Reversible Folding of UDP-Galactose-4-Epimerase from Yeast *Kluyveromyces fragilis*†

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ABSTRACT: UDP-galactose-4-epimerase from yeast *Kluyveromyces fragilis* is a dimeric molecule with one molecule of cofactor NAD per dimer. In presence of 8 M urea, the enzyme is inactivated with complete disorganization of its structure and dissociation of the subunits together with the cofactor. Dilution of the denaturant by sodium phosphate buffer (20 mM, pH 7.0) containing 1 mM NAD recovers the activity to the extent of 80–100%. At a monomer concentration of 0.8 μM, the reactivation follows second-order kinetics, $k = 1.48 \times 10^3$ M^{-1} s^{-1}, at 20 °C. The renatured enzyme resembles the native enzyme in terms of most of its physicochemical properties, e.g., circular dichroism spectrum, fluorescence spectrum, interaction with hydrophobic fluorophore 1-anilino-8-naphthalenesulfonic acid (ANS), hydrodynamic volume, K_m for the substrate UDP-galactose, etc. The reactivation process has an energy of activation of 15.2 kcal/mol. The folding pathway could be divided into four major parts: in the first phase (within 10 min), the secondary and tertiary structures of the monomers are formed as found by circular dichroism and fluorescence spectroscopy; in the second phase (within 20 min), the folded monomers associate to form an inactive dimer as observed by size-exclusion HPLC; in the third phase, the NAD binding site is formed, which is possibly induced by the cofactor; and finally, NAD assembles over the cofactor binding site to yield an active holoenzyme (within 120 min). The third and fourth steps have been detected kinetically. They are slow and rate-limiting, depending upon the concentration of extraneous NAD.

Folding of proteins into a biologically active conformation is the last step of post-translational processes. Evidence has been obtained that in some cases other proteins catalyze these processes, e.g., molecular chaperones, protein disulfide-isomerase, peptidylprolyl *cis,trans*-isomerase, etc. (Ellis & van der Vies, 1991), but their involvements are not universal. Exploration of the "rules" that guide the polypeptides to select a unique conformation is a major problem of modern biochemistry. The folding problem adds another dimension when the protein in question has a multimeric structure or is associated with a cofactor. In that case, proper folding of each subunit together with association of the monomers is required (Jaenicke & Rudolph, 1980). These two processes must be properly coordinated; otherwise, specific recognition by a subunit may not be achieved during folding (Jaenicke & Rudolph, 1986). This subunit association is considered to be entropy driven as the water molecules are excluded from the hydrophobic intermolecular surfaces leading to a tightly packed globular conformation of the multimeric proteins (Richards, 1977).

UDP-galactose-4-epimerase (EC 5.1.3.2; hereafter called epimerase) is an obligatory enzyme of galactose metabolism. It reversibly converts UDP-galactose (UDP-gal)[1] to UDP-glucose (UDP-glu) before the latter enters into the glycolytic pathway. Structurally, this enzyme is unique because it is made up of two possibly identical subunits and a cofactor NAD per dimer. Mechanistically, epimerase is an oxidoreductase rather than an isomerase. Unlike all classical multimeric dehydrogenases, the bound NAD acts as a true cofactor and not as a cosubstrate. Interestingly, a number of other oxidoreductases exist, e.g., UDP-glucuronic acid decarboxylase, *S*-adenosyl homocysteine hydrolase, TDP-glucose oxidoreductase, quinine synthetase, etc., which have NAD-bound dimeric structures and mechanisms of action similar to those of epimerase, but of entirely different specificity (Gabriel, 1978; Gabriel & Lanten, 1978; Frey, 1987). Epimerase is considered a prototype model of this class of enzymes.

The quaternary structure of epimerase thus presents an unusual architectural feature. Since only 1 mol of cofactor is bound per mole of the dimer, the enzyme obviously has one catalytic site per molecule of the dimeric holoenzyme, i.e., part of the active site is shared by each of the subunits. This is also a difference between an epimerase and a dehydrogenase. In contrast to the reconstitution and reactivation studies of different dehydrogenases (Zettlmeissl *et al.*, 1982; Hermann *et al.*, 1981, 1983; Jaenicke *et al.*, 1981), the question of assembly of holoenzyme structure and symmetry at the active site of the epimerase remains unsolved. In this article, the reconstitution pathway of epimerase from a totally denatured and dissociated state has been described. This is the first report of a folding pathway of an enzyme belonging to this important class of oxidoreductase.

MATERIALS AND METHODS

Enzyme Purification and Assay. Epimerase was purified from yeast strain *Kluyveromyces fragilis* (ATCC Strain No. 10022) after Darrow and Rodstrom (1968). In experiments concerning physical measurements, where higher purity of the enzyme was necessary, the final preparation was passed through an LKB-Ultropac TSK G3000SW size-exclusion HPLC column to remove trace contaminants of low molecular weight. Detailed experimental conditions of this chroma-

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonic acid; SE-HPLC, size-exclusion, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UDP-gal, uridine diphosphate galactose; UDP-glu, uridine diphosphate glucose; 5′-UMP, 5′-uridine monophosphate; UV, ultraviolet.

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tography will be described in the text. Purity was demonstrated by the observation of a single band of molecular weight 75 000 in SDS–PAGE (Laemmli, 1970). Enzyme activity was assayed according to the coupled assay procedure of Darrow and Rodstrom (1968), where the formation of UDP-glu from UDP-gal was monitored spectrophotometrically at 340 nm for 4 min by coupling with UDP-glucose dehydrogenase and NAD. The specific activity of the less pure preparation was 35 units/mg, while that of the HPLC-purified sample was between 40 and 45 units/mg of protein. One unit was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of UDP-gal per minute at 25 °C. The less pure preparation was used for reactivation experiments. It was verified that the kinetics of reactivation calculated on the basis of the active protein present in the original solvent was independent of the specific activity of the preparation between 35 and 45. Thus, the contaminants present in the less pure preparation had no role in determining the rate of reactivation. Epimerase was preserved as stock in sodium phosphate buffer (20 mM, pH 7.0) containing 1 mM EDTA and 5 mM 2-mercaptoethanol at −70 °C in small aliquots. Only two cycles of freezing and thawing were allowed. Epimerase contains no disulfide linkage but has essential thiol groups for catalysis (Bhattacharjee & Bhaduri, 1992). Therefore, a reducing condition was always maintained in dealing with the enzyme. Protein concentration was measured either by taking $E^{1\%}_{280nm} = 10.0$ (Darrow & Rodstrom, 1968) or by following Lowry (1951) in cases where UV-absorbing materials were present in the solution.

Denaturation and Renaturation. Epimerase (5 μL, 12 mg/mL) was diluted with 5 μL of sodium phosphate buffer (20 mM, pH 7.0) containing 5 mM 2-mercaptoethanol and 40 μL of 10 M urea in the same buffer. Denaturation was allowed for 10 min at 20 °C, by which time unfolding was complete. Renaturation was initiated by adding 100 μL of 10 mM NAD in water and 850 μL of buffer. Aliquots of 5–10 μL of the renaturing solution were directly transferred to the assay mixture from time to time to follow the kinetics of reactivation. If necessary, the renatured enzyme was diluted further with the renaturing buffer. The maximum concentration of urea that was carried over to the assay mixture was 0.02 M. This had no inhibitory effect on the system. Final concentrations of protein and denaturant during renaturation were 0.39 μM (dimer concentration) (60 μg/mL) and 0.4 M, respectively. To characterize the denatured state of the enzyme, it was diluted to 1 mL by 8 M urea at pH 7.0.

The assay of epimerase needs NAD, which influences the rate of reactivation, thus leaving the possibility of further reactivation of inactive epimerase during the assay. Such a possibility has been ruled out because of the following: (a) Under assay conditions, i.e., in the presence of 1 mM NAD, reactivation follows a second-order reaction with respect to epimerase concentration (described in the text, Figure 3). Since the protein was diluted 20–200-fold during the assay, it is expected that the rate of reactivation will be reduced by at least a factor of 400. (b) Experimentally, the assay always followed linear kinetics, thus excluding the possibility of formation of an active species during the assay, and (c) reactivation was not efficient at the assay condition of pH 8.8 (D. Bhattacharyya, unpublished observation).

Second-order kinetics of reactivation was followed using the relation, $C^2/P = 1/Kt + C$, where K is the second-order rate constant, P is the formation of the product, i.e., catalytically active protein that could be monitored against time t, and C is a constant. During reactivation, the concentration of the active protein was measured by monitoring the activity of the renatured state. It has been verified that the specific activity of the renatured protein was between 90 and 100% of that of native enzyme.

Gel Filtration on Bio-Gel P-200. Epimerase (1 mg) was loaded onto a Bio-Gel P-200 column (82 × 0.75 cm) which was equilibrated with sodium phosphate buffer (20 mM, pH 7.0) at 4 °C. The flow rate was 8 mL/h and the fraction size was 1 mL. The protein content in the fractions was detected using Bio-Rad Protein Assay dye reagent (Bio-Rad Laboratories), and absorbance was read at 595 nm. To obtain the chromatogram of the denatured enzyme, 2 mg of epimerase was diluted to 1 mL by 8 M urea and denatured for 2 h before it was loaded onto the column. The column could not be equilibrated in the presence of 8 M urea due to poor flow rate. It was precalibrated with the following marker proteins: aldolase (158 000), hemoglobin (64 000), chicken egg ovalbumin (45 000), and carbonic anhydrase (29 000).

Size-Exclusion HPLC. Epimerase (60 μg) in the native, denatured, or renatured state was passed through an LKB Ultropac-TSK G3000SW size-exclusion HPLC column (0.75 × 30 cm) equilibrated with sodium phosphate buffer (20 mM, pH 7.0). The flow rate was 0.5 mL/min, and the elution of protein was followed by measuring absorbance at 280 nm. This HPLC system can clearly differentiate between the dimeric and monomeric states of epimerase within a reasonably short time compared to the time of reassociation during reactivation. However, the molecular weight of the proteins could not be determined accurately under the conditions employed here, possibly because of nonspecific ionic interactions of charged groups of the gel matrix with the protein which are not neutralized by the low-salt buffer. This has been termed as "non-ideal SE-HPLC" (Regnier, 1983). Epimerase undergoes tetramerization in high-salt buffer. Dimeric epimerase was also eluted from the column at a position where resolution was not high.

Spectroscopic Methods. The enzyme assay was carried out using either a Hitachi 3200 U or a Beckman DU 6 recording spectrophotometer. Circular dichroism (CD) measurements between 200 and 300 nm were made with a Jasco V-500C spectropolarimeter using cells of either 2 mm or 1 cm path length at 20 °C. All fluorescence measurements were done with either an Aminco Bowman spectrofluorometer using a 100-μL quartz cuvette or a Hitachi F4020 spectrofluorometer with a standard 700-μL cuvette.

Estimation of NAD. Estimation of NAD bound to epimerase after denaturation with urea or in the native form was performed as follows: 2 mg of protein was diluted to 1 mL by sodium phosphate buffer (20 mM, pH 7.0) in presence and absence of 8 M urea and was incubated at 20 °C for 10 min or 2 h. Excess urea and any presumably dissociated NAD were removed from epimerase by passing the solution through a spin column containing Sephadex G-50 (Maniatis *et al.*, 1982). The eluted protein was denatured by 70% ethanol, and the solvent was removed by warming under vacuum. The residue containing denatured protein and NAD, if any, was extracted with 1 mL of 0.2 M glycylglycine buffer, pH 8.8. Protein was removed by centrifugation, and NAD in the supernatant was estimated spectrophotometrically at 340 nm by alcohol dehydrogenase (Ray & Bhaduri, 1976).

Reagents. Urea (AR, E. Merck, India) was further recrystallized from hot ethanol to remove possible contamination by cyanate ions (Fröhlich & Jones, 1987). Fine chemicals, e.g., UDP-gal, NAD, ANS, EDTA, alcohol dehydrogenase, 2-mercaptoethanol, etc., were purchased from Sigma (St. Louis, MO). All other laboratory reagents were of analytical grade and were purchased from a local market.

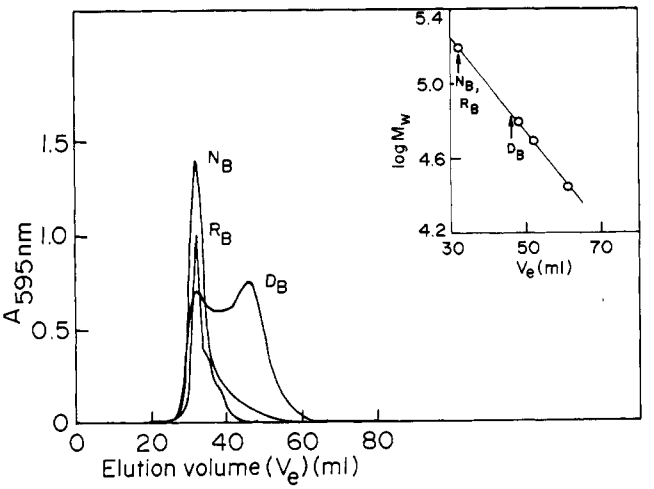


FIGURE 1: Determination of molecular weight of epimerase by chromatography on a Bio-Gel P-200 column. The elution profiles of epimerase are denoted as N_B (native), D_B (denatured), and R_B (renatured). The subscript B stands for Bio-Gel. The protein content of the fractions was determined by measuring absorbance at 595 nm after reaction with Bio-Rad Protein Assay dye reagent. Experimental conditions have been described in the text. Inset: Molecular weight calibration curve. The column was calibrated with standard molecular weight markers (from left to right): aldolase (158 000), hemoglobin (64 000), chicken egg ovalbumin (45 000), and carbonic anhydrase (29 000). Elution positions of native (N_B), denatured (D_B), and renatured (R_B) epimerase have been marked by arrows in the curve.

UDP-glucose dehydrogenase was partially purified from beef liver (Zalitis *et al.*, 1972).

RESULTS

Quaternary Structure and Dissociation of Subunits. The quaternary structure of epimerase has been reported to be dependent on the ionic strength of the medium. In very low-strength buffer (e.g., 1 mM Tris-HCl, pH 7.0), it is dissociated to monomer, in medium-strength buffer (e.g., 20 mM Tris-HCl, pH 7.0) it remains at the dimeric state, while in high ionic strength buffer (e.g., 100 mM Tris-HCl, pH 7.0), it is tetramerized (Darrow & Rodstrom, 1970). To avoid the complication of tetramerization, conditions have been selected for this study where the enzyme attains a dimeric state. This is also a reason for selecting urea as the denaturant instead of guanidinium hydrochloride, because the latter always contributes to the ionic strength of the medium while the former does not. In order to establish the dimeric state of the enzyme used in this study, and to exclude the possibility of the existence of other dissociated or oligomeric states of the enzyme formed during preparation and/or storage, it was passed through a precalibrated Bio-Gel P-200 column. The native protein was eluted as a single peak of apparent MW 158 000 and no protein was detected at or near the void volume of the column. This indicates that the preparation was free from aggregated multimeric products or tetramers. When denatured by 8 M urea and passed through the same column equilibrated with buffer in the absence of the denaturant, epimerase eluted as two overlapping peaks—one close to the native dimeric peak and the other of MW 69 000, which corresponds to about one-half of its native molecular weight. This clearly indicates the dimeric state of the native molecules. Since it took 8 h for the denatured protein to elute from the column, a part of the dissociated monomer appears to be dimerized (Figure 1). From the calibration curve, the Stokes radii of native dimeric epimerase and its dissociated state (presumably folded monomer) were 48.3 and 33.9, respectively. The usefulness of Bio-Gels in low-strength buffer has been mentioned by Ackers (1975).

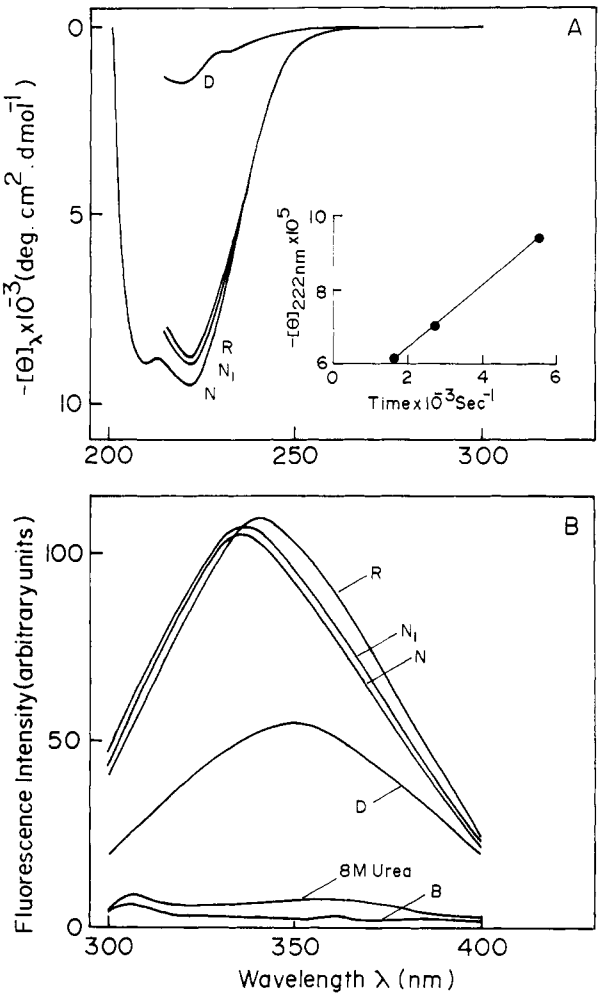


FIGURE 2: (A) Circular dichroism spectra of native, denatured, and renatured epimerase. Epimerase (10 μL, 6 mg/mL) was diluted up to 1 mL by sodium phosphate buffer (20 mM, pH 7.0) and was scanned (tracing N, native). Epimerase (10 μL) was diluted to 1 mL with the same buffer containing 8 M urea for denaturation. The loss of secondary structure was complete by 10 min, and no further change of the spectrum was observed even after incubation for 2 h (tracing D, denatured). Epimerase (10 μL) was incubated with 40 μL of 8 M urea for 10 min to ensure complete denaturation. Renaturation was initiated by diluting the denaturant to 1 mL with the same phosphate buffer in the presence and absence of 1 mM NAD. Recovery of secondary structure was at a maximum after 10 min in either case (tracing R, renatured). The concentration of urea in the renaturing solution was 0.4 M. Therefore, native epimerase was also incubated with 0.4 M urea for 2 h, and the spectrum was scanned to compare with the renatured enzyme (tracing N_I). Each spectrum was corrected using a base line having the same composition of solvent. When urea was present, the spectrum could not be scanned below 215 nm due to noise from the instrument. Inset: Kinetics of regain of secondary structure during renaturation. Renaturation of epimerase was performed as described above, and the gain of molar ellipticity at 222 nm, $[\theta]_{222}$, was followed against time. The double-reciprocal plot of the kinetics of increment of $[\theta]_{222}$ yielded a second-order rate constant K of 27.2×10^3 M^{-1} s^{-1}. Final protein and urea concentrations were 422.4 μM (0.64 mg/mL) and 0.4 M, respectively. The temperature was 20 °C. (B) Fluorescence spectra of native, denatured, and renatured epimerase with 280-nm excitation. Emission spectra of epimerase in sodium phosphate buffer (20 mM, pH 7.0) (tracing N), in the same buffer containing 0.4 M urea (tracing N_I), or containing 8 M urea (tracing D), and renatured by dilution from 8 to 0.4 M urea (tracing R) have been illustrated. The spectra of buffer (B) and 8 M urea are shown at the bottom and have not been corrected from the sample spectrum. During renaturation, the concentration of denaturant could not be reduced below 0.4 M because dilution of the enzyme restricted optical measurements.

Native and Denatured States of Epimerase. The circular dichroism spectrum of epimerase in the far-UV region (200–300 nm) in sodium phosphate buffer (20 mM, pH 7.0) is

shown in Figure 2A. It shows a maximum negative ellipticity at 222 nm, characteristic of a protein having an α-helix. A rapid estimation of the helix content using the relation, $[\theta]_{222} = 30000f_H - 2340$, where $[\theta]_{222}$ is molar ellipticity at 222 nm and f_H is fraction of helix content ($0 \leq f_H < 1$) (Chen & Yang, 1971), computes that epimerase contains 23.4% α-helix. The structure was stable for 4 h at 20 °C as no change of spectrum was observed. Incubation of epimerase by 8 M urea for 10 min reduces $[\theta]_{222}$ to only 12% of its native form. Thus, almost complete unfolding of the molecule is indicated. The residual 12% structure could not be melted by prolonged incubation with 8 M urea. A hydrophobic core of a protein that still does not melt under strong denaturing conditions is thought to be the site of nucleation during folding (Ghelis & Yon, 1982). The fluorescence emission spectra of native and denatured epimerase with 280-nm excitation have been shown in Figure 2B. Epimerase exhibits an emission maximum at 335 nm. For the denatured protein, the emission maximum shifted from 335 to 350 nm with 50% quenching of fluorescence intensity. These spectra are typical for a protein having tyrosine and tryptophan residues and going from a hydrophobic to a hydrophilic environment upon denaturation (Teipel & Koshland, 1971a,b). Denaturation followed by fluorescence or circular dichroism was complete by 10 min.

ANS (1-anilino-8-naphthalenesulfonic acid), a hydrophobic fluorophore, reacts nonspecifically with many proteins, with enhancement of fluorescence intensity (ex: 375 nm, em: 480 nm) together with a blue shift of emission maxima (520 → 480 nm) [see Slavik (1982) for a review]. Interestingly, ANS goes to the substrate binding subsite of the catalytic region of epimerase because it could be replaced by 5′-UMP, a strong competitive inhibitor for the substrate UDP-gal. Therefore, ANS can be used to monitor the substrate binding site of the enzyme even when the enzyme becomes inactive (Samanta & Bhuduri, 1982, 1983). The denatured protein fails to produce an enhancement of the fluorescence intensity with this probe, indicating complete disorganization of the subsite (Figure 3A). While epimerase was catalytically stable at 20 °C in the phosphate buffer for 4 h, it was completely inactivated by 8 M urea within 30 s at 0 °C. Estimation of bound NAD to the native and denatured states of the enzyme as described in Materials and Methods indicated that 0.89 and 0.05 mol of the cofactor were bound per mole of the enzyme, i.e., the cofactor moiety was completely dissociated from the enzyme. Consequently, the characteristic coenzyme fluorescence[2] was also completely lost (Figure 3B). Estimation of the dimeric structure by size-exclusion HPLC (will be described later) indicated that the denatured protein was constituted of only the monomer population. All of this evidence concludes unfolding of epimerase at the conditions employed.

Renatured State of Epimerase. Epimerase could be renatured close to its original structure within a time period of 2 h under the conditions described earlier. The dimeric structure and the same molecular shape of the renatured enzyme have been confirmed by chromatography on Bio-Gel P-200, where it eluted at the identical position as the native enzyme (Figure 1). The renatured enzyme restored 95% of its original secondary structure and had a characteristic maximum negative molar ellipticity at 222 nm (Figure 2A). The kinetics of renaturation monitored by the increase of $[\theta]_{222}$ for the first 10 min follows a straight line in a double-reciprocal second-order plot and has a rate constant of 27.2×10^3 M^{-1} s^{-1} (Figure 2A, inset). A blue shift of 10 nm (350 → 340 nm) of fluorescence emission maxima was observed with 280-nm excitation upon renaturation. This and the almost quantitative release of quenching of fluorescence intensity of the renatured state are indicative of a structure where the aromatic amino acid residues are in a buried environment (Teipel & Koshland, 1971a,b) (Figure 2B). The gain of fluorescence intensity at 335 nm followed complex kinetics (results not shown). Both processes, gain of $[\theta]_{222}$ and gain of fluorescence intensity at

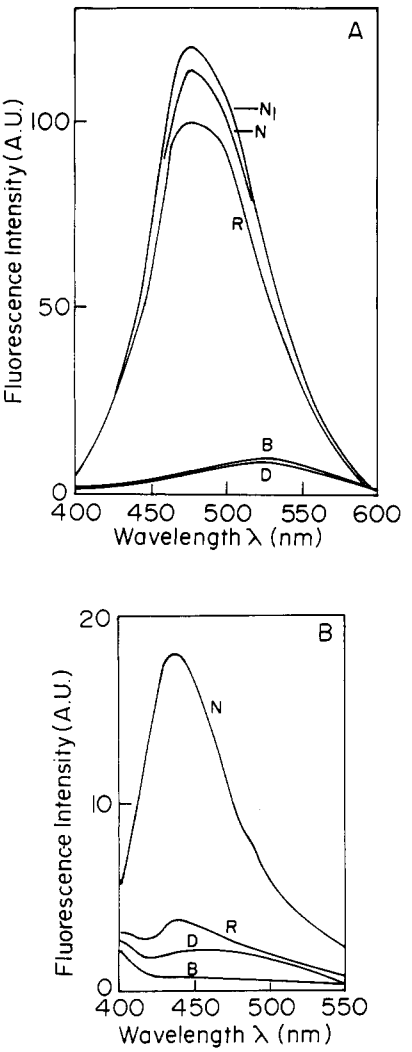


FIGURE 3: (A) Interaction of ANS with epimerase. Native (N), denatured (D), and renatured (R) epimerases were treated with 1-anilino-8-naphthalenesulfonic acid (ANS) in water at a final concentration of 100 μM. Excitation was done at 375 nm. Concentration of the protein was 60 μg/mL. Conditions for denaturation and renaturation have been described earlier. Identical emission spectra were obtained whether ANS was added after 10 min or 2 h of renaturation of epimerase (R). N_I is the emission spectrum of epimerase after interaction with ANS in the presence of 0.4 M urea, which was the denaturant concentration in the renaturing solution. (B) Coenzyme fluorescence of epimerase. The native (N), denatured (D), and renatured (R) forms of epimerase were generated as described in the text, except that in all cases the protein concentration was 250 μg/mL. Excitation was done at 353 nm. The base lines of the buffer in the presence and absence of 8 M urea were same and are presented as spectrum B. Because of its low intensity, base-line subtractions have not been done. The temperature was 20 °C.

[2] Epimerase has a very characteristic and unusual NADH-like fluorescence called "coenzyme fluorescence". Though the exact nature of the fluorophore still remains obscure, it is believed that the NAD^+ moiety at the catalytic site forms a charge-transfer complex with a cysteine residue, thus generating a NADH-like spectrum (ex: 353 nm, em: 435 nm) (Gabriel *et al.*, 1975). This fluorophore is extremely sensitive toward perturbation at the active site and has been successfully used to identify amino acids at the active site of the molecule (Ray & Bhaduri, 1980; Mukherjee & Bhaduri, 1986, 1992; Bhattacharjee & Bhaduri, 1992). However, assembly of NAD on the enzyme has no relation to the maintenance of coenzyme fluorescence and hence to activity. In fact, a preparation which is active but devoid of coenzyme fluorescence has been termed a "dark enzyme".

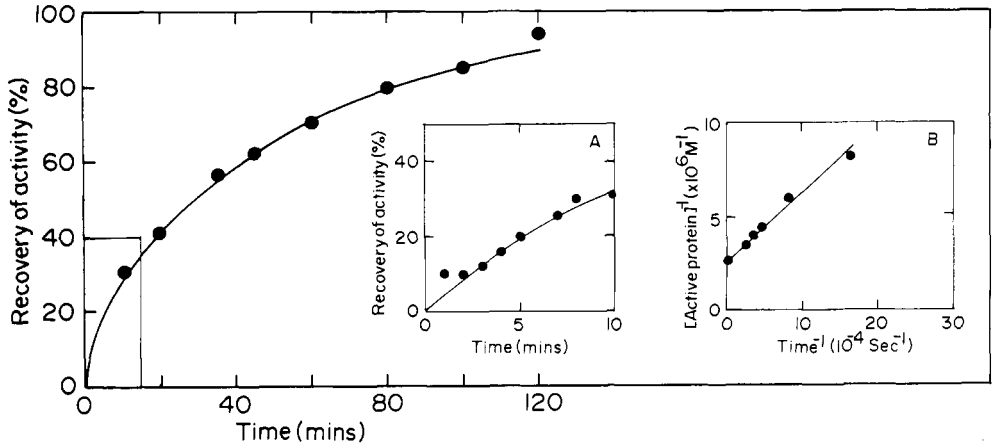


FIGURE 4: Kinetics of reactivation of epimerase. Conditions of denaturation and renaturation have been described in the text. The assay was followed spectrophotometrically for 4 min. It is assumed that, during the assay, further reactivation was inhibited (as discussed in the text) and therefore no correction has been made. Final concentrations of protein and denaturant were 0.396 μM (concn of dimer, 60 μg/mL) and 0.4 M, respectively. Inset A: Enlargement of the beginning of the kinetics showing the absence of the lag phase. The experiment was performed as described above, except that for each point a new renaturation mixture was made. Inset B: Double-reciprocal plot of the kinetics of reactivation. Points were obtained from this figure, and the value of the second-order rate constant, k, was 1.48×10^3 M^{-1} s^{-1}. If we assume that the native and renatured states have the same specific activity, the active protein concentration was estimated by measuring the activity.

335 nm, were complete by 10 min of renaturation and were independent of extraneous NAD concentration (0–1 mM) in the renaturing buffer. During renaturation the protein can fully interact with ANS from an early stage of folding (10 min) when the enzyme is inactive and can be replaced by 5′-UMP (Figure 3A). This interaction was independent of the extraneous NAD concentration and thus was independent of the rate of dimerization (will be described later). Therefore, it is likely that the folded monomer is sufficiently structured to have a site similar to its substrate binding site.

A remarkable difference between the properties of the native and renatured enzymes is that the latter poorly recovers the coenzyme fluorescence—a maximum of 16% within 10 min of renaturation (Figure 3B). This recovery was variable and was independent of the recovery of activity. It appears that, once disrupted, this fluorophore may not be regenerated *in vitro*. Dissociation of epimerase by (*p*-chloromercuri)benzoate and reconstitution by reduction also regenerated an active enzyme which was devoid of coenzyme fluorescence (Darrow & Rodstrom, 1966, 1970).

Reactivation of Epimerase. Epimerase could be reactivated from the completely unfolded state induced by 8 M urea by dilution with sodium phosphate buffer (20 mM, pH 7.0), provided extraneous NAD was added (1 mM). The time course of refolding of epimerase as measured by the reappearance of its activity is illustrated in Figure 4. As shown, within 2 h of renaturation, 90% of the activity could be recovered. Extrapolation to infinite time of renaturation kinetics shows that 100% of the original activity could be regained.[3] To check whether a lag phase is operating at the beginning of folding, the initial state of reactivation (within 10 min) was followed carefully. As described in Figure 4 (inset A), within experimental error no such lag phase of reappearance of activity could be detected.

The reappearance of the activity is governed by a second-order reaction. This is shown by the straight line obtained in a double-reciprocal, second-order plot (Figure 4, inset B). Therefore, the reaction which yields an enzymatically active species has a rate constant of 1.48×10^3 M^{-1} s^{-1} at 20 °C. This process is about 18.5 times slower than the rate of formation of secondary structure as monitored by circular dichroism. The renatured enzyme was not, however, thermally stable. Once it recovered maximum activity, it was slowly inactivated under the renaturing conditions. A drop of about 40% of activity was observed for a further incubation for 2 h.

The rate and also the extent of recovery of activity were found to be dependent on the protein concentration during folding. Approximately 10–60 μg/mL protein was optimum for reactivation. Higher or lower concentrations of protein led to poor recovery of activity, although no aggregate was formed in any case. In the presence of 1 mM NAD, the reappearance of activity was governed by a second-order reaction that was dependent on protein concentration only. This is shown by the straight lines obtained in a double-reciprocal, second-order plot as well as by the concentration dependence of the slopes of the straight lines (Figure 5).

The process of reactivation was found to be strongly dependent on temperature. The kinetic constants of reactivation at 0, 10, 20, and 30 °C were 0.18×10^3, 0.37×10^3, 1.48×10^3, and 2.88×10^3 M^{-1} s^{-1}, respectively, in the presence of 1 mM extraneous NAD (Figure 6). The energy of activation (E_{act}) of the process from an Arrhenius plot was estimated to be 15.2 kcal/mol (64.4 kJ/mol) (Figure 6, inset). The Lineweaver–Burk plot for the determination of K_m for the substrate UDP-gal yielded a value of 0.10 mM for the native and fully reactivated enzymes. The same value was reported earlier by Maxwell (1957) (0.11 mM) and by Ray and Bhaduri (1975) (0.13 mM). Since the renatured enzyme was fully catalytically functional, dimerization and assembly of a stoichiometric amount of NAD are obvious.

Dimerization of Monomer. The elution profiles of native, denatured, and renatured epimerase from an LKB TSK-G3000 SW SE-HPLC column equilibrated with sodium phosphate buffer (20 mM, pH 7.0) have been shown in Figure 7A. Since a silica gel based SE-HPLC column could not be calibrated in such a low ionic strength buffer (Regnier, 1983), the eluted peaks were passed through a Bio-Gel P-200 column to ascertain their molecular states, as described earlier. During rechromatography, the native and renatured enzymes were eluted at an apparent molecular weight of 158 000, and the denatured protein was eluted at a molecular weight of 69 000. This

[3] Although the recovery of activity was between 80–100%, sometimes it was as poor as 30%. The yield was dependent on the age of the purified enzyme and not on its specific activity. It is suspected that a trace amount of undetectable protease cleaves the molecule without affecting its activity, but interferes with folding. No elaborate study in this direction has been done.

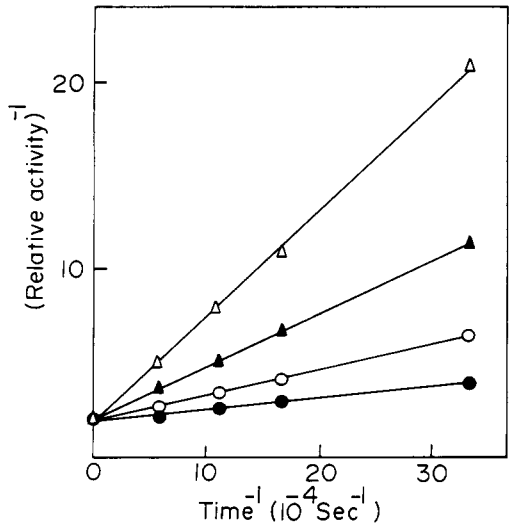


FIGURE 5: Dependence of kinetics of reactivation of epimerase on protein concentration. Epimerase was denatured and renatured as has been described in the text, except that the final protein concentration was 60 (●), 30 (○), 15 (▲), and 7.5 (△) μg/mL. All straight lines passed through the point of relative activity of 2 on the *y*-axis because 100% recovery of activity is assumed for infinite time of reactivation in each case.

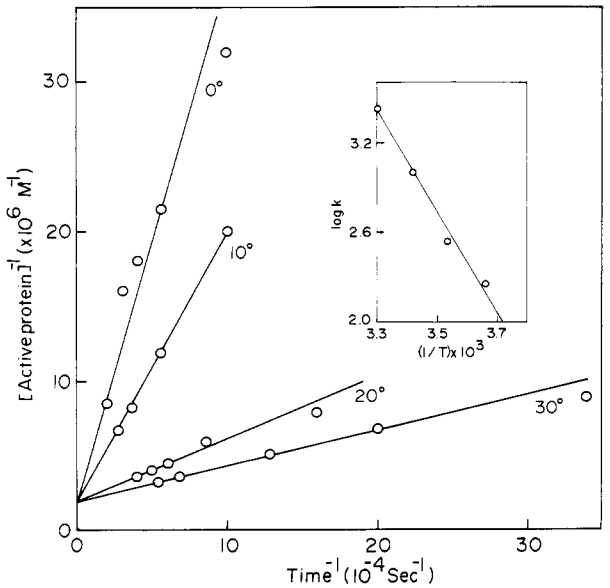


FIGURE 6: Dependence of the kinetics of reactivation of epimerase on temperature. Denaturation and renaturation of epimerase have been described in the text, except that the renaturing medium was maintained at 0, 10, 20, or 30 °C. The double-reciprocal plots of the reappearance of catalytic activity vs time have been shown. The second-order rate constants of reactivation were as follows: 0 °C, 0.18×10^3 M^{-1} s^{-1}; 10 °C, 0.37×10^3 M^{-1} s^{-1}; 20 °C, 1.48×10^3 M^{-1} s^{-1}; and 30 °C, 2.83×10^3 M^{-1} s^{-1}. Inset: Arrhenius plot for the energy of activation. Results presented are the average of two sets of data.

confirmed the dimeric and monomeric states of the enzyme once passed through an HPLC column (results not shown).

Aliquots of epimerase during renaturation (0–180 min) were passed through HPLC, and the formation of the dimer was quantitated by integrating the area of the first peak eluted from the column (Figure 7A). The rates of dimerization during renaturation in the presence and absence of extraneous NAD (1 mM) are different and are shown in Figure 7B. The rate constants of dimerization under these two conditions were calculated to be $\geq 25.0 \times 10^3$ and 2.28×10^3 M^{-1} s^{-1} at 20 °C. It was also observed that, in the presence of 1 mM NAD, the rate of dimerization was independent of the protein

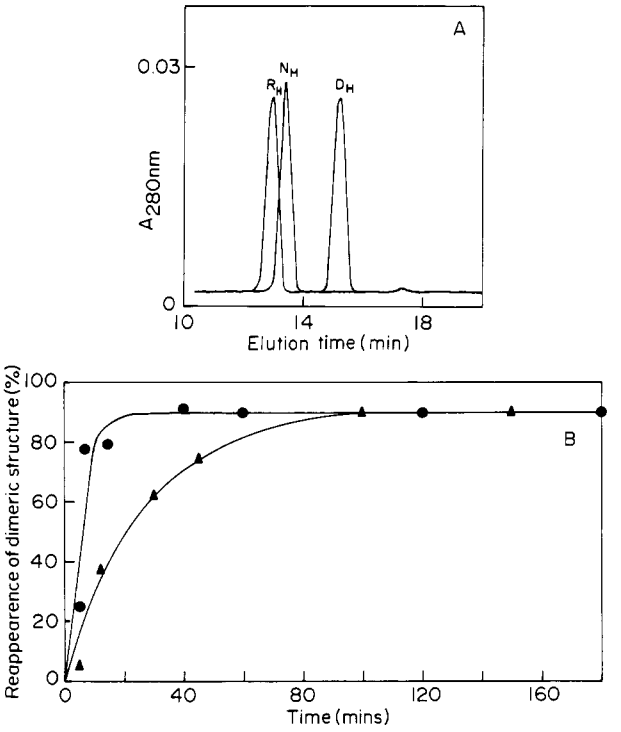


FIGURE 7: (A) Elution profiles of native, denatured, and renatured epimerase from SE-HPLC column. Epimerase was passed through a LKB-Ultropac TSK G3000SW size-exclusion HPLC column equilibrated with 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 mL/min. Elution of protein was followed by measuring the absorbance at 280 nm. The positions of epimerase (N_H), epimerase denatured by 8 M urea for 10 min (D_H), and epimerase renatured for 2 h (R_H) are illustrated in the chromatogram. The experimental conditions for denaturation and renaturation have been described in the text. Protein (60 μg) was loaded in each case. The subscript H in the figure stands for HPLC. (B) Kinetics of dimerization during renaturation of epimerase. Epimerase during renaturation was passed through the HPLC column to separate the monomeric and dimeric species, D_H and R_H, at stipulated intervals of time. The reappearance of the dimeric structure was measured by integrating the peak under R_H by one-half of the height multiplied by the base length. The 100% value was taken as a control run of the same amount of protein (60 μg) and measuring the area of the peak under N_H. The concentration of extraneous NAD during renaturation was 0 (▲) or 1 mM (●). It may be recalled that for R_H it takes 13 min to be eluted from the column, and any possible dimerization during chromatography has not been accounted for.

concentration (result not shown). It is worthwhile to mention here that it takes about 13 min to separate the dimer from the monomer in the HPLC column and any dimerization during that period has not been accounted for. Therefore, the *k* values reported here have some degree of overestimation. Since dimerization was slow in the absence of NAD (30% after 10 min), but secondary structure formation was complete by that time (CD measurements), the initial folding step seems to be reorganization between a single monomer. The slower rate of dimerization in the absence of NAD makes the HPLC technique more reliable as the cofactor is separated from the protein at the initial state of chromatography.

Assembly of NAD. Since the formation of structured monomer and dimerization were complete by 20 min, but the recovery of activity was a slow second-order process (~90% recovery in 2 h) during renaturation in the presence of 1 mM extraneous NAD, it was expected that the assembly of NAD to the apoenzyme might be a rate-limiting step. Reactivation in the presence of 0–5 mM NAD shows that the stoichiometric amount of NAD present in the renaturing solution which came from the dissociation of the holoenzyme was ineffective in generating activity. A linear increase in the second-order

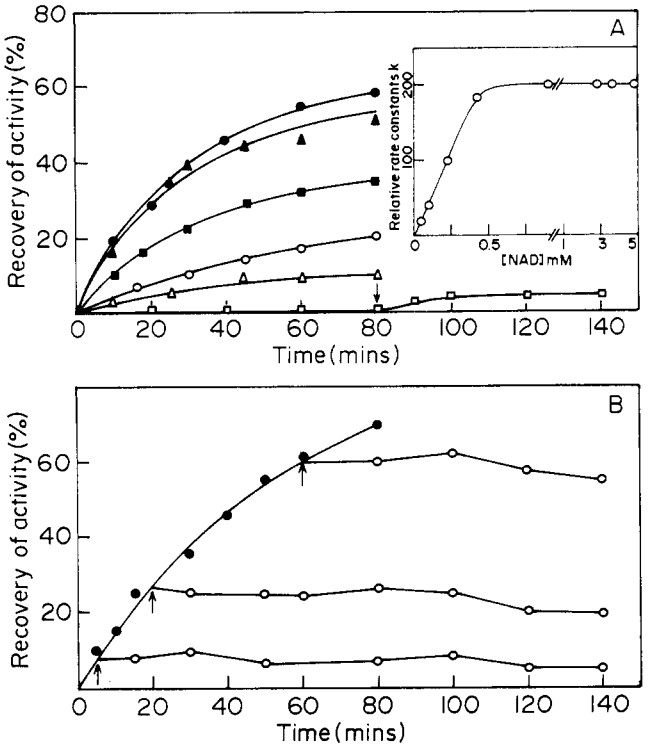


FIGURE 8: (A) Dependence of rate of reactivation on the concentration of extraneous NAD. Epimerase was denatured and renatured as has been described in the text, except that the NAD concentration was varied: 0 mM (□); 0.044 mM (△); 0.088 mM (○); 0.22 mM (■); 0.44 mM (▲); 0.88 mM or between 1 and 5 mM (●). NAD (1 mM) was added after renaturation for 80 min in the absence of the cofactor (marked by downward arrow), and the activity of the renaturing enzyme was followed for another 60 min. Inset: A double-reciprocal plot of the kinetics of reactivation for each set of experiment was drawn (See Figure 4, inset B). The relative rate constants obtained from the plots were drawn against the concentration of extraneous NAD. (B) Inhibition of reactivation by depletion of extraneous NAD during renaturation. Epimerase was denatured and renatured as described in the text. Kinetics of reactivation is been represented by ●. In parallel experiments during renaturation, at three different points (5, 20, and 60 min) (marked by upward arrows), samples were passed through Sephadex G-50 columns to remove any unbound NAD. Activity of the NAD-depleted proteins was measured for a period of 1 h or more (○). Removal of unbound NAD was done as follows: 1.6-mL columns (50 × 5 mm) were packed with preswelled Sephadex G-50 and equilibrated with sodium phosphate buffer (20 mM, pH 7.0) containing 5 mM 2-mercaptoethanol. After spinning in a low-speed laboratory centrifuge (Remi) for 2 min at 2000 rpm to remove excess buffer from the gel, 100-μL samples were loaded per column and were spun identically. The average recovery of protein was between 90 and 95%. Quantitative retention of small molecules was checked by using strongly absorbing colored materials as well as by radioactive compounds. All experiments were done at 20 °C.

rate constant of reactivation was observed with the increase in extraneous NAD concentration up to about 0.5 mM, and the increase finally reached a limit (Figure 8A and inset). Higher concentrations of NAD could not enhance the rate of reactivation, nor was a pseudo-first-order reaction observed. Therefore, if the association of NAD to the dimer was the rate-limiting step in the presence of a low concentration of NAD, there must be another second-order, rate-limiting reaction operating in the presence of a high concentration of NAD (1–5 mM). Thus it is likely that an intermediate exists between the inactive apoenzyme and the active holoenzyme, which have identical structures. The most probable answer appropriate to this situation is the formation of the NAD binding site of the apoenzyme. It has been verified that, although a low concentration of NAD slows the rate of dimerization, this inhibition is not sufficient to account for the slower rate of reactivation. For example, in the presence of 0.088 mM NAD, dimerization was complete by 1 h but recovery of activity was only 16%.

If the renaturation was performed in the absence of NAD for 1 h, abortive folding of the protein prevented reactivation, even if a higher concentration of NAD was added afterward (marked by a downward arrow in Figure 8A). This indicates that NAD binding site formation was a cooperative process and was influenced by NAD itself (like substrate induction).

Once NAD was bound to the dimer structure, it may not yield an active enzyme, but rather a further conformational readjustment might be necessary to express the activity. Such very minor but discrete adjustments of structure are not usually detectable by the methods used in this study. Therefore, the intermediate formed during renaturation was passed through a Sephadex G-50 column to remove unbound NAD, and the activity of the partially active enzyme was followed for 1 h or more. If any inactive enzyme was separated with bound NAD, it should be reactivated by that time. However, the regain of activity was totally stopped whenever the extraneous NAD was removed at any stage of renaturation (Figure 8B).

DISCUSSION

Under a defined set of conditions, functional properties of epimerase reappear from a completely unfolded and dissociated state induced by 8 M urea. The renatured enzyme resembles its native state in terms of most of its physicochemical properties, especially those which are associated with the active site of the molecule, e.g., activity, and therefore dimeric structure and assembly of NAD, K_m for the substrate UDP-gal, substrate binding site (ANS fluorescence), etc. The molecular properties of the renatured enzyme, e.g., molar ellipticity at 222 nm, protein fluorescence spectrum, hydrodynamic volume, etc., are also very similar to those of the native enzyme. These have been summarized in Table I. This strongly suggests that, in spite of minor differences, e.g., coenzyme fluorescence or thermal stability, the process could be followed to establish the folding pathway of the enzyme. A significant aspect of the renaturation process is that it is completely free of aggregation. This is in contrast to the folding pattern of many oligomeric proteins where aggregation of a folding intermediate enters into kinetic competition in the proper folding pathway (Jaenicke & Rudolph, 1980). The reactivation process in the presence of 1 mM NAD was temperature dependent, having an E_{act} of 15.2 kcal/mol. An E_{act} of 20 ± 2 kcal/mol is sometimes considered to be due to the cis–trans isomerization of proline residues during denaturation (Brandts *et al.*, 1975). The possible involvement of proline isomerization is now under investigation.

For several oligomeric proteins, it was observed that the reappearance of activity during folding followed biphasic kinetics—a first-order reaction preceding a second-order reaction. The first reaction, which yields an inactive species, therefore produces the lag phase. It is believed to be a folding step occurring within a single polypeptide chain (Rudolph *et al.*, 1977; Engelhard *et al.*, 1976; Jaenicke & Rudolph, 1980; Dautry-Varsat & Garel, 1978, 1981). The folded monomer subsequently associates into active dimer and oligomer. The absence of this lag phase in the case of epimerase indicates that there is a single rate-limiting step in expressing activity, and all preceding and subsequent steps must be rapid (Creighton, 1990).

Identification of various intermediates involved in the process of protein folding is suggestive of the fact that large conformational changes occur at the beginning of the process with minor readjustments at the end. These minor adjustments may be insignificant in amplitude in terms of structural

Table I. Properties of Native, Denatured, and Renatured Epimerase

	native protein	denatured by 8 M urea	renatured
catalytic activity (sp. act.)	100% (45)	0% (0)	80–100% (40–45)
K_m for UDP-gal at pH 7.6 (mM)	0.1	N/A[a]	0.1
thermal stability	catalytically stable for 4 h at 20 °C at pH 7.0	N/A	loses 40% activity in 2 h after maximum reactivation
molecular weight	158 000	69 000	158 000
Stokes radius[b]	48.3	33.9	48.3
$[\theta]_{222}$	100% (taken as control)	12%	95%
excitation at 280 nm (protein fluorescence)			
FI at 335 nm[c]	100	50	110[d]
emission maximum (nm)	335	350	340
excitation at 353 nm (coenzyme fluorescence)			
FI at 435 nm	100	7	16[d]
excitation at 375 nm (interaction with ANS)			
FI at 475 nm	100	5	80[d]
emission maximum (nm)	475	525	475

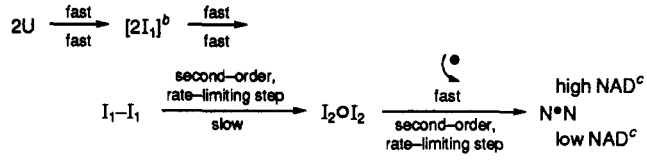
[a] N/A = not applicable. [b] The Stokes radius (r_S) was calculated from the straight line, log r_S vs elution volume, obtained from the gel filtration experiment (Figure 1) using the following values (r_S): aldolase (48.3), hemoglobin (33.2), ovalbumin (31.2), and carbonic anhydrase (23.6) (Corbett & Roche, 1984). [c] FI indicates fluorescence intensity in arbitrary units, and in each case a value of 100 is taken for the native protein. [d] The properties of the renatured protein are in 0.4 M urea. This concentration of denaturant causes minor (5%) perturbation in the structure of the enzyme.

parameters but of crucial importance in bringing back the functional properties, e.g., refolding of aspartokinase homoserine dehydrogenase (Vaucheret *et al.*, 1987). Therefore, in the case of epimerase, the assembly of NAD over a dimer structure *per se* may yield an inactive holoenzyme, and the inactive species may further isomerize to form the active holoenzyme. The moderately bulky size of dimeric epimerase and sharing of the active site by either of the subunits may possibly be reasons for such consecutive steps. To check this, epimerase during renaturation in presence of 1.0 mM extraneous NAD was passed through Sephadex G-50 (Figure 8B). In no case was any further gain in activity of the eluted enzyme observed. This indicates that, once NAD was assembled over the dimer, it yielded an active protein and there was no intermediate in between.

The regain of the catalytic activity of epimerase in the presence of excess extraneous cofactor NAD (1.0 mM) was a lengthy process of 2 h. Since almost quantitative recovery of secondary and tertiary structures was complete within a short time of renaturation (10 min), it is obvious that the major folding of the polypeptide chain was complete at any early stage. This appears to be true for many large polypeptide chains (Wetlaufer, 1973; Schulz & Schirmer, 1979; Fazel *et al.*, 1983; Müller & Garel, 1984). From the structure of epimerase, the rate-limiting step of the reappearance of activity appears to be any one of the following: (a) interaction between two monomers to form a dimer (apoenzyme); (b) the assembly of cofactor NAD on the dimer; or (c) interaction between two different parts of the same molecule (monomer or dimer). As a result, the activity of the molecule is expressed. The former two possibilities are bimolecular second-order association types of reactions, while the last one is a unimolecular second-order isomerization type of reaction.

SE-HPLC studies ruled out the first possibility because dimerization was complete by 10–15 min of renaturation. To explore the second possibility, renaturation was performed in the presence of varying concentrations of NAD (0–5 mM). Interestingly, it was observed that the rate of reactivation was dependent on NAD concentration only up to a limit (0.5 mM) (low NAD) and was independent in the range 1–5 mM (high NAD), although the kinetics of reactivation was second-order in either range (Figure 8A). This clearly indicates that there are two rate-limiting steps operating in the process of reactivation of the enzyme under two different conditions. It was also observed that the rate of dimerization was independent of protein concentration in the presence of 1 mM NAD. These

Scheme I[a]



[a] U is the unfolded monomer; I_1 is the folded monomer; $I_1 - I_1$ is the folded dimer; $I_2 \circ I_2$ is the folded dimer (apoenzyme) with NAD binding site; ○ is the NAD binding site; ● is NAD; and N•N is the renatured, active holoenzyme. [b] The existence of folded monomer during renaturation in the absence of NAD has been demonstrated. But as the concentration of the cofactor is raised, the kinetics of dimerization becomes comparable with folding. Intermediate I_1 has been put into brackets to indicate this fact. [c] Rates in the upper and lower set are valid in the presence of 1–5 and 0–0.5 mM NAD, respectively.

two observations in combination with previously discussed events strongly suggest that, *en route* to renaturation (at high NAD), the formation of the NAD binding site is the slowest step. This site is apparently formed by a second-order reaction between two subunits of the folded apoenzyme.

In the presence of low NAD, the slowest step of reactivation cannot be the process of dimerization because it is a diffusion-controlled reaction between two molecules of protein only. Also, at intermediate concentrations of NAD (e.g., 0.088 M NAD), dimerization was much faster than reactivation. For a similar reason, the NAD binding site formation, although a slow second-order process, cannot be rate-limiting. Therefore, in this case the final interaction of NAD with the apoenzyme appears to be the slowest step.

On the basis of the observations presented in this article, a preliminary model for the folding of epimerase has been proposed in Scheme I. This scheme does not include the intermediates formed, if any, at the early stage of renaturation, e.g., before 10 min. Therefore, the process of folding may be tentatively divided into four major parts.

(1) In the first phase of folding, circular dichroism and fluorescence studies indicate that the secondary and tertiary structures are mostly recovered within 10 min. The substrate binding site is also formed by this time. This process has a k of 27.2×10^3 M^{-1} s^{-1}.

(2) In the second phase, folded monomers reassociate to form the dimer. This process has been followed by SE-HPLC and has k values of $\geq 25.0 \times 10^3$ and 2.25×10^3 M^{-1} s^{-1} in the presence and absence of 1 mM NAD. The structured dimer is inactive apoenzyme.

(3) The NAD binding site is then formed on the dimeric structure, which is possibly induced by the cofactor. Kinetic studies of reactivation show that this is a second-order rate-limiting step in the presence of high concentrations of NAD (1–5 mM) and has a k of 1.48×10^3 M^{-1} s^{-1}. This step is independent of NAD concentration.

(4) Kinetic studies of reactivation also suggest that, once the cofactor binding site is formed, NAD assembles over it and yields an active holoenzyme. This is also a second-order process and is rate-limiting in the presence of low concentrations of NAD (0–0.5 mM).

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