Mechanism of Refolding and Reactivation of Lactic Dehydrogenase from Pig Heart after Dissociation in Various Solvent Media†

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ABSTRACT: Lactic dehydrogenase from pig heart (H_4) can be reversibly dissociated to the monomer using acid pH, 6 M guanidine hydrochloride at neutral or acid pH, or 6 M urea. Equilibrium studies suggest the catalytic activity of the enzyme to be correlated with the tetrameric quaternary structure; dissociation, denaturation, and deactivation parallel each other within a narrow range of denaturant concentration. The reconstitution of the enzyme to its native structure shows the same parallelism at lower denaturant concentration. In the intermediate range the tetrameric enzyme is metastable, while dissociated monomers polymerize to form inactive high aggregates. Removal of the denaturants and separation of irreversibly denatured products of aggregation lead to full recovery of enzymatic activity ($A_{sp} = 96 \pm 4\%$) with parallel reconstitution of all physicochemical and enzymological properties characterizing the native quaternary structure. Analyzing the kinetics of reactivation and reassociation, conditions far from the equilibrium of dissociation-association provide maximum yields (~60%; phosphate buffer, pH 7.6, $I = 0.2$ M, $c < 0.4$ μM, 20 °C). The sigmoidal kinetic traces may be described by a sequential first-order transconformation and second-order association reaction according to $2D \xrightarrow{k_1} 2D^* \xrightarrow{k_2} N^*$. Assuming the monomeric subunits to be inactive, the fitting of all data is possible for $k_1 = 1.45 \pm 0.45 \times 10^{-3}$ (s^{-1}) and $k_2 = 5 \pm 1$ ($mM^{-1}\ s^{-1}$). The two constants hold for reactivation experiments using all denaturants mentioned. This suggests the transformation of a common intermediate to a final state of renaturation to be rate limiting in the different pathways of reactivation.

The acquisition of the three-dimensional structure of proteins has been shown to be controlled by thermodynamic and kinetic constraints (Wetlaufer and Ristow, 1973; Baldwin, 1975; Anfinsen and Scheraga, 1975). Both the folding of the nascent polypeptide chain and the refolding of the "structureless" denatured molecule prove that no additional information beyond that contained in the amino acid sequence and its aqueous environment is necessary to generate the native structure and function. In the case of oligomeric proteins, favorable interchain interactions, in addition to the intrachain ones, provide a minimum on the energy surface characterized by a certain well-defined state of association. The particular biological significance of the assembly depends on whether or not the subunits of a specific enzyme contain sufficient information to provide catalytically active entities. For lactic dehydrogenase (LDH[1]), indirect evidence from a variety of experiments seems to prove that the monomeric enzyme is catalytically inactive (Jaenicke, 1970; Bartholmes et al., 1973; Jaenicke, 1974; Rudolph and Jaenicke, 1976; Chan and Mosbach, 1976). Other findings contradict this result (Levitzki, 1972; Cho and Swaisgood, 1974; Tenenbaum-Bayer and Levitzki, 1976). Previous reactivation experiments with the H_4 isoenzyme of LDH (Jaenicke, 1974) proved that a second-order reaction is involved in the reactivation process. This suggests association to be a necessary requirement for enzymatic activity. In the present study a more elaborate analysis of the complex reactivation process is given. Experimental results regarding the less complex kinetics of reassociation and reactivation of LDH-M_4 have been previously presented (Rudolph and Jaenicke, 1976).

Materials and Methods

LDH from pig heart, NADH, and NAD^+ were obtained from Boehringer (Mannheim), and dithiothreitol was from Calbiochem (Luzern). Ultra Pure guanidine hydrochloride and urea were supplied by Schwarz/Mann (Orangeburg, N.Y.). All other reagents were A-grade substances from Merck (Darmstadt); quartz twice-distilled water was used throughout.

Stock solutions of the enzyme (~4 mg/mL) were prepared by repeated dialysis at 4 °C against 0.2 M potassium phosphate buffer, pH 7.6, containing 1 mM EDTA and 0.1 mM dithiothreitol. LDH activity was measured in phosphate buffer, pH 7.0, $I = 0.1$ M (plus 0.74 mM pyruvate plus 0.2 mM NADH) using a recording Eppendorf spectrophotometer thermostated at 25 °C. The specific activity of the native enzyme was 400 IU/mg.

Enzyme concentration was calculated from $A_{280}^{0.1\%} = 1.4$ cm^2/mg (Jaenicke and Knof, 1968); this figure holds for the native and for the renatured enzyme using the Lowry method for comparison. Molar concentrations are based on a subunit molecular weight of 35 000.

Equilibrium measurements of the pH-dependent dissociation, deactivation-reactivation, and denaturation-renaturation were performed in 0.1 M phosphate buffer (plus 1 mM EDTA plus 0.1 mM dithiothreitol) at 20 °C. After incubating the enzyme in its native or dissociated state at a given pH, activity and protein fluorescence were determined.

Deactivation and dissociation of the native enzyme at 20 °C were achieved either by dilution (1:5, 1:10) with 1 M glycine/H_3PO_4 buffer, pH 2.3 (plus 1 mM EDTA plus 1 mM dithiothreitol) or by incubation in 0.2 M phosphate buffer, pH 2.3 or 7.6, in the presence of 6 M guanidine hydrochloride or

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[1] Abbreviations used are: LDH, lactic dehydrogenase; H_4 and M_4, the homologous isoenzymes from heart and skeletal muscle, respectively; NAD, nicotinamide adenine dinucleotide; *c*, enzyme concentration; CD, circular dichroism; D, N, N*, denatured, native, and renatured states, respectively.

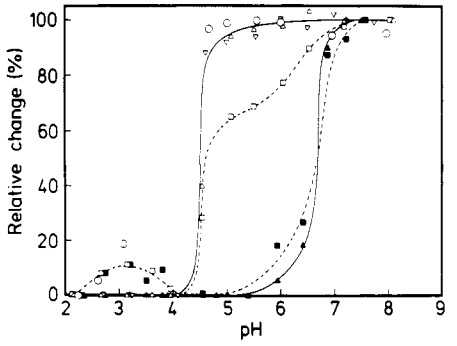


FIGURE 1: pH-dependent dissociation, deactivation, and denaturation of LDH-H_4. Incubation was for 24 h in 0.1 M phosphate, plus 1 mM EDTA, plus 0.1 mM dithiothreitol at 20 °C and a given pH. Open symbols: dissociation, deactivation, and denaturation of native tetramers; closed symbols: reactivation and renaturation of acid-denatured monomers. Relative changes refer to the state at pH 2.3 (0%) and neutral pH (100%). (○) Change of the sedimentation coefficient, $\Delta s_{20,w}$, for c = 0.22 mg/mL. At 3.5 < pH < 4.5, the enzyme represents a heterogeneous system, presumably due to the formation of wrong aggregates. (△,▲) Deactivation-reactivation: c = 7.5 μg/mL. (▽) Deactivation at c = 10.1 μg/mL in the presence of NAD^+ (60% saturation). (□,■) Denaturation-renaturation as monitored by the change of protein fluorescence: c = 7.5 μg/mL; λ_{exc} = 275 nm, λ_{em} = 340 nm.

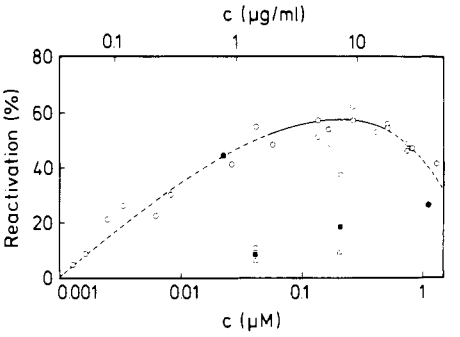


FIGURE 2: Effect of enzyme concentration on the extent of reactivation of LDH-H_4 after deactivation at 20 °C, relative to the initial enzymatic activity before denaturation. Reactivation was in 0.2 M phosphate, pH 7.6, plus 1 or 10 mM EDTA plus 0.1, 1, or 10 mM dithiothreitol. Reactivation time at low enzyme concentration was up to 550 h. (○,●) Deactivation was in 1 M glycine/H_3PO_4; pH 2.3, plus 1 mM EDTA, plus 1 mM dithiothreitol; filled symbols refer to reactivation after repeated denaturation-renaturation. (■,□) Denaturation was in 0.2 M phosphate, plus 1 mM EDTA, plus 0.1 mM dithiothreitol, and 6 M guanidine hydrochloride at pH 7.6 (filled symbols) and 2.3 (open symbols). (△) Denaturation was in 0.2 M phosphate, pH 2.3, plus 1 mM EDTA, plus 0.1 mM dithiothreitol, and 6 M urea.

6 M urea (plus 1 mM EDTA plus 0.1 mM dithiothreitol); the time of incubation was 5 min.

For reactivation and reassociation the aforementioned denaturation mixtures were diluted with 0.2 M phosphate buffer, final pH 7.6, in the presence of 1 or 10 mM EDTA and 0.1, 1, or 10 mM dithiothreitol.

The kinetics of reactivation were analyzed by taking aliquots at defined times; the optical tests were performed as described.

The recovery of native fluorescence was measured in a Hitachi-Perkin-Elmer MPF 2A spectrophotometer (λ_{exc} = 275 nm; λ_{em} = 340 nm). To further characterize the reactivated enzyme, solutions were concentrated in an Amicon diaflo with PM 10 filters. The renaturation mixture contained higher aggregates which were removed by centrifugation and subsequent Millipore filtration. The protein solution was then passed through a Sephadex G-100 column to separate remaining aggregated material from the renatured tetramers.

Conformational analyses of the native enzyme, the renatured tetramers, and the aggregates separated by gel chromatography made use of fluorescence spectroscopy and CD (Roussel-Jouan, Dichrographe II). Sedimentation velocity runs were performed in an analytical ultracentrifuge (Beckman, Model E) with a high-sensitivity photoelectric scanning system. Heat deactivation was applied as an additional criterion to compare the native enzyme to the renatured tetramer.

Results

Equilibrium Measurements

The application of reactivation and refolding kinetics to the problem of subunit activity in oligomeric enzymes is based on the reversibility of dissociation, unfolding, and deactivation which has to be verified by careful investigations of the denaturation and renaturation under varying concentration of the denaturants (H^+, guanidine hydrochloride, urea). On the other hand, the final product of renaturation and the enzyme in its initial state have to be compared directly using all available physicochemical or enzymological criteria.

Dissociation, Denaturation, and Deactivation. LDH-H_4 or -M_4 from a variety of sources and in the absence of high concentrations of denaturants or salts is tetrameric, even at enzyme concentrations of the order of μg/mL (Jaenicke and Knof, 1968; Markert and Massaro, 1968; Mire, 1969; Bartholmes et al., 1973). Acid denaturation as well as denaturation in guanidine hydrochloride or urea cause dissociation to monomers (Jaenicke and Knof, 1968; Jaenicke, 1974; Appella and Markert, 1961). Regarding the pH-dependent dissociation of LDH-H_4 (Figure 1), the monomer is predominant at pH < 3.5 (Jaenicke, 1974). At 3.5 < pH < 4.5, monomers, tetramers, and aggregates are present, while at pH > 4.5 homogeneous tetramers are the only detectable species. Deactivation and the main transition in the complex pH profile of fluorescence occur in a narrow pH range at pH 4.4 ± 0.3. Fluorescence changes beyond this range may reflect changes of solvation, conformational alterations, or ionization of groups in the vicinity of specific fluorophores. Formation of the binary complex LDH·NAD^+ does not show a stabilizing effect in the deactivation profile.

In summary, the pH-dependent dissociation, denaturation, and deactivation show a close parallelism, provided that equal experimental conditions are applied (cf. Rudolph and Jaenicke, 1976). Similar observations were made for the effects of guanidine hydrochloride and urea with LDH of different origin (Appella and Markert, 1961; Wassarman and Burgner, 1972).

As taken from Figure 1, the reactivation and the regain of native fluorescence after acid dissociation show strong "hysteresis"; i.e., reassociation at 4.4 < pH < 6.6 leads to high aggregates without detectable enzymatic activity, whereas the tetrameric enzyme is metastable in this pH range. At pH > 6.6, reactivation occurs with maximum yield at pH 7.6.

Reassociation, Renaturation, Reactivation. Optimum yields of reactivation are observed at enzyme concentrations of 0.05–0.5 μM (Figure 2, cf. Jaenicke, 1974). The decrease at c < 0.05 μM may be caused by concentration-dependent deactivation (Bartholmes et al., 1973) and/or insufficient time for reactivation. At c > 0.5 μM, aggregation obviously competes with refolding and tetramer formation. The maximum yield does not exceed 60% for the following possible reasons: (a) instability at low enzyme concentration, and kinetically

TABLE I: Comparison of Native and Acid Denatured LDH-H_4 with the Products of Renaturation Separated According to Figure 3.

State of the enzyme[a]	$s_{20,w}$ (S)	$M_w \times 10^{-3}$	Activity (IU/mg)	$10^5 \times k_h$[b] (s^{-1})	Fluorescence[c]		Circular dichroism[d]		
					F_{rel} (%)	λ_{max}	$\Delta\epsilon(282)$	$10^{-3} \times \theta_{222}$	$10^{-3} \times \theta_{207}$
Native tetramers (pH 7.6)	7.38 ± 0.08	142 ± 2	405 ± 16	14.5 ± 2.0	100 ± 5	339	38.2 ± 1.1	12.7 ± 0.6	10.8 ± 0.5
Renatured tetramers (pH 7.6)	7.38 ± 0.08	142 ± 2	388 ± 15	14.5 ± 2.0	94 ± 5	339	37.6 ± 1.6	12.9 ± 0.4	11.5 ± 0.4
Inactive aggregates (pH 7.6)	Nd	Nd	0		40 ± 2	335	4.1 ± 2.6	Nd	Nd
Dissociated monomers (pH 2.3)	2.0 ± 0.5	36 ± 2	0		39 ± 2	336	3.1 ± 1.9	9.8 ± 1.0	9.6 ± 1.0

[a] pH 7.6: 0.2 M phosphate, plus 1 mM EDTA, plus 0.1 mM dithiothreitol; pH 2.3: 1 M glycine/H_3PO_4, plus 1 mM EDTA, plus 1 mM dithiothreitol. [b] First-order rate constant of the heat deactivation at 58.5 ± 0.1 °C; enzyme concentration, 4.4 μg/mL. [c] F_{rel}: Relative intensity at 340 nm (% of native fluorescence), λ_{exc} = 275 nm, λ_{max} (nm); enzyme concentration, 6–9 μg/mL, 20 °C. [d] Near-UV CD (L mol^{-1} cm^{-1}) calculated for the tetramer (M_w = 140 000); enzyme concentration, 0.06–0.46 mg/mL, 20 °C. Far-UV CD (deg cm^2 $dmol^{-1}$) calculated for a mean residue weight of 113; enzyme concentration 0.46 mg/mL, 20 °C.

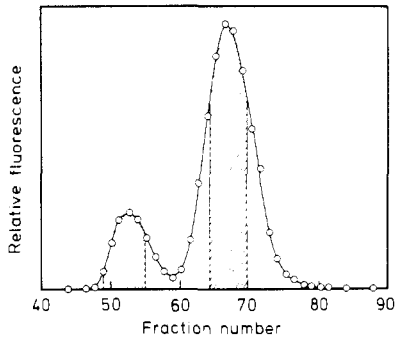


FIGURE 3: Separation of renatured tetrameric LDH-H_4 from aggregated material. Gel chromatography was on Sephadex G-100 (85 × 1.6 cm) with fluorescence detection (0.2 M phosphate, pH 7.6, plus 1 mM EDTA, plus 0.1 mM dithiothreitol). Hatched areas indicate fractions further characterized as higher aggregates and N*.

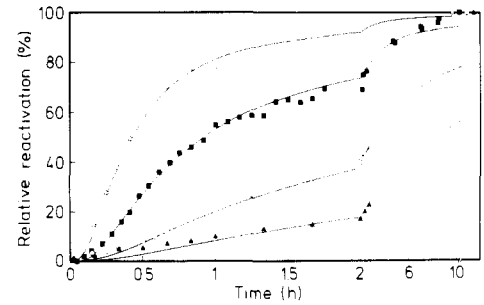


FIGURE 4: Kinetics of reactivation of LDH-H_4 after deactivation in 1 M glycine/H_3PO_4, pH 2.3, plus 1 mM EDTA plus 1 mM dithiothreitol at 20 °C. Reactivation was in 0.2 M phosphate, pH 7.6, plus 1 mM EDTA, plus 0.1 or 10 mM dithiothreitol at 20 °C and varying enzyme concentrations (nM): (△) 367, (■) 91, (○) 19, and (▲) 7.0. Reactivation was calculated relative to final values, determined after a reactivation time of up to 160 h. Solid lines are calculated according to an irreversible unimolecular-bimolecular kinetic mechanism with $k_1 = 1.45 \times 10^{-3}$ (s^{-1}) and $k_2 = 5$ (mM^{-1} s^{-1}).

determined competition of reactivation and aggregation at high enzyme concentration; (b) instability of the denatured and dissociated enzyme; (c) refolding to a partially active conformer; (d) heterogeneity of the starting material in terms of fractions with different capacity for reversible dissociation (e.g., partial proteolysis of the native enzyme).

The last two alternatives can be excluded by the experimental finding that a second denaturation–renaturation cycle leads to a yield of reactivation comparable to the regain after the first denaturation–renaturation. In both cases, reactivation refers to the initial activity before denaturation (Figure 2).

Denaturation in guanidine hydrochloride or urea at pH 7.6 or 2.3 causes the reactivation to be decreased compared to acid dissociation under optimum conditions; incubation in guanidine hydrochloride or urea at pH 7.6 leads to even lower yields as compared to guanidine hydrochloride or urea at pH 2.3.

To compare the products of reactivation with native LDH (N), renatured tetramers (N*) were separated from higher aggregates (Figure 3). The specific activity of the two fractions amounts to 96 ± 4% and 0% of the initial activity of the native enzyme. Similarly, the spectroscopic properties and the heat stability coincide for N* and N within the limits of error (Table I). The spectral characteristics of the inactive aggregates closely resemble those of the acid-dissociated monomers. As suggested by the amplitudes of the double minimum in the far-UV CD spectrum (Table I), an appreciable amount of residual secondary structure is preserved in the dissociated monomers.

Kinetic Measurements

The aforementioned optimum conditions of renaturation lead to a final product of reactivation indistinguishable from the fully active enzyme in its native state (N* = N). Therefore, reactivation kinetics can be used to analyze the correlation between association, refolding, and catalytic function. In the range of the transition tetramer ⇌ monomer, dissociation and deactivation are slow first-order processes, the rate of which is strongly enhanced with increasing concentration of the denaturants (cf. Anderson and Weber, 1966; Wassarman and Burgner, 1972). Under the present experimental conditions, 5-min incubations are sufficient to fully denature and dissociate the enzyme.

The reconstitution of the native tetramer includes first-order transconformation and second-order association reactions (Jaenicke et al., 1975). A distinction between monomeric and oligomeric active species can be made if the reactivation process turns out to be higher than first order. Similar evidence is expected from an enzyme-concentration-dependent regain of protein fluorescence, as in this case major conformational changes should follow the association of the subunits.

In this context, it is important to know whether the varying degree of denaturation after dissociation in acid media, guanidine hydrochloride, urea, etc. (Tanford, 1968; Tiffany and Krimm, 1973; Kugimiya and Bigelow, 1973; Snape et al., 1974; Tsong, 1975; Gerschitz et al., 1977) has an influence on the time course of reactivation.

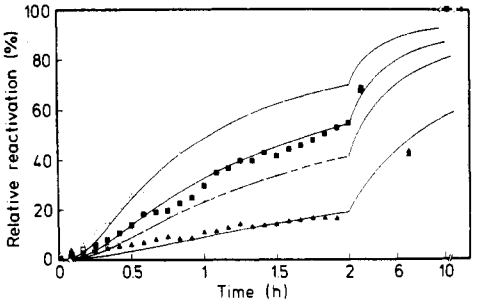
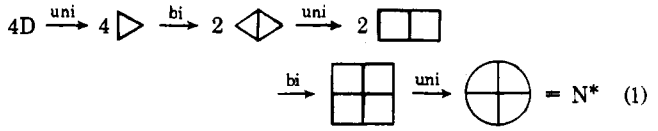


FIGURE 5: Kinetics of reactivation of LDH-H_4 after deactivation in various denaturants at 20 °C. Deactivation in 0.2 M phosphate, plus 1 mM EDTA, plus 0.1 mM dithiothreitol, containing 6 M guanidine hydrochloride, pH 2.3 (△), 6 M guanidine hydrochloride, pH 7.6 (■,▲), and 6 M urea, pH 2.3 (○). Reactivation in 0.2 M phosphate, pH 7.6, plus 1 mM EDTA plus 0.1 mM dithiothreitol, 20 °C, at varying enzyme concentrations (nM): (△) 77, (■) 39, (○) 23, and (▲) 7.7. Reactivation was calculated relative to the final values of reactivation, determined after a reactivation time of up to 170 h. Solid lines are calculated according to an irreversible unimolecular-bimolecular kinetic mechanism with the rate constants derived from the reactivation kinetics after acid dissociation (Figure 4).

Reactivation. Recovery of enzymatic activity after acid dissociation was monitored by sampling aliquots at defined time intervals. As suggested by the sigmoidal kinetic traces (Figure 4), the reactivation cannot be described by one single rate-determining step. The decrease of the rate at decreasing enzyme concentrations proves that an association reaction must be involved in the process of reactivation. From the foregoing equilibrium data it is evident that the present reactivation kinetics refer to essentially irreversible conditions. Removing the denaturant, e.g., by shifting the pH from 2.3 to 7.6, leads to a shift of the equilibrium D ⇌ N* toward the native tetramer.[2] As a consequence, at least some of the single elementary steps in the D → N* transition will proceed in an irreversible fashion. For the formation of tetramers, the following transconformation and reassociation reactions have to be considered as a minimum scheme



(1)

Since the kinetics of reactivation show sigmoidal profiles, a single elementary process cannot be rate determining in the overall reaction. Therefore, an attempt has been made to fit the kinetic traces with an irreversible two-step consecutive reaction. Because of the observed concentration dependence, bimolecular processes must be involved.

Concentration-dependent sigmoidal traces can be expected on the basis of the following reaction sequences

$$2\,D \xrightarrow{uni} 2\,D^* \xrightarrow{bi} N^* \qquad (2)$$

$$4\,D \xrightarrow{bi} 2\,D^* \xrightarrow{bi} N^* \qquad (3)$$

with D* as one of the hypothetical intermediates in the D → N* transition of eq 1. A complete analytical solution of irreversible unimolecular-bimolecular and bimolecular-bimolecular reaction sequences has been put forward by Chien (1948). In the present context, irreversibility means that the reverse reactions have rate constants much smaller than the constants for the forward reactions; i.e., physical irreversibility is not demanded by the mathematical approximation used. For both models, a whole spectrum of kinetics was calculated by varying all rate constants. No single pair of second-order rate constants could be obtained to describe the kinetic data based on the bimolecular-bimolecular mechanism which implies a pronounced concentration dependence. On the other hand, a first-order rate constant k_1 and a second-order rate constant k_2 are sufficient to reproduce the kinetics for all concentrations measured, in accordance with the unimolecular-bimolecular mechanism (2). Both constants had to be in a defined range. From the given experimental data it cannot be decided which of the uni- and bimolecular steps in the overall reaction scheme (1) corresponds to k_1 or k_2. The second-order rate constant was calculated using the concentration of monomers. If the rate-limiting bimolecular reaction belongs to the dimerization of the dimer after a rapid dimerization of monomers, k_2 has to be corrected accordingly.

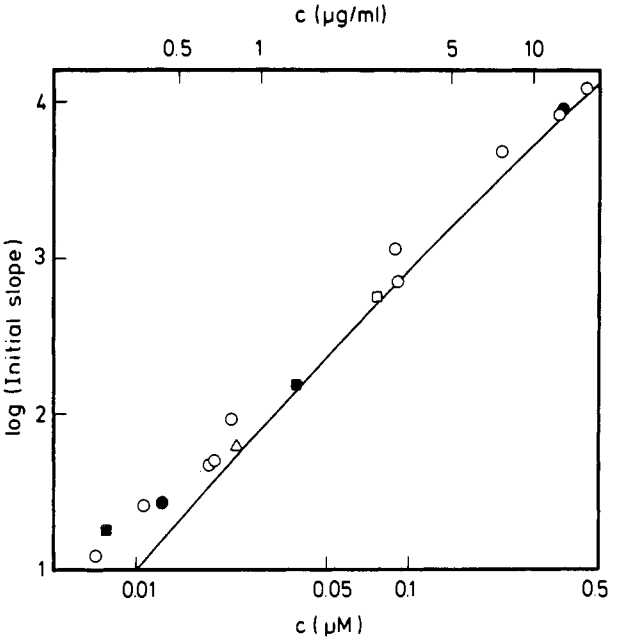


FIGURE 6: Reaction order of the reactivation of LDH-H_4 calculated from the regain of activity after 5 and 15 min (20 °C). Deactivation was in 1 M glycine/H_3PO_4, pH 2.3, plus 1 mM EDTA plus 1 mM dithiothreitol (○,●); 0.2 M phosphate, plus 1 mM EDTA plus 0.1 mM dithiothreitol plus 6 M guanidine hydrochloride, pH 7.6 (■); 6 M guanidine hydrochloride, pH 2.3 (□); 6 M urea, pH 2.3 (△). Reactivation was in 0.2 M phosphate, pH 7.6, plus 1 or 10 mM EDTA, plus 0.1, 1, or 10 mM dithiothreitol; (●) refers to the reactivation after repeated denaturation-renaturation. The solid line was calculated for an irreversible unimolecular-bimolecular kinetic mechanism with the rate constants given in Figure 4.

The solid lines in Figure 4 represent the profiles calculated according to eq 2 for $k_1 = 1.45 \times 10^{-3}$ (s^{-1}) and $k_2 = 5$ ($mM^{-1}\,s^{-1}$). The same set of rate constants describes the kinetics of reactivation for a sequence of two cycles of deactivation-reactivation. Comparing the results for the reactivation after acid dissociation with the reactivation kinetics after dissociation in guanidine hydrochloride or in various other denaturants (Figure 5) proves that these kinetics follow the same mechanism with identical rate constants k_1 and k_2. Figure 6 illustrates the determination of the reaction order using the initial slopes of the kinetics after dissociation in various denaturants. The solid line has been calculated ac-

[2] The experimental condition far from equilibrium cannot be avoided because in the range of transition N → D or D → N* irreversible association to inactive aggregates leads to complete deactivation.

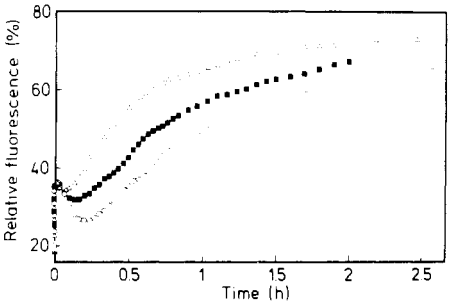


FIGURE 7. Recovery of native fluorescence of LDH-H_4 after denaturation in 1 M glycine/H_3PO_4, pH 2.3, plus 1 mM EDTA plus 1 mM dithiothreitol at 20 °C. Renaturation was in 0.2 M phosphate, pH 7.6, plus 1 mM EDTA, plus 0.1 mM dithiothreitol at 20 °C and varying enzyme concentrations (nM): (△) 320, (■) 138, (○) 66. Final values of renaturation were calculated from the reactivation at ~50 h. The kinetics were repeated two times for each concentration; λ_{exc} = 275 nm, λ_{em} = 340 nm.

cording to the unimolecular-bimolecular model using the rate constants obtained from Figure 4. The systematic deviations at low enzyme concentrations are caused by the fact that the final values of reactivation cannot be reached because of insufficient time of reactivation, or inactivation at high dilution.

Renaturation. As shown in Table I, the intrinsic fluorescence of acid-denatured LDH-H_4 is decreased to ~40% of the fluorescence of the native enzyme. The time course of fluorescence return after readjusting to neutral pH is characterized by a triphasic profile (Figure 7): after a fast increase (which amounts to ~38% of the total change in fluorescence), a slow decrease is observed followed by a slow increase to the value characteristic for N*. The rate of the slow processes is of the order of the rate of reactivation. Attempts to fit the renaturation kinetics according to the unimolecular-bimolecular mechanism with a unimolecular decrease of fluorescence and a bimolecular increase lead to qualitative agreement. Due to the wide range of error, it cannot be excluded that there are more elementary processes involved in the regain of native fluorescence which do not contribute to the kinetics of reactivation.

Discussion

It is well established that LDH like other oligomeric enzymes is reversibly dissociated at low pH or by treatment with guanidine hydrochloride, urea, high salt concentrations, or freeze-thaw under specific conditions of the solvent (Jaenicke, 1970; Rudolph and Jaenicke, 1976).

The different denaturants lead to different final states of unfolding. At acid pH, appreciable residual secondary structure is preserved (Table I), while in guanidine hydrochloride the enzyme, like other proteins, is considered to be completely unfolded (Tanford, 1972). There is clear evidence for certain proteins that even in concentrated solutions of guanidine hydrochloride or urea the molecules do not represent "structureless" polypeptide chains (Tiffany and Krimm, 1973; Snape et al., 1974). Maximum unfolding is obtained by superimposing the effects of guanidine or urea with the effect of protonation at pH ~2 (Tsong, 1975).

The relevance of the kinetic analysis of the refolding process with respect to the structure-function relationship of the enzyme (LDH) depends (a) on the clear distinction and description of the native and dissociated states of the enzyme, (b) on the reversibility of dissociation, denaturation, and deactivation, and (c) on whether the initial native state and the final state after reassociation of the enzyme are comparable.

(a) As shown in Figure 1, the breakdown of the native quaternary structure occurs in a narrow pH range of ~0.5 pH unit which points to a high cooperativity of the processes of dissociation, denaturation, and deactivation. In contrast to earlier observations using different solvent conditions (Jaenicke, 1974), the three processes run parallel, provided the experimental conditions are comparable. The fluorescence profile clearly indicates local conformational changes, characterized by additional transitions, which precede the deactivation as well as the breakdown of the native tertiary and quaternary structure of the enzyme. The initial and final states are fully described by the homogeneous native tetramer, on one hand, and the homogeneous inactive and (partially) unfolded monomer, on the other.

(b) Renaturation of the latter system leads to a mixture of active tetramers (up to 60%) and inactive higher aggregates which consist of polypeptide chains fixed in their denatured state by "wrong" interchain interactions. Regarding the limiting pH of reactivation and renaturation, the observed "hysteresis" clearly indicates the presence of intermediate states in the transition $N \rightleftharpoons D$ (Figure 1). These are corroborated by the fact that rapid changes of the medium (e.g., by dilution) lead to higher yields of reactivation as compared to slow changes (e.g., by dialysis) (Rudolph, unpublished results). As pointed out, kinetic experiments under solvent conditions causing the equilibrium constant of deactivation-reactivation to be close to 1 cannot be performed due to the formation of "wrong aggregates" under this condition (Teipel, 1972).

(c) According to all available biochemical and physicochemical criteria, the product of reconstitution N* is indistinguishable from LDH in its native state (N) (Table I). This result confirms earlier results with LDH-M_4 (Rudolph and Jaenicke, 1976) and LDH from other species (Anderson and Weber, 1966; Chilson et al., 1966). Variant findings (Teipel and Koshland, 1971a,b; Levi and Kaplan, 1971) most probably are caused by insufficient separation of "wrong aggregates" or by chemical modification. The observation that N* and N are indistinguishable proves that both states refer to the same energy minimum. From this finding, no conclusion can be drawn regarding the relative weight of thermodynamic and/or kinetic constraints in the folding of the polypeptide chain, because of the structural elements present at acid pH which may provide nucleation centers.

As mentioned before, the inactive aggregates show spectral properties similar to the acid-denatured monomers. Therefore, changes in fluorescence during reactivation can be ascribed to the process $D \rightarrow N$ (Figure 7). It is obvious that no unequivocal correlation of the observed fluorescence changes to specific fluorophores can be given. The analysis of the reaction order may, however, be used to prove the mechanism of refolding taken from the detailed investigation of the kinetics of reactivation.

In its acid-denatured state (pH 2.3), LDH is fully dissociated and inactive, but only partially unfolded; whether part of the native structure is preserved or a (solvent dependent) new secondary structure is established remains to be analyzed.

At $4.5 < pH < 6.5$, LDH is in a metastable state characterized by a tendency to form inactive high aggregates. Whether there exists a dissociation-association equilibrium cannot be decided under the conditions of the present experiments. The kinetics of denaturation ($N \rightarrow D$) and renaturation ($D \rightarrow N^*$) refer to essentially irreversible processes, because

N, D, and N* are measured outside the aforementioned pH range.

The decrease of the yield of reactivation at $c > 0.5\ \mu M$ obviously reflects the enzyme-concentration-dependent increase of the rate of formation of inactive aggregates relative to the rate of tetramerization.

One rate-limiting step of the reactivation reaction turns out to be an irreversible second-order process in agreement with earlier findings (Anderson and Weber, 1966; Jaenicke, 1974; Rudolph and Jaenicke, 1976). Variant results with first-order kinetics may be explained by erroneous final values of reactivation or high enzyme concentrations (Vallee and Williams, 1975; Tenenbaum-Bayer and Levitzki, 1976). Earlier results indicated that the reactivation profile is sigmoidal (cf. Teipel and Koshland, 1971a; Jaenicke, 1974; Vallee and Williams, 1975). A more detailed kinetic analysis proves that an irreversible consecutive unimolecular-bimolecular mechanism is sufficient to describe all the kinetic data (Figures 4-6). This is in contrast to the reactivation of the M_4 isoenzyme, where a single irreversible bimolecular process is sufficient to describe the kinetics of reactivation (Rudolph and Jaenicke, 1976). According to the rate-limiting bimolecular process involved in the reactivation of both isoenzymes, monomeric H and M must be enzymatically inactive. Earlier experiments using covalent binding of subunits to a solid matrix led to contradicting results (Cho and Swaisgood, 1974); however, more recent evidence from this approach (Levi, 1975; Chan and Mosbach, 1976) supports the conclusion of the present kinetic investigation. This result is not at all trivial, especially because within the tetramer the individual subunits of LDH are kinetically independent (Holbrook et al., 1975). For aldolase which (like LDH) is not allosterically regulated and tetrameric under nondenaturing conditions, previous experiments have shown that artificially generated isolated monomers have at least partial activity (Chan and Mawer, 1972; Chan et al., 1973; Rudolph et al., 1976, 1977).

It is evident from the kinetic experiments that enzymatic activity is not generated unless both the uni- and bimolecular processes have taken place; i.e., as in the case of the M_4 isoenzyme, the formation of active centers necessarily needs an association step. Whether this step corresponds to the formation of active dimers or whether the tetramer is the active entity cannot be answered on the basis of the given experimental data. The two rate constants (k_1, k_2) calculated from the reactivation data after acid dissociation hold equally well for experiments using other denaturants (Figures 5 and 6). Since the various states of denaturation (ϑ_i) differ widely regarding the secondary and/or tertiary structure of the subunits, the reactivation reactions $\vartheta_i \rightarrow N^*$ must contain a common intermediate D which then obeys the irreversible unimolecular-bimolecular reaction scheme; i.e., both transconformation and association of D must be rate determining in the overall reactivation. In other words, the various extent of residual structure has no significant influence on the kinetics of reactivation in terms of rate-limiting nucleation steps. Accordingly, the simple sequential model (eq 2) has to be modified to

$$2\vartheta_1 \rightarrow 2\vartheta_2 \rightarrow 2\vartheta_3 \rightarrow 2D \xrightarrow{k_1} 2D^* \xrightarrow{k_2} N^* \qquad (4)$$

or to an alternative mechanism

$$\begin{matrix} 2\vartheta_1 \\ 2\vartheta_2 \\ 2\vartheta_3 \end{matrix} \rightarrow 2D \xrightarrow{k_1} 2D^* \xrightarrow{k_2} N^* \qquad (5)$$

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Limited Degradation of the Third Component (C3) of Human Complement by Human Leukocyte Elastase (HLE): Partial Characterization of C3 Fragments†

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ABSTRACT: The third component of human complement (C3) was digested with human leukocyte elastase (HLE). Digestion proceeds in two stages under controlled conditions of proteolysis. The initial event of HLE attack produces cleavage of the C3 α chain near the amino terminus forming an α' chain and releasing an 8500 molecular weight fragment (α4) which is immunologically and chemically similar to the C3a anaphylatoxin. The purified α4 fragment is lacking in both anaphylatoxic or chemotactic activity. More extensive digestion of C3 with HLE results in a selective degradation of the α' chain to form three principle fragments of 36 000 (α1), 28 000 (α2), and 24 000 (α3) molecular weight. Digestion of C3 with HLE either for extended periods of time (2 h) or at high ratios of enzyme to C3 (1:20) does not affect the covalent integrity of the β chain. Specificity of HLE cleavage sites on the protein substrate is consistent with that previously observed with model synthetic substrates. However, the ability of HLE to cleave the peptide linkage between aliphatic residues and a cationic residue was not predicted from model substrate studies. Fragments α1 to α4 account for approximately 90% of the total α-chain structure. Fragments α2 and α4 are not covalently bonded to the disulfide-linked complex of α1, α3, and β chain. In addition, the α2 fragment cross-reacts with anti-C3d; C3d is a natural catabolite of C3 formed by the action of a plasma enzyme C3b inactivator on C3b. The C3d fragment contains the labile C3 binding site. Distribution of the carbohydrate on C3 is divided between both α and β chains. After extensive HLE digestion, all of the stainable carbohydrate on the α chain is detected on the α3 fragment. The carbohydrate moiety represents a significant marker for future structural analysis. Characterization of the HLE fragments from the α chain includes identification of NH_2- and COOH-terminal residues and the respective amino acid compositions. A corresponding characterization of the isolated α chain of C3 permits a tentative alignment of the major HLE fragments from the α chain. A molecular model derived from an alignment of the HLE fragments of C3 compares favorably with the tentative C3 model reconstructed from fragments produced by plasma enzymes. C3 is selectively cleaved into C3a and C3b during complement activation and further degradation of C3b by plasma enzymes produces α-chain fragments closely resembling the degradation products obtained after HLE digestion of C3.

Crude lysates of lysosomal granules from human peripheral leukocytes have been found to induce alterations in a number of serum proteins involved in inflammation and tissue injury. Lysosomal extracts incubated with high- and low-molecular-weight kininogens cause the release of a kinin (Movat et al., 1973). This kinin-generating activity has been purified recently and is thought to be due to leukocyte elastase (Movat et al., 1976), a lysosomal protease comprising approximately 16% of the total granular extract (Janoff, 1973; Taylor and Crawford, 1975). Crude lysosomal lysates have also been shown by Taubman et al. (1970) and Ward and Zvaifler (1971) to produce a factor from serum and from the fifth component of human complement which is chemotactic for rabbit polymorphonuclear (PMN) leukocytes. Goldstein and Weissman (1974) noted that a C5a[1]-like fragment produced by the lysosomal proteases acts to release additional lysosomal enzymes from isologous, cytochalasin-B-treated human PMN leukocytes. Proteolytic degradation of other components of the complement system such as $C\overline{1s}$ (Taubman and Lepow, 1971) and C2 (Taubman et al., 1970) has previously been described. However, none of the proteolytic alterations mentioned above have been well characterized biochemically. Such a study would help clarify the molecular events occurring during the inflammatory process as well as elucidate the substrate specificity of the proteases contained in leukocyte lysosomes.

Human leukocyte elastase (HLE) is a serine protease consisting of a single polypeptide chain with a molecular weight between 22 000 (Taylor and Crawford, 1975) and 32 000 (Ohlsson and Olsson, 1974a). A highly basic glycoprotein, HLE, appears as a characteristic set of four isozymes after

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[1] Symbols used for the complement components conform to the recommendations of the World Health Organization Committee on Complement Nomenclature (1968). A bar above the complement component (e.g., C1s) signifies an enzymatically active form.