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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of these conformational changes were then compared to the rates of regain of activity reported in the previous paper.

Experimental Section

Enzymes. Fumarase, lactic dehydrogenase, and malic dehydrogenase from pig heart, and enolase, aldolase, and glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle were obtained from commercial sources as described in the preceding paper (Teipel and Koshland, 1971).

Reagents. *N*-Acetyl-L-tryptophanamide and *N*-acetyl-L-phenylalaninamide were obtained from Cyclo Chemical Corp. and *N*-acetyl-L-tyrosinamide from Mann Research Laboratories. Dithiothreitol was purchased from Calbiochem and guanidine hydrochloride (Ultra Pure) was obtained from Mann Research Laboratories.

Optical rotation measurements were made with a Cary 60 recording spectropolarimeter at 27° in 1- and 5-cm path-length cuvets. Protein concentrations ranged from 0.21 to 0.40 mg per ml for solutions of native and denatured enzyme and from 0.01 to 0.04 mg per ml for solutions of renatured enzyme. Solutions of native enzyme were prepared in 0.01 M Tris-acetate, 1×10^{-3} M dithiothreitol, and 1×10^{-3} M EDTA (pH 7.5). Solutions of denatured enzyme were prepared by diluting 4:1 solutions of native enzyme into 8 M guanidine hydrochloride, 1×10^{-3} M dithiothreitol, and 1×10^{-3} M EDTA (pH 7.0; final concentrations of guanidine hydrochloride were 6 M). Enzyme–guanidine hydrochloride solutions were allowed to incubate at room temperature for 2 hr before reactivation or before optical rotation measurements of the denatured enzyme were made.

Enzymes denatured in guanidine hydrochloride were renatured by diluting slowly (over a period of 30 sec) 0.1 ml of the denatured enzyme into 6 ml of 0.01 M Tris-acetate, 1×10^{-3} M dithiothreitol, and 1×10^{-3} M EDTA (pH 7.5; 61-fold dilution). Changes in optical rotation with time were measured at 233 mμ within 1 min after the dilution of the enzyme. The optical rotatory dispersion curve for the renatured enzyme was measured approximately 30 min later on this same renatured solution of enzyme.

Mean residue rotations (m') were calculated as a function of wavelength from

$$[m'] = \frac{3[\alpha]_\lambda MRW}{(n_\lambda^2 + 2)100}$$

where $[\alpha]_\lambda$ is the specific rotation at a particular wavelength, MRW is the mean residue weight, and n_λ is the refractive index of the solvent at a particular wavelength. For buffers of low ionic strength the values of refractive index as a function of wavelength were assumed to be equal to those of pure water (International Critical Tables, 1930). The refractive index of 6 M guanidine hydrochloride solutions as a function of wavelength was calculated from a form of the Sellmeier equation (Tanford *et al.*, 1967b): $n^2 = 1 + 0.9934\lambda^2/(\lambda^2 - 15067)$.

Mean residue weights, MRW, calculated from the amino acid composition of the enzymes, were as follows: fumarase, 108.2 (Kanarek and Hill, 1964); enolase, 108.9 (Holt and Wold, 1961); aldolase, 109.1 (Penhoet *et al.*, 1969); glyceraldehyde phosphate dehydrogenase, 108.2 (Harris and Perham, 1964); lactic dehydrogenase, 109.3 (Wachsmuth *et al.*, 1964); and malic dehydrogenase, 104.7 (Thorne, 1962).

Fluorescence Measurements. Fluorescence measurements were made with a Hitachi MPF-2A recording fluorescence spectrophotometer equipped with a constant-temperature circulating bath. Measurements were made at 25° in 1-cm path-length cuvets. Protein concentrations ranged from 0.01 to 0.04 mg per ml. Solutions were excited at 280 mμ and fluorescent light was detected at right angles to the incident radiation after passing through a 290-mμ cutoff filter. The excitation slit width was set between 4 and 6 mμ and the emission slit width between 12 and 16 mμ. Under these conditions no appreciable photodecomposition of the protein samples were detected. All fluorescence spectra were uncorrected.

Solutions of native and denatured enzyme were prepared as described above, except that the concentration of Tris-acetate was 0.05 M and the concentration of dithiothreitol 0.01 M. Renaturation was effected by rapidly diluting 0.05 ml of the denatured enzyme directly into a fluorescence cuvet containing 3 ml of 0.05 M Tris-acetate, 0.01 M dithiothreitol, and 1×10^{-3} M EDTA (pH 7.5) at 25 ± 1° (61-fold dilution). Changes in fluorescence with time were monitored within 20 sec after dilution of the enzyme. These changes were generally measured at the emission wavelength where the greatest difference in fluorescence between native and denatured enzyme was observed. The emission spectrum of the renatured enzyme was measured approximately 15 min after initiation of reactivation.

Results

Optical Rotation. Optical rotatory dispersion curves were measured for native enzyme, enzyme denatured in 6 M guanidine hydrochloride, and renatured enzyme. The results of these experiments for the enzymes fumarase, enolase, aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase are shown in Figure 1.

The dispersion curves for the native enzymes all displayed the characteristic negative cotton effect, with a minimum trough between 232 and 234 mμ. Mean residue rotations at these wavelengths varied from −3650° for glyceraldehyde phosphate dehydrogenase to −6650° for fumarase. The dispersion curves for the enzymes in 6 M guanidine hydrochloride were generally featureless and typical of those observed for unordered proteins. Mean residue rotations for the denatured enzymes at 235 mμ varied from −2000° for glyceraldehyde phosphate dehydrogenase to −2760° for enolase. These values lie close to those observed by Tanford *et al.* (1967b) for a group of proteins estimated by other physical criteria (Tanford *et al.*, 1967a; Lepanje and Tanford, 1967) to be randomly coiled. Although there has been no rigorous attempt to demonstrate that the enzymes denatured in this study are also in a randomly coiled state, nevertheless the optical rotatory dispersion spectra (and, as will be described below, fluorescence measurements) suggest at least no appreciable regions of ordered structure remain.

The dispersion curves for the renatured species (measured approximately 45 min after initiation of renaturation) differed significantly among the six enzymes investigated. In the cases of fumarase, enolase, and aldolase, for example, the dispersion curves of the renatured enzyme were very similar to those observed for the native enzyme. The dispersion curves of lactic dehydrogenase and malic dehydrogenase, on the other hand, differ dramatically from the rotatory spectra of their native counterparts. The full dispersion curve of renatured glyceraldehyde phosphate dehydrogenase

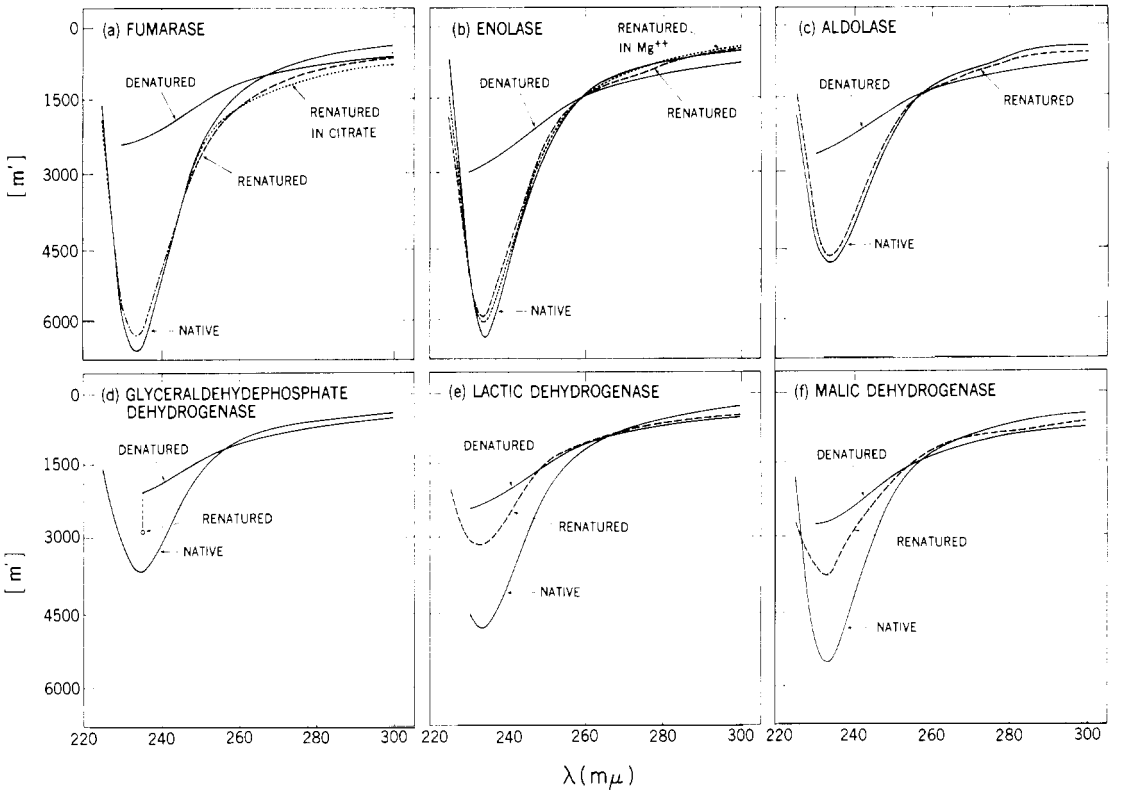


FIGURE 1: Optical rotatory dispersion curves for native, denatured, and renatured enzymes. Solutions of native, denatured, and renatured enzyme were prepared as described in the Experimental Section. Fumarase and enolase were also renatured in the added presence of 5×10^{-3} M citrate and 2×10^{-3} M $MgSO_4$, respectively. Dispersion curves for the renatured enzymes were measured approximately 45 min after initiation of renaturation. Final enzyme concentrations for the native and denatured species were: (a) fumarase, 0.4 mg/ml; (b) enolase, 0.23 mg/ml; (c) aldolase, 0.25 mg/ml; (d) glyceraldehyde phosphate dehydrogenase, 0.25 mg/ml. Final protein concentrations for the renatured enzyme solutions were: (fumarase, 0.035 mg/ml; (b) enolase, 0.03 mg/ml; (c) aldolase, 0.034 mg/ml; (d) glyceraldehyde phosphate dehydrogenase, 0.01 mg/ml; (e) lactic dehydrogenase, 0.038 mg/ml; (f) malic dehydrogenase, 0.041 mg/ml.

was not measured, since the enzyme solution slowly became turbid after dilution into the reactivation buffer. (The lower concentration of dithiothreitol or acetate may be responsible for the precipitation of glyceraldehyde phosphate dehydrogenase here which was not observed at similar enzyme concentration in the reactivation studies.) The rotation at 233 mμ, measured 1 min after dilution, however, differs significantly from that of native glyceraldehyde phosphate dehydrogenase. This enzyme may, therefore, be tentatively classified with lactic dehydrogenase and malic dehydrogenase, as a protein regaining only limited native structure after renaturation.

Enolase and fumarase were also renatured in the presence of Mg^{2+} and citrate, respectively. Mg^{2+} is a cofactor in the reaction catalyzed by enolase and citrate is a competitive inhibitor of fumarase. These compounds were previously shown to increase significantly the regain of enzymatic activity when added to the renaturation medium (Teipel and Koshland, 1971). As shown in Figure 1a,b, the dispersion curves for fumarase and enolase renatured in the presence of these effectors differ little from the dispersion curves of the enzyme renatured in their absence.

Renaturation was also monitored by optical rotation at 233 mμ as a function of time. These experiments revealed that the total increase in optical rotation at 233 mμ on going from the denatured to renatured state occurred within 1 min of the initiation of renaturation. For all the enzymes investigated (with the exception of glyceraldehyde phosphate dehydrogenase, due to its precipitation) no change in rotation at 233 mμ was detected between 1 min and 45 min after dilution of the enzyme into the renaturation medium. (The time involved in diluting the enzyme, filling the cuvet, and waiting for polarimeter stabilization prevented measurement of rotational change before 1 min.) The optical rotation of the renatured enzyme at 233 mμ, measured 45 min after dilution and shown in Figure 1 thus also represents the rotation measured at 233 mμ after 1 min. Since, over the period indicated, no changes in rotation were observed at 233 mμ, a wavelength very sensitive to changes in the optical rotatory properties of proteins, it is likely that the full dispersion curves in Figure 1 also represent the optical rotatory spectra of the renatured enzymes 1 min after initiation of renaturation.

Fluorescence Spectra. The fluorescence emission spectra of native, denatured, and renatured enzyme were determined and rates of renaturation, as judged by changes in fluorescence intensity with time, were measured. The results of these experiments for the enzymes fumarase, enolase, aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase are shown in Figure 2.

The emission spectra for the native and renatured enzymes have been normalized with respect to the emission spectra of the denatured enzyme. Fluorescence intensities have been expressed in units relative to the intensity of the denatured species at its λ_{max}. The latter has arbitrarily been assigned a value of 10 for all the enzymes. Several features of the emission spectra of the native, denatured, and renatured enzyme become apparent when the spectra are so normalized.

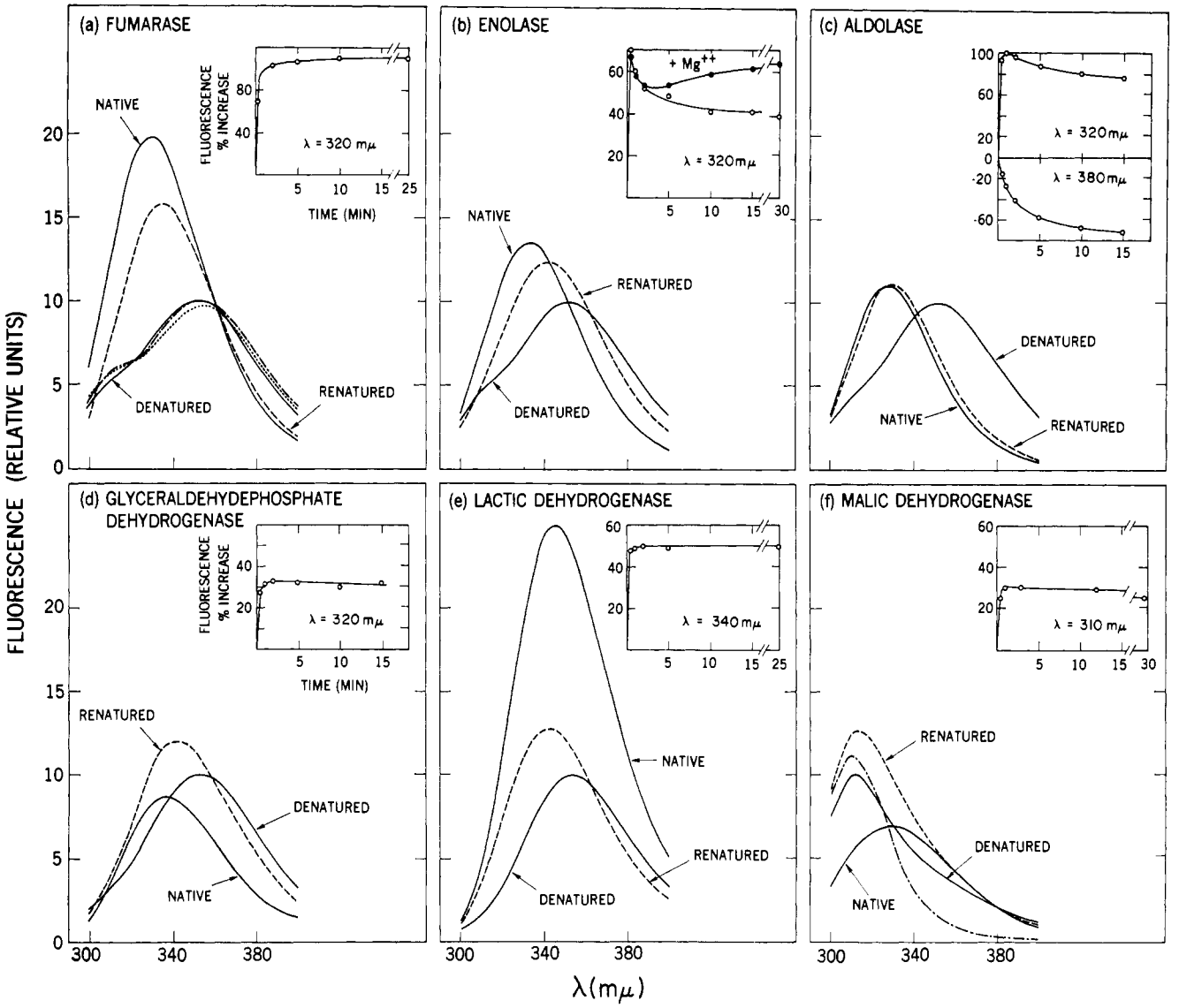


FIGURE 2: Fluorescence emission spectra of native, denatured, and renatured enzymes. Solutions of native, denatured, and renatured enzymes were prepared as described in the Experimental Section. Solutions were excited at 280 mμ. The spectra of the renatured enzymes were measured approximately 15 min after initiation of renaturation. Final enzyme concentrations for the native, denatured, and renatured species were: (a) fumarase, 0.023 mg/ml; (b) enolase, 0.024 mg/ml; (c) aldolase, 0.025 mg/ml; (d) glyceraldehydephosphate dehydrogenase, 0.01 mg/ml; (e) lactic dehydrogenase, 0.021 mg/ml; (f) malic dehydrogenase, 0.038 mg/ml. Emission spectra for a mixture of *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide, and *N*-acetyl-L-phenylalaninamide in 1×10^{-2} M sodium phosphate, pH 7.5 (–·–·–), and in 6 M guanidine hydrochloride, 1×10^{-2} M sodium phosphate, pH 7.5 (·····), are compared to spectra of denatured fumarase in part a. Emission of *N*-acetyl-L-tyrosinamide (6.45×10^{-6} M) in 0.05 M Tris-acetate, pH 7.5 (–·–·–), is compared to spectra of malic dehydrogenase in part f. Initial rates of renaturation, as monitored by the change in fluorescence intensity are shown in the upper right insert for each of the enzymes. Changes in fluorescence at the wavelengths indicated have been represented as a per cent increase or decrease in the intensity of the denatured enzyme. Initial changes in fluorescence with time are also included for the enzyme enolase when renatured in the presence of 2×10^{-3} M $MgSO_4$.

First, the similarity in shape between the fluorescence spectra of the enzymes after denaturation in guanidine hydrochloride is emphasized. It may be observed that all of the enzymes, with the exception of malic dehydrogenase, display a fluorescence maximum from 352 to 354 mμ. The differences in spectral shape due to the small shoulders at 310 mμ may be attributed to the relative tyrosine content of the enzymes. In Figure 2a the emission spectra of denatured fumarase is compared to the spectra of a mixture of *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide, and *N*-acetyl-L-phenylalaninamide, the latter both in buffer and in 6 M guanidine hydrochloride. The molar concentration of the aromatic derivatives were equal to molar concentrations of the tryptophan, tyrosine, and phenylalanine residues, respectively, in the denatured fumarase sample, as determined from its amino acid composition (Kanarek and Hill, 1964). It may be observed that the general shape, λ_{max}, and quantum yields of the three spectra are nearly identical. These results indicate that (1) the aromatic residues of fumarase in 6 M guanidine hydrochloride are fully exposed to solvent, suggesting the enzyme is extensively unfolded, and (2) the presence of 6 M guanidine hydrochloride does not appreciably alter the fluorescence properties of the exposed aromatic residues. The similarity between the fluorescence

spectra of denatured fumarase and the other denatured enzymes (containing tryptophan) suggests that the latter proteins are also without significant structured regions.

The emission spectrum of denatured malic dehydrogenase, unlike the other enzymes, displayed a maximum at 310 mμ. This anomaly is due to the fact that pig heart mitochondrial malic dehydrogenase, employed in these studies, contains little (Chan and Schellenberg, 1968) or no (Thorne, 1962) tryptophan. Comparison of the spectra of the denatured enzyme to an equal residue concentration of L-acetyl-L-tyrosinamide (Figure 2f) reveals that the spectrum of the denatured enzyme may be attributed chiefly to its tyrosine fluorescence.

While the fluorescent properties of the denatured enzymes are all very similar, it may be seen from Figure 2 that the emission spectra for the native enzymes differ significantly, both with respect to relative intensity and position of λ_{max}. A comparison of the fluorescence spectra of the native and denatured enzymes, however, reveals two features which the tryptophan-containing enzymes share in common. Firstly, the maximum fluorescent intensity of the native species is consistently greater than that of its denatured counterpart and secondly the λ_{max} of the native form is shifted to shorter wavelengths relative to the denatured form. The maximum fluorescent intensity of native glyceraldehyde phosphate dehydrogenase seen in Figure 2d is less than that of denatured glyceraldehyde phosphate dehydrogenase due to quenching of the fluorescence of the native form by bound DPN. The maximum intensity of the native apo enzyme (not shown), however, is greater than that of denatured glyceraldehyde phosphate dehydrogenase.

For malic dehydrogenase, containing principally tyrosine, the fluorescent intensity of the native enzyme is less than that of the denatured form, and its λ_{max} is shifted to longer wavelengths. The lower fluorescent intensity of the native species is typical for proteins containing only tyrosine (Konev, 1967). The red shifted λ_{max} of the native relative to the denatured enzyme is due to a contamination of the commercial enzyme preparation (Thorne and Kaplan, 1963).

The fluorescence spectra of the renatured species are as varied as those observed for the native enzyme. With the exception of malic dehydrogenase, however, the change in fluorescent spectra accompanying the transition from the denatured to renatured state, are all in the direction of the native enzyme. In the case of the tryptophan-containing enzymes, for example, there is an increase in maximum fluorescent intensity and a shift in λ_{max} toward shorter wavelengths with renaturation. This shift in λ_{max} toward shorter wavelengths has also been demonstrated for the spectra of model compounds when the dielectric constant of the solvent is decreased. This correlation suggests that, upon refolding, tryptophan residues are transferred from a hydrophilic to a hydrophobic environment. For malic dehydrogenase there is an increase in fluorescent intensity with renaturation. As noted above, however, the intensity of the native species for this enzyme is less than that of the denatured form. The behavior of malic dehydrogenase, therefore, differs from that of the other enzymes in that the spectral change upon renaturation is not in the direction of the native enzyme.

It is also interesting to note that only the spectra of renatured fumarase passes through the isosbestic point shared by the native and denatured forms of the enzyme. This observation indicates that the renatured species of all the enzymes investigated, with the possible exception of fumarase, do not represent an equilibrium mixture of only native and denatured enzymes, but rather are composed of appreciable concentrations of intermediate and structurally distinct form(s) of the enzyme. Intermediate conformational forms are also indicated in the case of fumarase since the optical rotatory dispersion spectrum of the renatured species does not share a common isosbestic point with the native and denatured enzyme.

Rates of Fluorescent Changes. Figure 2 also contains plots of the rate of renaturation of each enzyme as measured by fluorescence. In these experiments, a small aliquot of the denatured enzyme was rapidly diluted into an excess of the renaturation buffer, and changes in fluorescent intensity at a particular wavelength monitored with time. Changes in fluorescence have been expressed as the per cent increase or decrease in intensity relative to the intensity of the denatured enzyme at the same wavelength.

Several observations may be made with respect to the results of these rate studies. Firstly, the initial rate of increase in fluorescent intensity observed for all of the enzymes was very rapid. Half-times of 30 sec or less were measured for the initial reaction. Secondly, in the cases of aldolase and enolase a significant decrease in fluorescent intensity followed the initial increase in intensity. A less pronounced, though decidely real decrease was observed for malic dehydrogenase. These results are indicative of at least two kinetic processes, occurring at different rates and perhaps involving the environments of different aromatic residues. Thirdly, the effect of Mg^{2+} on the renaturation of enolase is clearly discernable (Figure 2b). The presence of Mg^{2+} was observed to increase appreciably the regain of enzymatic activity (Teipel and Koshland, 1971) of enolase upon renaturation and, as discussed above, altered slightly the optical rotatory dispersion curve of the renatured protein. Here, monitoring renaturation by fluorescence, it may be seen that the effect of Mg^{2+} on the environment of the aromatic residues is first expressed approximately 2 min after initiation of renaturation. Finally as may be seen in Figure 2c, the kinetic profiles for the renaturation of aldolase differ significantly depending on whether fluorescence changes are monitored at 320 or 380 mμ. These observations again suggest that intermediate conformational states are produced during the process of renaturation.

Discussion

Structural Studies of Renaturation. Two major conclusions may be drawn from the optical rotation studies of renaturation. Firstly, a rough correlation is seen between the regain of native structure and the regain of enzymatic activity. The optical rotatory dispersion curves for renatured fumarase, enolase, and aldolase, which displayed a high regain of activity, were observed to be very similar to their native counterparts. Conversely, the dispersion curves of the renatured glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase, which gave low levels of reactivation, differed markedly from those of the native enzyme. Secondly, the gross structural changes accompanying renaturation as measured by optical rotation were complete within 1 min, whereas very little enzyme activity was regained within this interval (Teipel and Koshland, 1971). These observations suggest that some polypeptide chains rapidly refold (within 1 min) to an inactive structural species similar, but not identical, in conformation to the native enzyme and these precursors then slowly regain biological activity as a

result of minor structural alterations. Other chains refold to less native-like conformations which do not subsequently rearrange to yield active enzyme within physiologically significant intervals. Thus the proportion of "correctly" *vs.* "incorrectly" folded precursors initially formed is related to the final level of reactivation.

These conclusions are supported by the fluorescence studies. Although the correlation between regain of structure and regain of activity was not as dramatic as in the optical rotation measurements, the emission spectra of renatured fumarase, enolase, and aldolase were observed to resemble the spectra of the native enzyme far more closely than the corresponding spectra of glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase. Furthermore the greatest changes in fluorescent intensity accompanying renaturation were complete within 30 sec—before an appreciable degree of reactivation had occurred. Finally, small fluorescence changes were measured between 1 and 30 min after renaturation for the enzymes aldolase, enolase, and malic dehydrogenase. These changes in fluorescence are indicative of structural alterations in the vicinity of the aromatic residues. The absence of any change in optical rotation at 233 mμ during the same interval, however, suggests these alterations are minor and do not affect the gross conformation of the enzyme. This interpretation is consistent with the refolding mechanism discussed above, whereby the slow regain in activity is mediated by minor conformational changes.

General Conclusions of in Vitro Renaturation Studies. A general mechanism for the refolding of denatured enzyme molecules *in vitro*, derived from the renaturation studies reported here and in the previous paper (Teipel and Koshland, 1971), is schematically represented in Figure 3. The structural species depicted in Figure 3 are not intended to represent all the intermediate or final conformational states produced on renaturation. Numerous other structural isomers are undoubtedly formed during the refolding of a randomly coiled polypeptide chain, however, since inclusion of such intermediates does not alter the basic postulates of the mechanism, these species have been omitted for the sake of simplicity. The following major conclusions may be drawn with reference to Figure 3. (1) There is a rapid folding of the unstructured polypeptide chain (I) to structured, but inactive conformations (II_a, II_b, and II_c). As judged by changes in optical rotation and fluorescence, this initial folding process is essentially complete within 1 min. In addition to the enzymes reported here, rapid rates of renaturation have recently been observed for staphylococcal nuclease (Schechter *et al.*, 1970), lysozyme (Tanford *et al.*, 1966; Yutani *et al.*, 1968), and collagen (Hauschka and Harrington, 1970). The correlation observed between the structural properties of these species and the final level of reactivation suggests that conformations II_b and II_c, which are precursors of the active enzyme, are more highly ordered than II_a, which is a precursor of the inactive enzyme. (2) Following the rapid regain of gross conformational properties, species II_a, II_b, and II_c undergo minor structural alterations to form either active enzyme (III_c and III_d) or inactive enzyme (III_a and III_b). The conformational changes leading to the regain of activity occur relatively slowly, with half-times of up to 75 min for some of the enzymes investigated. The rate of conformational change leading to inactive enzyme, III_a, has not been determined. The formation of inactive aggregates, III_b, however, occurs relatively rapidly, as judged by the rate of appearance of precipitate, when concentrated solutions of enzyme are

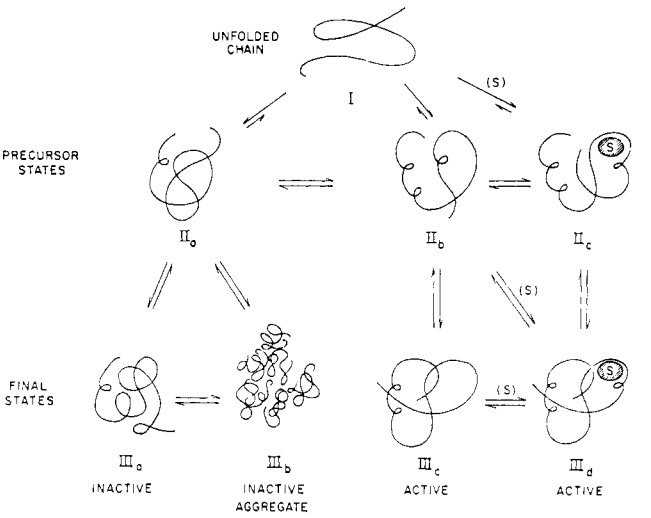


FIGURE 3: Schematic representation of the refolding of a denatured protein *in vitro*. The significance of the conformational states I, II, and III and details of the refolding mechanism are described in the text.

renatured. (3) The relatively rapid regain of structure compared to the slow regain of activity indicates that intermediate conformational states, *i.e.*, species structurally distinct from either the randomly coiled polypeptide or the native enzyme are formed in appreciable concentrations during renaturation. The observation that neither the optical rotatory dispersion nor fluorescence spectra of the refolded proteins pass through the isosbestic point common to the native and fully denatured enzyme indicate that intermediate species are also present at equilibrium. The formation of intermediate conformational states has also been reported for the renaturation of carbonic anhydrase B (Wong and Tanford, 1970), whereas only the native and randomly coiled protein are found in significant amounts on denaturation of lysozyme (Tanford *et al.*, 1966) and ribonuclease (Salahuddin and Tanford, 1970) by guanidine hydrochloride. The absence of intermediate states in appreciable concentrations suggests that the transition between denatured and native enzyme is a more cooperative process for ribonuclease and lysozyme than for carbonic anhydrase B or the enzymes studied here. (4) Environmental conditions, *i.e.*, the concentration of specific metabolites, the ionic strength and the protein concentration of the renaturation medium, influences both the rate and final extent of reactivation. Higher yields of active enzyme were invariably observed when renaturation was conducted in the *initial* presence of substrate or cofactor. Addition of substrate *after* initial folding had occurred increased the levels of reactivation of the enzymes fumarase, enolase, and aldolase, but did not further enhance the reactivation of glyceraldehyde phosphate dehydrogenase or lacticed hydrogenase. These results suggest that refolding of the former group of enzymes is determined primarily by thermodynamic considerations, whereas refolding of the latter group of enzymes is at least partly influenced by kinetic factors.

For those enzymes under thermodynamic control it is apparent that the interconversion between all intermediate conformational states is relatively mobile, such that equilibration to the thermodynamically most stable state(s) occurs within physiologically significant periods of time (a few hours). Thus substrate present initially or added after initial refolding will increase the level of reactivation by binding

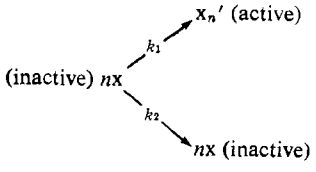
to one or more of the intermediate (II_b) or final (III_c) conformational states, displacing the equilibrium in favor of the active species III_d.

For enzymes under kinetic control it is proposed that the rate of equilibration between the intermediate conformational states II_a, II_b, and II_c is slow relative to the rates of formation of the final conformational states. The relative *rates* of folding to II_a, II_b, and II_c will thus determine the distribution between potentially active (II_b and II_c) and potentially inactive (II_a) precursor and thereby also determine the yield of active enzyme subsequently realized. Renaturation in the initial presence of substrate increases the *rate* of formation of the potentially active precursor II_c, relative to II_a, enhancing the final level of reactivation. Mechanistically the substrate may be visualized as binding to a semistructured form of the enzyme (some intermediate conformation between the random coil and II_c), thereby providing a nucleus or center about which the remaining polypeptide chain may more rapidly fold to the correct conformation. This hypothesis is supported by the observation that DPN enhanced the reactivation of glyceraldehyde phosphate dehydrogenase only if it interacted with the refolding protein during the first 30 sec of renaturation (before II_c, much less an active species of the enzyme is formed). Addition of substrate after the initial formation of II_a, II_b, and II_c will not appreciably affect the level of reactivation since the relative distribution of potentially active or inactive precursors is already determined. Under other conditions, the intermediates were different and DPN had a lesser effect.

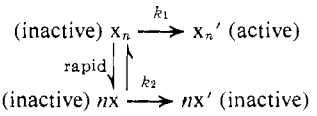
If the inactive enzyme is thermodynamically more stable than the active enzyme, then active enzyme may still be formed because of kinetic control but this enzyme will be metastable. This might explain why certain native enzymes, under conditions which should not alter their covalent structure, gradually lose activity, aggregate and finally precipitate. Thus the results support the conclusion of Anfinsen and coworkers on the importance of primary structure and thermodynamic factors. They also provide some clarification of the role of kinetic factors.

(5) Finally, it may be recalled that the renaturation studies reported here are concerned with subunit proteins. Thus, although omitted from the simplified refolding mechanism in Figure 3, the reassociation of individual polypeptide chains to form the native quaternary structure is an important step in the renaturation process. It is not known whether this reassociation takes place during the rapid phase of refolding (I → II) or during the slower rearrangement process (II → III). A certain degree of structural regain, however, must precede correct reassociation since the intersubunit contact sites of the native enzyme presumably possess a specifically ordered conformation.

The effect of polypeptide chain association on the kinetics of renaturation is reflected in the dependence of reactivation on enzyme concentration described in the previous paper (Teipel and Koshland, 1971). The level of reactivation was observed to increase with increasing protein concentration for the enzymes fumarase, enolase, and aldolase. This increase in relative rate may arise from either (a) the law of mass action, *e.g.*



where $d([active]/[inactive])/dt = (k_1/k_2)[x]^{n-1}$ or (b) the dependence of a rapid equilibrium between potentially active and inactive precursors on enzyme concentration, *e.g.*



where $d([active]/[inactive])/dt = (k_1/k_2)[x]^{n-1}$ and K is the association constant for the rapid equilibrium between active and inactive species. The level of reactivation is not due to a change in the equilibrium between native and denatured enzyme, since the activity of the native enzymes does not vary as a function of protein concentration. The decrease in reactivation with increasing protein concentration observed for the enzymes glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, and malic dehydrogenase may be explained by a similar kinetic mechanism, except that in the latter case, the rate of formation of a higher ordered inactive polymeric species increases relative to the rate of formation of the native quaternary structure.

Relevance to in Vivo Folding. Several conclusions drawn from the *in vitro* renaturation studies are relevant to the *in vivo* assembly of proteins. In the first place, the rate of regain of gross conformational structure accompanying renaturation *in vitro* is faster than the rate of polypeptide chain synthesis *in vivo*. The initial folding process occurs in less than a minute whereas the synthesis of a polypeptide chain of 35,000 molecular weight requires at least 5 min, as estimated from the rate of hemoglobin synthesis in mammalian cells (Hunt *et al.*, 1969). This result suggests that folding will occur during the ribosomal translation process.

Secondly, the nascent polypeptide chain, folding from its amino terminal end as it is being synthesized *in vivo*, will most likely assume an *initial* structure distinct from that produced by the folding of a complete polypeptide chain *in vitro*. This difference may be attributed to the subsequent influence of residues nearer the carboxyl terminus on the conformations formed before their addition to the peptide chain. The role of the initial conformations with respect to the final conformation assumed by the protein will depend on whether thermodynamic or kinetic factors are more important in controlling the folding process. If folding is under thermodynamic control, the initial folding may be incorrect but the protein will quickly equilibrate to the active and most stable conformation as for fumarase, enolase, and aldolase. For enzymes under kinetic control, however, such as glyceraldehyde phosphate dehydrogenase and lactic dehydrogenase, the initial conformation assumed by the protein will be important in specifying its final conformation. In this case the native structure derived from folding of a partially complete chain *in vivo* might differ from that produced after refolding of a complete protein *in vitro*. Such a difference may account for the facts (a) that several enzymes when renatured *in vitro* are biologically active but structurally different from their native counterparts (Chilson *et al.*, 1966; Pfumm and Beychok, 1969; Dawson *et al.*, 1965; Teipel and Koshland, 1971) and (b) that many proteins when denatured and refolded under seemingly physiological conditions regain little or none of their original activity.

It is also observed that even for proteins which regain nearly full biological activity when renatured *in vitro*, the rate of regain of activity is considerably slower than that estimated for the synthesis of active enzyme *in vivo*. This

increased *rate* of activation *in vivo* would be explained by assuming that folding of the partially complete chain excludes the formation of precursors which rearrange only slowly to the active enzyme.

The effect of environmental conditions on the assembly of proteins *in vivo* is probably much the same as that observed *in vitro*. The presence of substrate, for example, might increase the yield of active enzyme either by thermodynamically stabilizing the active conformation of the enzyme or by interacting with the still incomplete polypeptide chain on the ribosome and increasing its rate of folding to the correct conformation. Whether environmental conditions play an important role in the regulation of active protein synthesis still remains to be determined, but such a mechanism could provide an additional control device in the living system.

It is interesting to note the close analogy between the effect of metabolite on protein renaturation and the evidence for ligand-induced changes in an active, fully folded protein. In each case the ligand effects a new conformational state by including a conformational rearrangement and stabilizing the final state. Most ligand-induced changes in the native enzyme are so rapid that they can be examined only by kinetic techniques for fast reactions. The fact that half-times for ligand-induced folding processes of the precursor to the native enzyme are of the order of minutes or hours, however, is in accord with slow conformational changes in fully folded proteins (Frieden, 1970; Ray and Hatfield, 1970).

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