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The Two-Step Reversible Denaturation of Lactate Dehydrogenase at Low pH†

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ABSTRACT: Upon exposure to conditions of low pH, beef B_4 lactate dehydrogenase rapidly loses enzymatic activity, but this process can be completely reversed yielding 100% of the original activity if the enzyme is immediately returned to neutral conditions. As the time of exposure to low pH is increased, the fraction of activity recovered declines to a value of 50–60% and remains nearly constant over a period of many hours. Correlated with this behavior is a change in the kinetics of the recovery of activity. Recovery of activity has been shown to be a second-order process for enzyme exposed to low pH for brief periods of time (Anderson, S., and Weber, G. (1966), *Arch. Biochem. Biophys. 116,* 207). After several minutes at low pH recovery of activity is found to become first order and to occur at a considerably slower rate. Gel filtration chromatography at low pH separates the protein into two fractions. The lower molecular weight fraction is found to be primarily monomeric, as indicated by equilibrium ultracentrifugation, and is capable of recovering enzymatic activity. The higher molecular weight fraction is generated from the lower molecular weight fraction, and is incapable of recovering activity. These results are interpreted to indicate that the enzyme exists sequentially in three denatured forms at low pH, the first two capable of being restored to the native state, and the third irreversibly denatured.

Lactate dehydrogenase is a tetrameric protein of molecular weight equal to about 140,000 (Appella and Markert, 1961; Jaenicke and Knof, 1968; Adams et al., 1970). It appears to undergo reversible dissociation to subunits under a variety of conditions, as indicated by the technique of isozyme hybridization (Markert, 1963; see reviews by Jaenicke, 1970; Everse and Kaplan, 1973). In general, dissociation is accompanied by irreversible denaturation of the protein, behavior which has complicated the interpretation of direct physicochemical data on the dissociated state. A number of reports have been published indicating that fully reversible dissociation of lactate dehydrogenase may be induced by exposure of the enzyme to low pH (Anderson and Weber, 1966; Levitzki, 1972; Levitzki and Tenenbaum, 1975). Loss of catalytic activity at low pH may be completely reversed upon neutralization; and, if two isozymes are exposed together to such treatment, the formation of hybrid isozymes results. Recovery of activity was found to follow second-order kinetics in the case of beef A_4[1] and B_4 lactate dehydrogenase (Anderson and Weber, 1966; Levitzki and Tenenbaum 1975; S. Anderson, personal communication), further evidence that these isozymes become dissociated at low pH. For all isozymes examined, full recovery of the native condition may be obtained only if exposure of the enzyme to acidic conditions is of very short duration, exposure for longer than several minutes resulting in irreversible loss of activity. This suggests that the dissociated state is unstable and indicates that interpretation of physicochemical data must still be approached with caution.

Direct characterization of the enzyme at low pH has shown the molecular weight to be lower than that of the native tetramer (Deal et al., 1963; Jaenicke and Knof, 1968; Anderson and Weber, 1966; Millar et al., 1969). Millar and coworkers using enzyme prepared from beef heart presented evidence from equilibrium ultracentrifugation indicating the existence of a number of different molecular weight species at low pH in rapidly reversible equilibrium. The minimum molecular weight observed was approximately 18,000, about half the previously reported monomer molecular weight (Appella and Markert, 1961). The centrifugation data were found to be consistent with a scheme involving the reversible association of the 18,000 species to polymeric forms of a size as large as the native tetramer. In contrast to the full recovery of activity obtained with enzyme briefly exposed to low pH, enzyme used in this study was found to be incapable of recovering activity. This has suggested to us that two different denatured forms of the ezyme may have been under investigation in the different studies, a short-lived form capable of complete renaturation, and an irreversibly denatured form present after the

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[1] Abbreviations used are: LDH, lactate dehydrogenase; A_4 and B_4 LDH refer to the homopolymeric isozymes, composed of four A or four B subunits, and are equivalent to M_4 and H_4 LDH, respectively.

long exposure to acidic conditions involved in the equilibrium studies. We report here results confirming this hypothesis and showing, in addition, that before becoming irreversibly denatured, the enzyme exists sequentially in two distinct forms that are both capable of being restored to the native state.

Materials and Methods

The B_4 isozyme of lactate dehydrogenase (EC 1.1.1.27) from beef heart was obtained from the Worthington Biochemical Corp. The preparations used were judged to be pure by vertical starch gel electrophoresis (Smithies, 1955) using a stain specific for LDH activity (Massaro, 1967), and by acrylamide disc gel electrophoresis (Davis, 1964) using Amido Black. All chemicals were reagent grade and solutions were prepared using glass-distilled water. pH was determined at room temperature.

Enzyme stored in the crystalline state in 55% saturated ammonium sulfate was prepared for use by extensive dialysis against several changes of 0.002 *M* sodium phosphate buffer (pH 7.4). Changes in pH were accomplished by dilution unless noted otherwise. Enzyme was contained only in glassware treated with "Siliclad" (Clay-Adams), or in polypropylene or Teflon vessels. Siliconized glass or polypropylene-tipped micropipets were used for all transfers involving denatured or renaturing enzyme. Mixing of solutions of enzyme to initiate renaturation was done gently since it was found that recovery of activity can be reduced significantly by excessive stirring (see Results).

Protein concentration was determined spectrophotometrically, using an extinction coefficient of 1.50 for a 1-mg/ml solution (Pesce et al., 1964). The extinction coefficient was corrected for the loss of enzyme-bound nucleotide (Wieland et al., 1962) which occurred during chromatography at pH 2 or 3. Catalytic activity was determined at 25° from the rate of change in absorbance at 340 nm due to oxidation of NADH, after the method described by Pesce et al. (1964). The assay solution contained 1.4×10^{-4} *M* NADH and 5×10^{-4} *M* sodium pyruvate, in 0.1 *M* sodium phosphate buffer (pH 7.0) plus a sufficient quantity of enzyme to produce a change in absorbance of from 0.1 to 0.2 per min.

Gel-filtration chromatography was performed at 4°. Sephadex G-100 and G-150 were products of Pharmacia. Native lactate dehydrogenase, yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, sperm whale myoglobin, Blue Dextran 2000, and sodium chloride were used as molecular weight standards. The molecular weight of the acid-denatured enzyme for the assumed case of a globular conformation was estimated according to the method of Andrews (1964, 1965).

Analytical Ultracentrifugation. Centrifugation was performed with the use of a Beckman Model E analytical ultracentrifuge equipped with Rayleigh and scanner optics, electronic speed control, and an RTIC temperature control unit, and with a helium–neon laser light source (Williams, 1972) for the interference optical system. The equilibrium sedimentation method of Yphantis (1964) was used for the determination of molecular weight as a function of protein concentration. Six-channel centerpieces were used, and the height of the individual solution columns was 2.5 mm. Blank runs using distilled water were done along with each experimental run. Data were reduced with a computer program (Roark and Yphantis, 1969) which smooths the raw data and calculates values for the apparent weight-average molecular weight at a series of concentrations throughout the solution column. A value of 0.75 ml/g was used for the partial specific volume (Markert and Appella, 1961). Samples were run at two or three initial concentrations. Plots of molecular weight vs. protein concentration for different initial concentrations will coincide for a homogeneous or reversibly associating system, but will fail to do so for a noninteracting mixture of protein species (Yphantis, 1964; Roark and Yphantis, 1969).

Short (0.7 mm) solution columns were used to decrease the time required to reach equilibrium and permit determination of molecular weight as a function of time. The method of Yphantis (1960) was used. The sedimentation coefficient of renatured LDH was measured at a single low concentration of protein with the use of the Model E photoelectric scanner.

Fluorescence measurements were made with a Baird-Atomic Fluorispec SF-1 fluorescence spectrophotometer equipped with a water-jacketed cuvet holder. Uncorrected excitation and emission peaks for native LDH were found to be located at 288 and 352 nm, respectively. Peak positions for denatured and renatured enzyme were not seen to differ significantly from these values.

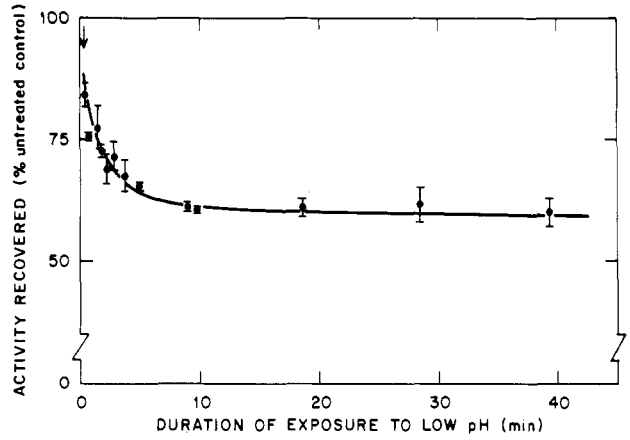

FIGURE 1: Recovery of enzymatic activity as a function of the duration of exposure to low pH. Beef B_4 LDH was diluted 20-fold into 0.1 *M* sodium phosphate buffer (pH 3.0) containing 0.002 *M* dithiothreitol at 0°, at time zero, and then 20-fold to a protein concentration of 0.021 mg/ml in 0.2 *M* sodium phosphate buffer (pH 7.3) containing 0.002 *M* dithiothreitol at 21°, at the times indicated. After 4 hr the samples were assayed as described under Materials and Methods for catalytic activity, which is plotted as the percent of the activity of an untreated control. Enzyme exposed to pH 3.0 was found to have lost all measurable activity by the time indicated with the arrow.

Results

Renaturation as a Function of Time at Low pH. Catalytic activity is no longer detectable after 15-sec exposure of beef B_4 LDH to pH 2.0 or 3.0 sodium phosphate buffer at 0°. If the inactivated enzyme is transferred into pH 7 sodium phosphate buffer at room temperature activity reappears. The percent of the original activity that may be recovered as a function of the duration of exposure of the enzyme to pH 3.0 is shown in Figure 1. It can be seen that the recoverable activity is initially quite high, but rapidly declines and after approximately 10 min levels off at a value of about 60%. Some slow decline in recovered activity may be seen to occur over the remaining period of time shown. In a separate experiment it was found that the activity recovered after 20 hr at pH 3.0 at a temperature of 0° was equal to 87% of that recovered after 1.5 hr.

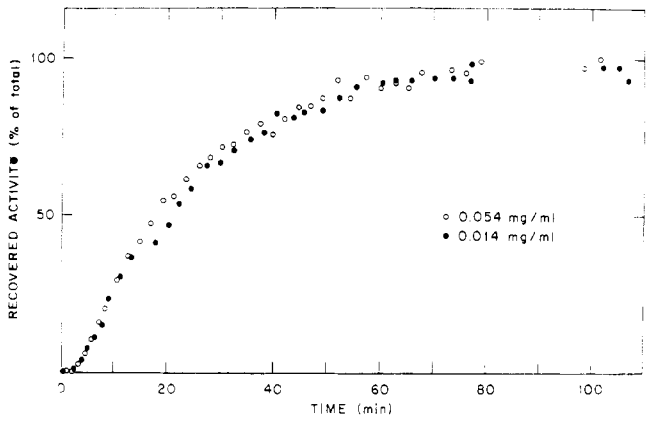

FIGURE 2: Time course of the recovery of enzymatic activity. Beef B_4 LDH was incubated for 20 min in 0.1 *M* sodium phosphate buffer (pH 2.0) containing 0.002 *M* dithiothreitol at 0°, and then diluted into 0.2 *M* sodium phosphate buffer (pH 7.3) containing 0.002 *M* dithiothreitol at 20°. The final concentration of enzyme was 0.014 (●), and 0.054 (○) mg/ml. Data are plotted as the percent of the final activity recovered, 43 and 30% of the activity of an untreated control, respectively, for the lower and higher concentration samples.

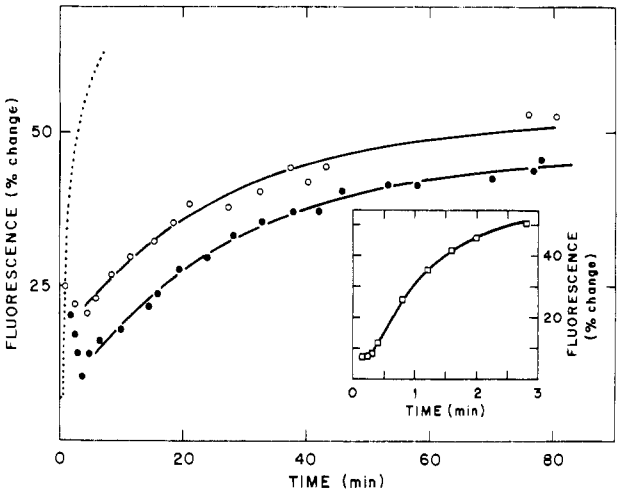

FIGURE 3: Time course of the recovery of fluorescence intensity. Main graph: buffers as described in Figure 2, denaturation for 40–50 min at 0° at a concentration of 0.37 mg/ml, renaturation begun at time zero at 20° at a concentration of 0.015 (○), and 0.0076 (●) mg/ml. Insert and dotted line: buffers as above except acidic buffer at pH 3.0, denaturation for 18 sec at 0° at a concentration of 0.35 mg/ml, renaturation begun at time zero at 20° at a concentration of 0.030 mg/ml. Data plotted as the percent of the difference in the intensity of the fluorescence of the native and of the denatured enzyme at 350 nm, both values determined at 20°.

Preliminary kinetic measurements indicated the rate, as well as the extent, of recovery of activity to be a function of the duration of exposure to acidic conditions, the rate declining with increasing time of exposure to pH 2 or 3. The time course of recovery of activity by enzyme exposed to pH 2.0 sodium phosphate buffer for 20 min, sufficient time for the rapid decrease in the extent and rate of recovery to be complete, and then transferred to pH 7.3 is shown in Figure 2 for two values of protein concentration. It may be seen that there is a distinct lag in the appearance of activity. The overall time course of reactivation appears to be independent of protein concentration, though a trend toward an increasing lag period with decreasing concentration has been noted. Semilogarithmic plots of the data shown in Figure 2 were linear following the lag in recovered activity, yielding an apparent first-order rate constant of $7.5 \pm 0.2 \times 10^{-4}$ sec^{-1}. The overall rate of reactivation is considerably slower than the second-order rate previously reported for beef B_4 LDH exposed to pH 3.0 for 30 sec (Anderson and Weber, 1966; Levitzki and Tenenbaum, 1975). Thus, it appears that the mechanism for renaturation changes with the duration of exposure of the enzyme to low pH. In contrast to the renaturation process for enzyme exposed to acidic conditions for brief periods of time, the renaturation of enzyme exposed to low pH for 20 min would appear to be rate limited by a first-order step (or several first-order steps) throughout most of its time course. The existence of more than one renaturation step preceding the appearance of an active species is suggested by the lag in the appearance of activity.

The time course of recovery of activity was found to become independent of the duration of exposure to pH 2 or 3 after 5–10 min under these conditions. It would appear from this observation and from the nearly constant level of recovered activity seen after prolonged exposure to acidic conditions that the protein attains a relatively stable denatured state at low pH. That further slow changes in the state of the protein do occur is indicated by the slow loss in recovered activity observed over a period of many hours under acidic conditions (Figure 1 and see below).

Changes in Physical Properties during Renaturation. Upon exposure to pH 2.0 or 3.0 sodium phosphate buffer at 0° the enzyme showed a 40–50% loss in the intensity of fluorescence at 350 nm. The decrease in fluorescence was found to be complete within 5–10 sec of exposure to acidic conditions. Fluorescence intensity was found to increase upon subsequent dilution of the enzyme into phosphate buffer at pH 7.3 (Figure 3). It may be seen that for enzyme exposed to acidic conditions for 40–50 min, following some initial rapid changes, the time course of the recovery of fluorescence intensity is similar to that for the recovery of activity seen in Figure 2, and appears to be independent of the concentration of enzyme used for renaturation. The rate of recovery of fluorescence intensity for enzyme exposed to pH 3.0 sodium phosphate buffer for 18 sec before being neutralized may be seen to be considerably more rapid.

Beef B_4 LDH was found to be capable of forming hybrids with the A_4 isozyme of glut herring after prolonged incubation of both enzymes at pH 3.0 (Vallee, 1974). The capacity to form hybrids was found to decrease as renaturation progressed. The presence of free subunits throughout renaturation was indicated by the formation of hybrids of the type A_3B_1 when the two enzymes were combined after recovery of activity by the B_4 isozyme had nearly reached completion.

Effect of Renaturation Conditions. A number of variables of the renaturation process were found to have a strong influence on the extent of activity recovered by enzyme exposed to pH 2 or 3 for a sufficient period of time (longer than 20 min) for the initial rapid loss in recovered activity to be complete. Recovered activity is markedly affected by the pH of the renaturation buffer in the range 6.0–7.0, the percent of the original activity recovered increasing from near zero below this range to about 55% above pH 7.0 (Figure 4A). The activity of enzyme that had not been exposed to low pH was found to be unaffected by incubation at the values of pH examined. The concentration of the buffer salt (potassium phosphate) was found to have no apparent effect on recovered activity in the range 0.02–0.4 *M*. At concentrations above 0.4 *M* recovery of activity

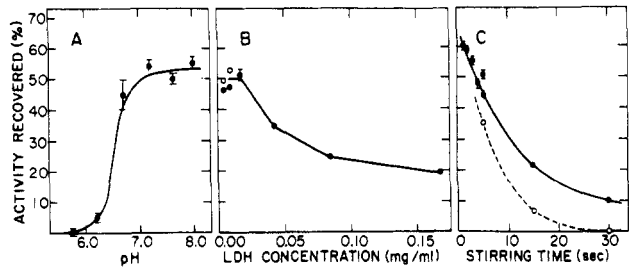

FIGURE 4: Effect of renaturation conditions on extent of activity recovered. Denaturation was carried out in 0.1 *M* sodium phosphate buffer containing 0.002 *M* dithiothreitol and renaturation in 0.2 *M* sodium phosphate buffer containing 0.002 *M* dithiothreitol, other conditions as noted. Following dilution of the denatured samples into renaturation buffer, renaturation was allowed to proceed for a period of 4–5 hr, at which time samples were assayed for catalytic activity. Recovered activity is expressed as a percent of the activity of control solutions not exposed to low pH. (a) Recovery of activity as a function of pH. Denaturation conditions: 0.28 mg/ml of LDH, pH 3.0, for 2 hr. Renaturation conditions: 0.014 mg/ml of LDH, the final pH as indicated, at 25°. (b) Recovery of activity as a function of LDH concentration. Denaturation conditions: 0.84 mg/ml of LDH, pH 2.0, for 30 min. Renaturation conditions: LDH concentration as indicated, final pH 7.0, 20°, in absence (●), and presence (○) of 1 mg/ml of bovine serum albumin. Control activity at lowest concentration was 75% of that at the highest concentration in absence, and 93% in presence of bovine serum albumin. (c) Recovery of activity as a function of the duration of stirring. Denaturation conditions: 0.43 mg/ml of LDH, pH 2.0, for 30 min. Renaturation conditions: 0.0086 mg/ml of LDH, pH 7.3, at 22°. Upon dilution into the renaturation buffer, samples were stirred using a Vortex-Genie mixer for the period of time indicated, and then allowed to renature for 5 hr. (●) 4-ml polypropylene test tube; (○) 6-ml siliconized glass test tube.

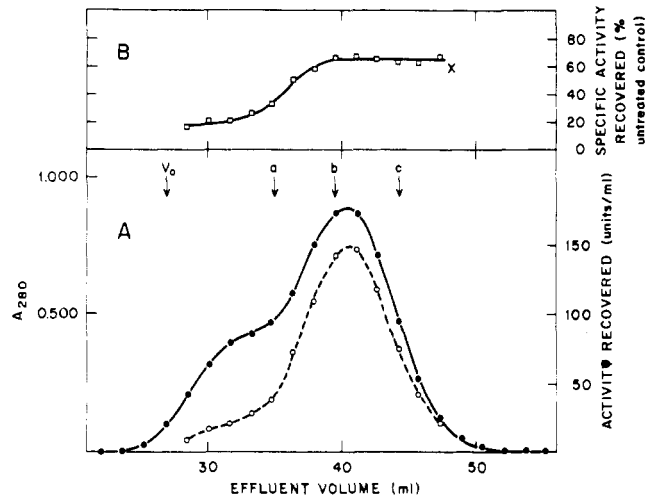

FIGURE 5: (A) Sephadex G-100 column chromatography of beef B_4 LDH under acidic conditions. A 0.9 × 90 cm glass column was used, preequilibrated with 0.1 *M* sodium phosphate buffer, pH 2.0, containing 0.002 *M* dithiothreitol, and run at 4°. The protein was brought to pH 2.0 by addition of ¼ volume of 0.5 *M* sodium phosphate buffer (pH 2.0) containing 0.01 *M* dithiothreitol 40 min before application to the column; 1.1 ml of sample (9.5 mg/ml) was loaded onto the column. The entire sample was recovered. Protein from each of the collected fractions was diluted tenfold into 0.2 *M* sodium phosphate buffer (pH 7.3) containing 0.002 *M* dithiothreitol, for renaturation at 16°. Samples were assayed for catalytic activity after 24 hr. A small correction for the dependence of recovered activity on protein concentration was made (see Figure 4B) giving a somewhat increased peak height. (●—●) A_{280}. (○ - - - ○) Recovered activity, expressed as units of activity recovered per milliliter of column eluate. Molecular weight standards: V_0, Blue Dextran 2000; a, LDH; b, bovine serum albumin; c, ovalbumin. Standards were run at pH 7.0, under which condition the beef B_4 LDH showed a single symmetric peak. (B) Recovered activity per milliliter of eluate divided by protein concentration. Also shown is the percent of the original activity recovered by an unchromatographed sample of enzyme that had been exposed for 2 hr to pH 2.0 and then allowed to renature (X).

was seen to decline, approaching zero at 1 *M* potassium phosphate. The percent of the native activity recovered was found to increase with decreasing enzyme concentration, reaching a maximum value at about 0.02 mg/ml (Figure 4B). A similar effect has been seen in the renaturation of aldolase denatured in guanidine hydrochloride (Teipel and Koshland, 1971), and may be due to irreversible aggregation of the protein during renaturation (Teipel, 1972). Below 0.02 mg/ml recovery of activity appears to level off, suggesting that irreversible aggregation is no longer significant at these lower concentrations. Stirring of a solution of enzyme at the beginning of renaturation results in a loss of recovered activity (Figure 4C) suggesting that the protein may be irreversibly denatured at the air–solution interface or at the test tube wall. Zero time of stirring appears to result in about 65% recovery of the native activity. Native enzyme shows no loss in activity after 1 min of stirring.

Addition of NADH to a concentration of 1.4×10^{-4} *M* to the renaturation buffer was found to result in an increase of only 2% in the activity recovered. A similar small increase was obtained by raising the concentration of dithiothreitol from 0.002 to 0.02 *M*. NADH at a concentration of 7×10^{-3} *M* was found to increase the overall rate of recovery of activity.

State of Association at Low pH. The apparent stability of the denatured state reached after 5 to 10 min at low pH (Figure 1) suggested that characterization by equilibrium methods might be possible. Initial attempts to determine the molecular weight of the protein at low pH by equilibrium sedimentation showed that it was composed of a mixture of noninteracting species of different molecular weights. To separate these components the protein was subjected to Sephadex G-100 gel-filtration chromatography. Figure 5 shows that the protein is separated into two major fractions. The elution position of the peak of the trailing fraction corresponds to that for a globular protein of 65,000 ± 3000 molecular weight, while the position of the peak of the leading fraction was found to be somewhat variable. The proportion of the total protein in the two fractions was found to depend on the period of incubation at low pH preceding application of the sample to the column, the ratio observed for a 1-hr preincubation at pH 2.0 as shown in Figure 5 being reversed after a 25-hr preincubation under the same conditions. Thus, it would appear that the leading fraction represents an aggregated form of protein derived from the trailing fraction.

To assess the ability of the chromatographic fractions to recover the native state, aliquots from each of the collected samples were diluted into pH 7.3 sodium phosphate buffer and allowed to recover activity (Figure 5, dotted line). It can be seen that the ability to recover activity is a property of the trailing fraction. Skewing of the peak of recovered activity toward higher values of molecular weight may indicate that the aggregated protein is capable of some limited recovery of activity, but this feature could also be the result of a rapidly reversible, concentration-dependent association of the renaturable protein (see below). Sixty-five percent of the maximum expected activity, based on the specific activity of the native enzyme, was recovered by protein in the trailing fraction. Similar results were obtained when chromatography was carried out at pH 3.0, though the relative size of the leading peak was considerably more pronounced

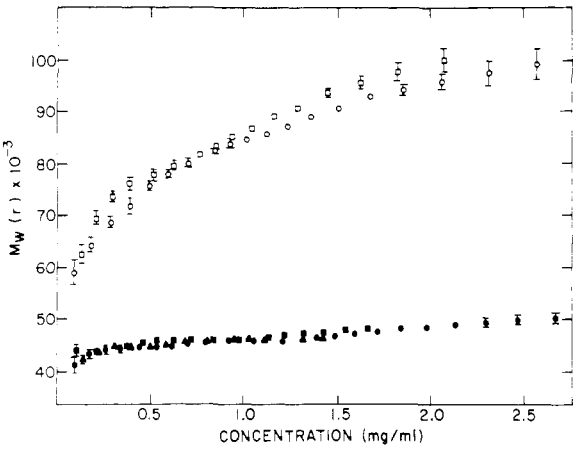

FIGURE 6: Equilibrium sedimentation of protein from the trailing and leading chromatographic fractions. Locally determined values of the weight-average molecular weight are plotted as a function of protein concentration. Concentration in mg/ml was calculated from the Rayleigh fringe displacement using a value for the refractive increment derived from data provided by Perlmann and Longsworth (1948). Closed symbols: Protein from the peak sample of the trailing fraction after chromatography at pH 2.0, conditions for chromatography as in Figure 5. Centrifugation was carried out for 18 hr at 30,000 rpm, at a temperature of 5°. An external loading centerpiece (Ansevin et al., 1970) made of Rexolite and equipped with sapphire windows was used. Concentrations loaded: (●) 0.74; (■) 0.37; and (▲) 0.22 mg/ml. Open symbols: Protein from the peak sample of the leading fraction after chromatography at pH 3.0. The enzyme was in 0.1 *M* sodium phosphate buffer (pH 3.0) containing 0.002 *M* dithiotreitol. Centrifugation was carried out for 24 hr at 26,000 rpm, at 3°. A charcoal-filled Epon cell equipped with sapphire windows was used. Concentrations loaded: (○) 0.62; (□) 0.31 mg/ml.

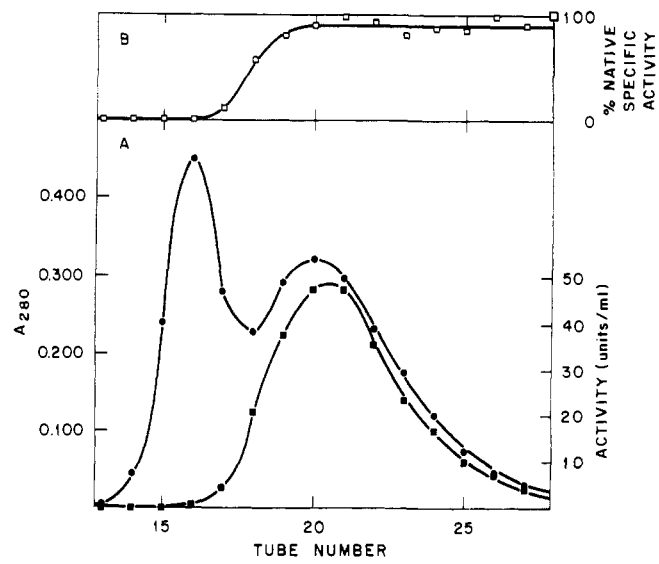

FIGURE 7: Sephadex G-150 gel filtration chromatography of renatured beef B_4 LDH. Denaturation was for 1 hr in 0.1 *M* sodium phosphate buffer (pH 3.0) containing 0.002 *M* dithiothreitol at 0°, at a concentration of 0.66 mg/ml of LDH, and renaturation was for 14 hr in 0.2 *M* sodium phosphate buffer (pH 7.2) containing 0.002 *M* dithiothreitol at room temperature, at a concentration of 0.031 mg/ml. The renatured enzyme was concentrated by ultrafiltration and vacuum dialysis and applied to a 1.5 × 60 cm column packed with Sephadex G-150 and preequilibrated with 0.01 *M* sodium phosphate buffer (pH 7.2). (A) (●) Optical absorbance; (■) catalytic activity; (B) (□) specific activity of the renatured sample and (larger square) of the native enzyme.

at pH 3.0 than at pH 2.0.

Reduction of the concentration of protein applied to the column from 9.0 to 4.5 mg/ml resulted in a marked decrease in the size of the leading fraction. This result suggests that at the considerably lower values of concentration used for the renaturation studies described earlier (Figures 1–4) the enzyme may be almost entirely in a form corresponding to that making up the trailing fraction. Short-column equilibrium centrifugation indicated the rate of aggregation of the protein at pH 3.0 to be strongly dependent on protein concentration. In addition, the rate of aggregation was seen to increase markedly with increasing temperature.

Protein from the trailing and leading peaks was subjected to analysis by equilibrium sedimentation. The results (Figure 6) reveal several aspects of the acid-denatured state. First, the molecular weight of the protein from the trailing fraction (closed symbols) is close to that previously reported for the LDH monomer (Appella and Markert, 1961). The extrapolated molecular weight of about 42,000 at infinite dilution is probably somewhat high due to the binding of buffer ions to the highly charged protein (Williams et al., 1958). The data were found to be inconsistent with a monomer–dimer association scheme (Adams and Fujita, 1962) involving a monomer of molecular weight equal to 35,000. Cleavage of the subunit into 18,000 molecular weight units as reported by Millar and coworkers (1969) is not seen. Second, plots of the apparent weight-average molecular weight vs. concentration for three different initial concentrations of LDH are seen to coincide. This result indicates that the small observed increase in molecular weight with protein concentration is due to a rapidly reversible association of the monomer. Third, the molecular weight of protein from the leading chromatographic fraction (open symbols) is greater than that of the material from the trailing fraction at all values of concentration. Similarly to the trailing fraction, it appears to represent a reversibly associating system, but with a stronger tendency to associate. In this case a small amount of noninteracting contaminant may also be present, as indicated by the imperfect overlap of the points from two initial concentrations.

Characterization of the Renatured Protein. Enzyme that had been incubated at pH 3.0 for an hour and then allowed to recover activity was concentrated and analyzed by gel-filtration chromatography on Sephadex G-150 (Figure 7). The reactivated enzyme elutes at a position corresponding to that of the native LDH tetramer and shows 89% of the native specific activity. (It is not clear whether this represents a significant deviation from the properties of the native enzyme, as has been reported for LDH renatured after exposure to concentrated lithium chloride (Levi and Kaplan, 1971).) A second peak of inactive protein is observed at the void volume. To determine whether the active fraction had been enriched in the capacity to regenerate the native structure a sample of this material was subjected to an additional cycle of denaturation and renaturation. This treatment resulted in recovery of 42% of the maximum expected activity, a value similar to that obtained for enzyme denatured and renatured only one time. The void volume fraction failed to regenerate activity when subjected to the same treatment.

The $s_{20,w}$ of the renatured enzyme was determined at a concentration of 0.25 mg/ml to be 7.1 S, a value consistent with that previously reported for the native enzyme (Markert and Appella, 1961).

Discussion

The results indicate the existence of three denatured forms of beef B_4 LDH at low pH. The relationship of these

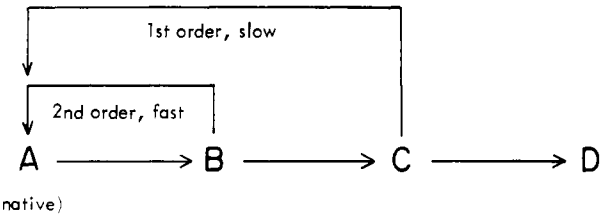

FIGURE 8: Interrelationship of denatured and native forms.

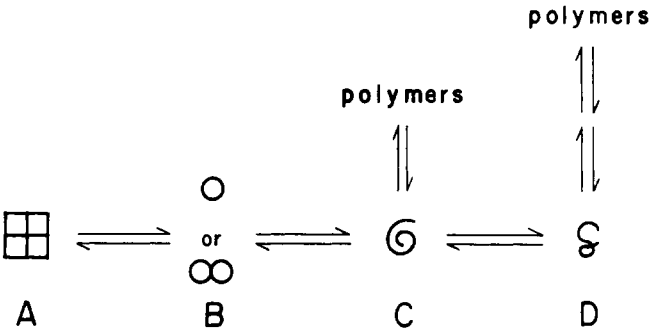

FIGURE 9: Schematic representation of the molecular events involved in the denaturation of beef B_4 lactate dehydrogenase.

forms is represented schematically in Figure 8 where only those forms of the enzyme for which direct evidence has been obtained are depicted. As shown previously (Anderson and Weber, 1966; Levitzki and Tenenbaum, 1974) brief exposure of the enzyme to low pH results in an inactive product, designated here as B, which is capable of complete renaturation following second-order kinetics. We find that more prolonged exposure to low pH results in the appearance of a second form, C, which is characterized by the more complex and concentration-independent kinetics of reactivation shown in Figure 2. Enzyme in this form has shown close to 100% recovery of the native specific activity (Figure 7) but no more than about 60% of the activity of untreated enzyme. This could indicate either that C represents more than one molecular species, or that a fraction of the population of enzyme molecules is subject to irreversible inactivation during the renaturation process. These possibilities are discussed further below. C appears to represent a rather stable state of the protein since following the first few minutes at pH 2 or 3 further changes in the kinetics of recovery of activity are not observed, and the rate of decrease in recovered activity is slow (Figure 1). Chromatography of the denatured protein under acidic conditions (Figure 5) shows the presence of two major fractions, one that is capable of recovering activity and which, therefore, would appear to represent C, and a second that is incapable of recovering activity. This latter material is generated slowly from the lower molecular weight fraction, and is designated as a third nonnative form, D. Under the conditions of low enzyme concentration, and in the short time interval involved in the experiments described in Figures 1-4, only a very small fraction of the enzyme would be expected to be in the form of D.

A possible model for the changes in molecular structure occurring during denaturation is shown in Figure 9. B appears to be a dissociated form of the enzyme, since renaturation proceeding from B follows second-order kinetics. However, it cannot be said whether the enzyme is fully dissociated to monomers. B may also be characterized by some degree of disruption of the native three-dimensional conformation of the subunit, since the enzyme is inactive in this form, and shows a large change in fluorescence intensity (Figure 3) and in the polarization of the fluorescence (Anderson and Weber, 1966) relative to the native enzyme. Complete disruption of the three-dimensional conformation of the subunit is possible but is considered unlikely since this would require refolding of the protein to occur quite rapidly, at a rate at least as fast as the observed second-order reactivation rate. In comparison, the rate of renaturation of LDH that has been denatured in guanidine hydrochloride, under which conditions the protein would presumably exist in a form approximating a random coil, is considerably less rapid (Chilson et al., 1965; Teipel and Koshland, 1971). Conversion of the native enzyme to B would appear to be essentially complete before any significant conversion of B to C takes place, since after 30-sec exposure to pH 3.0 no slow first-order phase is apparent in the renaturation of B (Anderson and Weber, 1966; Levitzki and Tenenbaum, 1974). It would also appear to be the case that subsequent conversion of B to the form or forms designated by C goes at least nearly to completion, since no initial rapid phase in the time course of the reactivation of C is observed.

Enzyme in the form of C is dissociated to subunits (Figure 6). Some association to species larger than the monomer is evident but appears to involve only a small fraction of the total protein at low concentrations. There is no indication in either the equilibrium sedimentation or the chromatography results of the presence of molecular species of a size smaller than that of the monomer, as has been observed previously in acid and guanidine hydrochloride denatured LDH preparations (Millar et al., 1969; Huston et al., 1972; Fosmire and Timasheff, 1972). In order to explain the failure of 100% of the protein molecules to regain the native state the possibility must be considered that C represents a mixed population of molecules of identical or nearly identical molecular weight with differing ability to recover the native state. The dissociated subunits might exist, for example, as a mixture of conformational isomers not all of which were capable of recovering activity within the time span of a reactivation experiment (see Garel and Baldwin, 1973). Another possibility is that a fraction of the subunits undergo the loss of a few amino acid residues, a change that might not be detected by equilibrium sedimentation, but which would result in inactivation. This explanation is considered less likely since enzyme submitted to a second cycle of denaturation and renaturation (Figure 7) shows a loss in activity similar to that seen for enzyme that has undergone only a single such cycle. Alternatively, C may be homogeneous both with regard to conformation and to polypeptide chain length, and the observed loss in activity may occur entirely during renaturation.

The simplest interpretation for the difference in the kinetics of renaturation of B and C is that while little refolding of B is required to attain the native conformation, C is of a sufficiently altered conformation to make refolding, rather than reassociation, rate limiting. That C may indeed be partially or fully unfolded is suggested by its early elution on Sephadex G-100 (Figure 5) relative to the elution position expected from the results of equilibrium sedimentation. B may, thus, represent a stable intermediate in the pathway from native to dissociated and unfolded LDH. Recent work has suggested the existence of stable intermediates in the unfolding of some single subunit enzymes (see, for example, Tsong et al., 1972; Ikai et al., 1973; Wong and Tanford, 1973; but see Garel and Baldwin, 1973). In the

present case the intermediate B might represent either a partially unfolded or a partially associated intermediate. The possibility of a stable dimeric intermediate is particularly interesting and would be consistent with the notion raised by X-ray crystallographic studies, of the LDH tetramer as a dimer of dimers structurally homologous with the malate dehydrogenase dimer (Adams et al., 1972).

The structural change involved in the conversion of C to D is not fully understood. The molecular weight data shown in Figure 6 suggest that D is in rapidly reversible equilibrium with monomer, while the chromatography and short-column centrifugation results indicate a slow conversion of C to D. Therefore, some change in the structure of the subunit is probably involved in the conversion of C to D, possibly a change in conformation or perhaps even cleavage of the peptide chain. The enzyme was probably in the form designated by D in an earlier study of the physical properties of beef heart LDH at low pH conducted by Millar and coworkers (1969). In addition to the fact that the enzyme was incapable of recovering activity, it also showed a strong reversible association similar to that indicated by the results shown for D in Figure 6. It is interesting to note that despite the resemblance of the centrifugation pattern seen for D to that expected for a concentration-dependent dissociation of the native LDH tetramer, D appears to be only distantly related structurally to the native state.

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