain in enzymatic activity. Thus, it is clear that the polypeptide chain rapidly refolds to an inactive conformational state which then slowly rearranges to an active state, and that the structure of this initial state is affected by the presence or absence of DPN. In the presence of DPN it may be postulated that the nucleotide enhances reactivation by binding to an early semistructured form of glyceraldehyde 3-phosphate dehydrogenase, thereby creating a correctly folded nucleus about which the remaining unstructured regions are more easily induced to assume a biologically active conformation. In the absence of DPN a nucleated center is not formed and a

greater concentration of the polypeptide chains rapidly refold to an incorrect conformation which subsequently polymerizes to an inactive aggregate. This mechanism does not apply to all enzymes, however, since some appear to be in rapid mobile equilibrium, with the substrates stabilizing the same final states regardless of when they are added to the medium.

These reactivation studies support the work of Anfinsen indicating that the sequence of amino acids determines the structures of the protein and that thermodynamic factors in many cases control the final structure. They also demonstrate that the kinetics of the folding process may play a key role in the amount of activity recovered and that environmental conditions and metabolite concentrations can affect the rate and extent of this process. The significance of these results to the biological system will be discussed after the structural studies on renaturation are evaluated in the following paper.

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# Kinetic Aspects of Conformational Changes in Proteins. II. Structural Changes in Renaturation of Denatured Proteins*

John W. Teipel and D. E. Koshland, Jr.

ABSTRACT: The renaturation of the enzymes fumarase, enolase, aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase was monitored by optical rotation and fluorescence. As judged by these criteria, the major regain of polypeptide chain structure accompanying renaturation was complete within 1 min. Further minor structural alterations, associated with the regain of enzymatic activity, occurred over much longer periods of time. Renaturation thus proceeds through stable precursor conformations which subsequently rearrange to active or inactive species. The initial rapid regain of structure observed during renaturation *in vitro* suggests that the nascent polypeptide chain folds as it is being synthesized on the ribosome *in vivo*.

The results of these structural studies in conjunction with the reactivation experiments reported in the precdeing paper are discussed in terms of a general mechanism for polypeptide chain folding.

In the preceding paper (Teipel and Koshland, 1971) an investigation of the influence of environmental factors on the *in vitro* reactivation of denatured enzymes was reported. In the present study refolding of the same enzymes was followed by the regain of ordered structure, as determined by optical rotatory dispersion and fluorescence. The rates

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