Reconstitution of Lactic Dehydrogenase. Noncovalent Aggregation vs. Reactivation. 1. Physical Properties and Kinetics of Aggregation†

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ABSTRACT: The reconstitution of lactic dehydrogenase after dissociation in 6 M guanidine hydrochloride or at acidic pH leads back to active tetramers indistinguishable from the native enzyme. In addition, aggregates of "irreversibly denatured" enzyme are formed to a certain extent. It has been shown that the ratio of both fractions depends on such factors as the extent of denaturation and the concentration of the enzyme in the process of reconstitution. The electron microscopical analysis of the aggregates shows a broad distribution of high molecular weight particles ($M_r > 10^6$). As suggested by circular dichroism measurements in the far-UV and by degradation in strong denaturants, these particles are composed of individual monomeric chains with partially restored secondary structure. The aggregates are stabilized by noncovalent interactions. For determination of the factors responsible for the competition of reactivation and aggregate formation, the kinetics of aggregation were analyzed by stopped-flow laser light scattering experiments and compared with the kinetics of reactivation [cf. Rudolph, R., & Jaenicke, R. (1976) *Eur. J. Biochem. 63*, 409–417]. Aggregation is determined by a process with a reaction order greater than 2, competing with a fast first-order folding reaction in the pathway of reactivation. Reactivation and aggregate formation seem to be kinetically controlled; the ratio of both fractions depends solely on the kinetics of formation and not on the relative conformational energies of the native enzyme and its aggregates. A general model for the competition of reactivation and aggregation is proposed, including the influence of denaturing and labilizing solvent conditions.

The great majority of regulatory enzymes represents oligomers composed of subunits which are attached to each other by noncovalent interactions. As a consequence, reconstitution of oligomeric enzymes after dissociation with denaturants, such as guanidine hydrochloride or acid, involves folding and association. In the case of monomeric enzymes, the folding transitions occurring during the formation of the three-dimensional structure have been analyzed in great detail by denaturation–renaturation studies in vitro (Anfinsen & Scheraga, 1975). On the other hand, the reactions involved in oligomer formation are far less characterized (Jaenicke, 1979).

The acquisition of the quaternary structure of a given enzyme comprises highly specific association reactions which must be subtly interconnected with folding transitions. Like the unfolding of monomeric enzymes, the dissociation and unfolding of oligomers have been shown to be reversible in many cases. Therefore, the parameters determining the in vitro refolding and reassociation can be analyzed in a quantitative way by transferring denatured and dissociated enzymes to quasi-physiological conditions. Under optimum conditions of denaturation–renaturation, yields close to 100% can be achieved, corresponding to complete reassociation, refolding, and reactivation to the native state of the enzyme. It has been previously shown for a number of enzymes that the product of reconstitution is indistinguishable from the initial native enzyme as far as the hydrodynamic, spectroscopic, and enzymatic properties are concerned (Jaenicke et al., 1975, 1979). In cases where only partial reactivation is observed, the latter statement holds only if "irreversibly denatured" material is separated from the native enzyme (Rudolph et al., 1977). No systematic studies have been reported so far regarding the physical properties of the aggregates and the mechanism of their formation. The aggregated material must be trapped in a minimum of conformational energy distinct from the one present in the native enzyme. Reactivation and aggregation compete with each other (Teipel & Koshland, 1971; Jaenicke & Rudolph, 1977); their relative proportions depend on both the denaturing and renaturing conditions. Increased denaturation of the dissociated monomers, e.g., by guanidine hydrochloride instead of low pH, increases the amount of aggregates. The same effect is observed after prolonged incubation in the range of dissociation $\rightleftharpoons$ association induced by the denaturant (Rudolph et al., 1977). In addition, the yield of reactivation depends on the renaturing conditions, since an increase in protein concentration causes an increase in aggregate formation (Jaenicke & Rudolph, 1977).

In the present investigation, the aggregation reaction accompanying denaturation and renaturation is characterized regarding the physical properties of the aggregation products and the kinetics of their formation. The aim is a better understanding of aggregation in order to avoid this side reaction during folding and association of oligomeric enzymes. This may be of general interest in connection with preparative approaches aimed at the separation and purification of enzymes.

Materials and Methods

Lactic dehydrogenase (LDH-M_4)[1] from pig muscle and NADH were obtained from Boehringer Mannheim GmbH (Mannheim); dithioerythritol was from Calbiochem (Luzern). All other reagents were A-grade substances from Merck

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[1] Abbreviations used: LDH-M_4, porcine lactic dehydrogenase from skeletal muscle; DTE, dithioerythritol; NAD, nicotinamide adenosine dinucleotide; c and c_G, concentration of enzyme and guanidine hydrochloride, respectively; ϑ_i, denatured state; A, aggregate; D, monomeric intermediate state; N, tetrameric native state; x, average degree of aggregation.

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(Darmstadt). Quartz bidistilled water was used throughout.

Stock solutions of the enzyme (~5 mg/mL) were prepared by repeated dialysis (4 °C) against 0.1 M standard potassium phosphate buffer, pH 7.6, containing 10 mM EDTA and 1 mM DTE. Enzyme activity was measured in phosphate buffer, pH 7.0 and I = 0.1 M (plus 0.74 mM pyruvate and 0.2 mM NADH), by using a recording Eppendorf spectrophotometer thermostated at 25 °C. The specific activity of the enzyme was found to be 640 ± 40 IU/mg.

Enzyme concentration was calculated from $A_{280}^{0.1\%} = 1.40$ cm²/mg; at low enzyme concentrations the Bio-Rad method and the Lowry method were applied by using the native enzyme as the standard. Molar concentrations are based on a protomer molecular weight of 35000.

Denaturation of the native enzyme was achieved by dilution (1:20–1:1000) with 0.1 M H_3PO_4 plus 1 mM EDTA plus 1 mM DTE, final pH 2.5. Incubation time at 20 °C was 5–15 min.

Renaturation, reactivation, and reassociation (plus aggregation) were achieved by mixing (1:2) with 0.1 M phosphate buffer plus 1 mM EDTA plus 1 mM DTE, final pH 7.0 and I = 0.1 M, in a stopped flow laser light scattering apparatus equipped with an argon laser (Spectra Physics, Darmstadt, Model 165-03); for the underlying principle cf. Riesner & Bünemann (1973).

Detection of the scattered light made use of an EMI 6094B multiplier with a Keithley Model 245 amplifier. A Hewlett-Packard 1801A dual-channel oscilloscope or a Metrawatt recorder (Servogor 200) was applied to monitor the signal. For calculation of the relative scattering as a function of time, the constant final signal for each concentration was taken as 100%:

$$R_{rel}\ (\%) = \frac{\text{signal at time } t}{\text{final signal}} \times 100 \qquad (1)$$

$$\text{yield of reactivation } (\%) = \frac{\text{final reactivation}}{\text{initial activity}} \times 100 \qquad (2)$$

Due to the stability of the enzyme, no correction for time-dependent deactivation of the native enzyme was necessary during the time course of reactivation.

Isolation of aggregates was achieved by dilution of the denatured enzyme (2:5) in 0.1 M phosphate buffer, pH 6.8, plus 1 mM EDTA plus 1 mM DTE (30 min) and centrifugation (20 min at 48000g). After the aggregates were washed with standard phosphate buffer and another centrifugation, the aggregates were suspended in standard buffer or water (final concentration ~3 mg/mL).

For characterization of the *physical properties of the aggregates*, absorption, fluorescence, and circular dichroism spectra were measured by using a Cary 118 spectrophotometer, a Hitachi Perkin-Elmer MPF 44A spectrofluorometer (λ_{exc} = 275 nm; λ_{em} = 340 nm), and a Roussel-Jouan Dichrographe II with high-sensitivity equipment and temperature control (20 °C). Electron microscopy was performed on a JEOL 100C instrument (Japan Electron Optics Ltd., Tokyo), using negative staining (4% uranyl acetate) at 15600–92000× magnification. Sedimentation analysis was carried out by using a Beckman Model E ultracentrifuge.

Results

It has been previously shown that the yield of reactivation of oligomeric enzymes depends on the concentration of the enzyme (Jaenicke & Rudolph, 1977). For LDH-M_4, a constant value of reactivation is observed only at concentrations below a limiting value of about 10 nM (Rudolph & Jaenicke, 1976); at higher concentrations, a drastic decrease of the yield of reactivation is observed, and aggregation becomes predominant. Figure 1 illustrates this effect for both reactivation and aggregate formation.

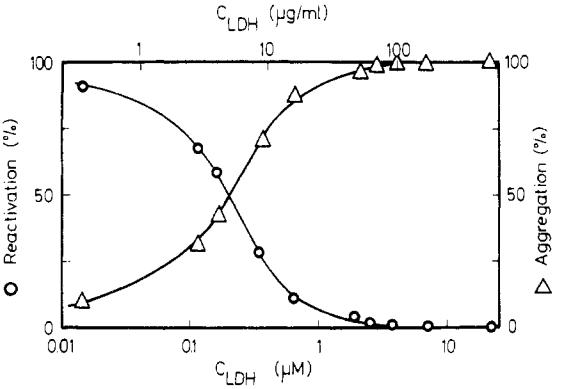

FIGURE 1: Effect of enzyme concentration on the extent of reactivation and aggregation of lactic dehydrogenase (LDH-M_4) after denaturation in 0.1 M H_3PO_4, pH 2.5, plus 1 mM EDTA plus 1 mM DTE (20 °C). Reactivation in 0.1 M phosphate buffer, pH 7.0, plus 1 mM EDTA plus 1 mM DTE (20 °C) was determined after up to 192 h. At c < 10 nM, maximum reactivation of ca. 90% is achieved.

The result provides a simple method to obtain aggregates on a preparative scale by renaturation of the enzyme above the critical upper limit of concentration where reactivation drops to low values and finally vanishes. For preparative purposes in connection with the separation of closely related enzymes, the method does not seem to be applicable because such enzymes do not differ exceedingly in the profiles of concentration-dependent aggregation [cf. Jaenicke & Rudolph (1977)]. For different families of enzymes, previous experiments (Teipel & Koshland, 1971) suggest the method to be applicable.

In order to follow the kinetics of aggregation, the conditions in the stopped-flow laser light scattering experiments were chosen such that the final concentration in the renaturation–aggregation system varied between 0.05 and 2.4 μM (cf. Figure 1). Accordingly, the yield of aggregate amounts to 10–100%; reactivation reaches a maximum value of ca. 90% at the lowest concentration (0.05 μM). Even under this condition, the light-scattering signal caused by aggregation exceeds the one caused by tetramer formation. By comparison of the change in light scattering due to the tetramer → monomer transition (pH 7.6 → pH 2.3) with the signal observed for the reaction monomer → aggregates in the given concentration range, the latter turns out to be higher by about 2 orders of magnitude. Therefore, the influence of tetramer formation on the light-scattering signal of aggregation can be neglected. In addition, the result clearly shows that the kinetics of tetramer formation cannot be monitored by light scattering, due to perturbations caused by "wrong aggregates". Since light scattering measures the weight-average molecular weight, a low percentage of aggregated material will affect the signal in an overproportional way. On the other hand, it has been shown that cross-linking of intermediates of association is a suitable method to study oligomer formation (Hermann et al., 1979).

The kinetics of aggregation are characterized by sigmoidal and concentration-dependent relaxations consisting of more than one exponential. Figure 2 illustrates the time course for various enzyme concentrations. Determination of the apparent reaction order by plotting the logarithm of the initial slopes vs. the logarithm of the concentration gives a value of n = 2.5 ± 0.1 (Figure 3). Attempts to fit the data with a consecutive bi-bimolecular process have been unsuccessful. As indicated by the marked sigmoidicity, however, several rate-limiting

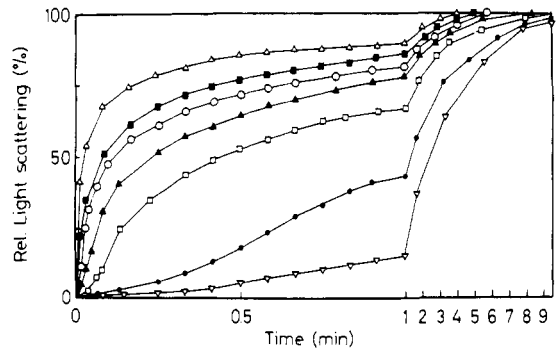

FIGURE 2: Effect of enzyme concentration of the kinetics of aggregation of lactic dehydrogenase (LDH-M_4). Denaturation and renaturation conditions are as given in Figure 1. Aggregation relaxation measured at 20 °C under varying enzyme concentrations (μM): 2.4 (△); 1.6 (■); 1.2 (○); 0.5 (▲); 0.2 (□); 0.1 (●); 0.05 (▽).

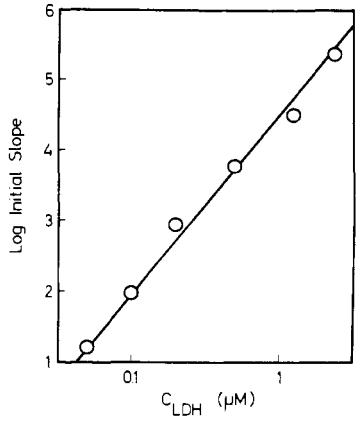

FIGURE 3: Determination of the apparent reaction order of the aggregation reaction of lactic dehydrogenase (LDH-M_4) (Figure 2) by double-logarithmic representation of the initial slopes (0–2 s) vs. enzyme concentration. $n = 2.5 \pm 0.1$.

reactions must be involved in the process of aggregation.

For characterization of the product of aggregation, the spectral properties were analyzed and compared to those of the native and denatured enzyme. As in the case of the heart isoenzyme (Rudolph et al., 1977), there are distinct differences in the dichroic and fluorescence data for the native and denatured enzyme which are completely reversed under conditions providing total reconstitution (Table I). While the reactivated enzyme turns out to be indistinguishable from the enzyme in its original native state, the aggregates resemble the denatured enzyme regarding their fluorescence properties. On the other hand, the far-UV circular dichroism suggests the secondary structure of the enzyme to be partially restored. As illustrated in Figure 4, the aggregates show a slightly changed CD spectrum which may reflect the effect of turbidity on (dichroic) absorption (Duysens, 1956; Urry, 1972). Turbidity artifacts may be caused by differential absorption flattening, differential light scattering, and concentration obscuring. Since all three effects (aside from slight shifts of the Cotton effects) tend to decrease the amplitude, the previously mentioned statement needs no correction in the given context.

From the given data, no conclusion can be drawn with respect to the quaternary structure of the aggregates. For this purpose, electron micrographs were taken by using negative staining with uranyl acetate to optimize the contrast. As shown in Figure 5, aggregation leads to an irregular network of high molecular weight particles which form filaments with structural elements 10–15 times the size of the native tetramer. The average particle size is in the order of several million. A more detailed evaluation is not justified because a closer inspection

Table I: Characterization of LDH-M_4 in Its Native, Denatured, and Aggregated States[a]

	native state (pH 7.6)	denatured state (pH 2.3)	aggregated state (pH 7.6)
sp act. (IU/mg)	639 ± 40	0	0
λ_{max} (nm)	399 ± 1	333	331
$-[\Theta]_{222}$ (deg cm^2 dmol^{-1})	15 300 ± 1000	10 800	14 700
$s^0_{20,w}$ (S)	7.6 ± 0.2	2.7	~20
M_r	140 000 ± 1500	35 000	~10^6

[a] Sp act., specific activity; λ_{max}, maximum fluorescence (λ_{exc} = 280 nm); $[\Theta]_{222}$, ellipticity at 222 nm (cf. Figure 4); $s^0_{20,w}$, sedimentation constant; M_r, molecular weight from s,D or sedimentation equilibrium.

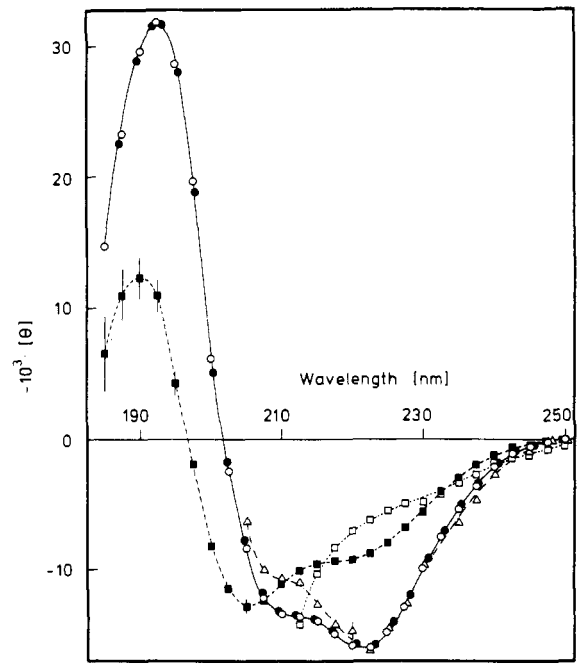

FIGURE 4: Far-UV circular dichroism spectra of LDH-M_4 in its native, denatured, and aggregated states (20 °C). (● and ○) Native and renatured LDH-M_4, respectively; 0.1 M phosphate buffer, pH 7.6, plus 1 mM EDTA and 1 mM DTE; c = 1.5 mg/mL. (■ and □) Monomeric LDH-M_4 denatured in 1 M glycine–H_3PO_4, pH 2, or 6 M guanidine hydrochloride, pH 2, respectively (1 mM EDTA and 1 mM DTE); c = 1.5 mg/mL. (△) Aggregated LDH-M_4 dispersed by 5–10-s low-energy sonication (Branson sonifier); c = 0.05 mg/mL, determined gravimetrically from the dry weight at 105 °C.

of the electron micrographs shows that there exists a broad distribution of particle sizes. Under no conditions could structural monomers be detected as separable entities.

Discussion

The competition of renaturation and aggregate formation is a general phenomenon characterizing the reconstitution of oligomeric enzymes. Up to date, high yields of renaturation have been achieved only by empirically establishing "optimal renaturing conditions".

Aggregation and quaternary structure formation lead to distinct minima of conformational energy, the absolute values of which are expected to be different. As a consequence, the ratio of aggregation vs. quaternary structure formation must be determined by the relative rates of both processes; i.e., they must be kinetically controlled. An analytical approach to optimize quaternary structure formation (which is the final goal of reconstitution experiments) can make use of this "kinetic determinism", either by increasing the rate of qua-

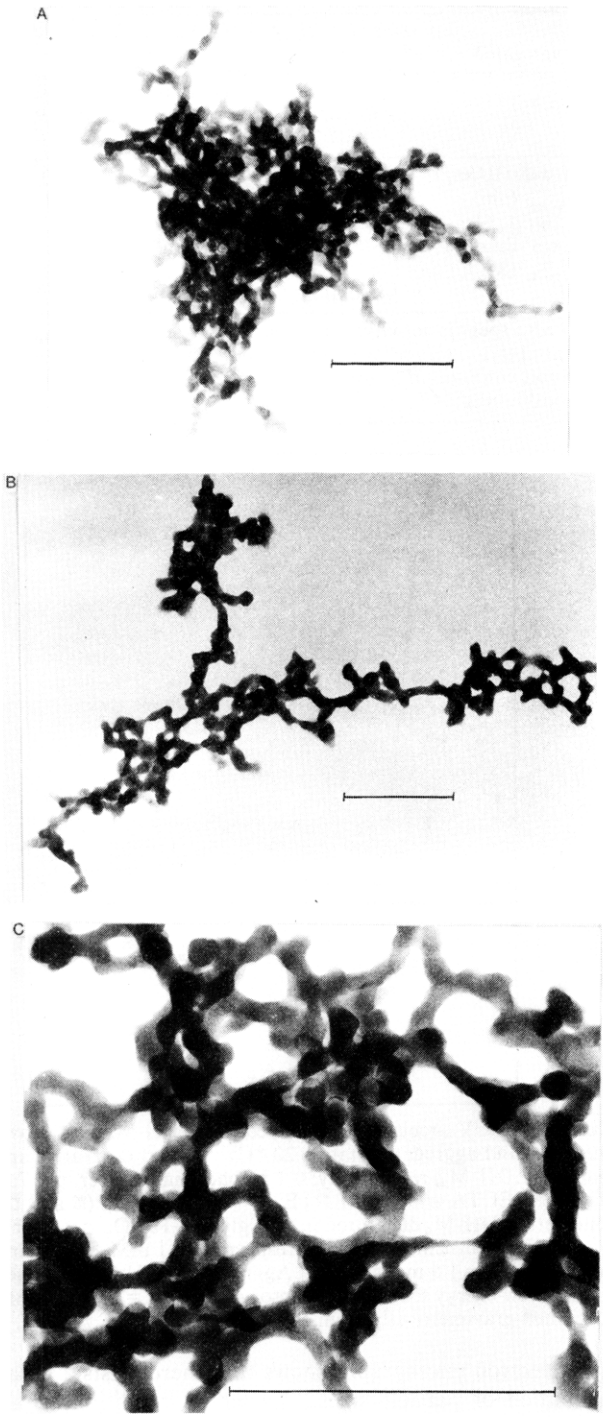

FIGURE 5: Electron micrographs of aggregates of LDH-M_4 obtained as described under Materials and Methods and stained with 4% uranyl acetate, pH 4.7. Primary magnification (A) 6240×, (B) 16 000×, and (C) 36 800×, respectively. The length of the bars corresponds to 1 μm.

ternary structure formation or by decreasing the rate of aggregation during reconstitution.

Aggregation is considered a general feature of proteins or enzymes in solution. Instead of being restricted to the specific conditions favoring reconstitution, it is also observed under moderate or even strong denaturing conditions. As shown by column chromatography and ultracentrifugation, aggregation of heart muscle lactic dehydrogenase is detected even at pH values as low as pH 2 (Vallee & Williams, 1975; Rudolph & Jaenicke, 1976). Observations like this may be taken to explain the constant, denaturant-dependent plateau of the yield of reactivation at low concentrations of the reactivating enzyme (Rudolph & Jaenicke, 1976; Rudolph et al., 1977). If we postulate an equilibrium between denatured monomers and aggregates at denaturing conditions, we may write

$$\vartheta_i \overset{K'}{\rightleftharpoons} \frac{1}{x}\mathrm{A} \tag{3}$$

In this equation, ϑ_i represents the denatured monomer characterized by a denaturant-dependent degree of unfolding [Figure 4; cf. Rudolph et al. (1977)], while A stands for aggregates with an average degree of aggregation. Upon reconstitution under optimum conditions, ϑ_i is converted to the native enzyme while the aggregates remain in their inactive conformation, provided that no additional aggregation occurs during reconstitution, e.g., at high concentrations of the reactivating enzyme. By application of eq 3, the yield of aggregation is expected to depend only on the equilibrium constant K'. The high yield after acid denaturation may be assumed to be caused by the residual structure pertaining at acid pH. On the other hand, the reported slow decrease of the reactivation after a long incubation time (Vallee & Williams, 1975; Rudolph & Jaenicke, 1976) may reflect a slow equilibration according to eq 3.

In the range of dissociation of the native quaternary structure, an additional equilibrium between denatured monomers and native oligomers has to be considered:

$$\mathrm{N} \overset{K}{\rightleftharpoons} \vartheta_i \overset{K'}{\rightleftharpoons} \frac{1}{x}\mathrm{A} \tag{4}$$

At denaturant concentrations where the transition oligomer $\rightleftharpoons$ monomer occurs, the equilibrium constant K will be close to 1.

In the given context, dissociation is considered a prerequisite to the conversion of oligomers to aggregates and vice versa (Rudolph et al., 1979). Therefore, an additional equilibrium between aggregates and native oligomers can be excluded. If, under the conditions of free equilibration at moderate denaturant concentrations, aggregates (A) represent the most stable species, aggregation will predominate. This is observed for both the heart and skeletal muscle isoenzymes of lactic dehydrogenase (Rudolph & Jaenicke, 1976; Rudolph et al., 1977), as well as a variety of other oligomeric enzymes (Jaenicke, 1978).

For a number of systems, different equilibration rates of the forward and backward reaction give rise to hysteresis effects of the $\mathrm{N} \rightleftharpoons \vartheta_i$ transition. These are strongly affected by ions which are known to stabilize or labilize the native structure; e.g., the phosphate ion has been demonstrated to show a stabilizing effect (Rudolph & Jaenicke, 1976).

The increased stability of aggregates at moderate denaturant concentrations cannot be generalized since it has been reported that under these conditions aggregates may as well be destabilized (Orsini & Goldberg, 1978).

For certain enzymes, no aggregation is observed whatsoever, which suggests an unperturbed equilibrium of the enzyme in its native and denatured state; in this case 100% reactivation is obtained. The dissociation of lactic dehydrogenase from pig muscle (M_4) at high hydrostatic pressure illustrates this limiting case (Schade et al., 1978).

In the present experiments where denaturant-dependent stabilization of aggregates has been observed, extensive incubation in the range of transition has to be avoided; for this reason renaturation was initiated by *fast* dilution to conditions favoring the native state. Denaturant present during renaturation in concentrations well below the range of transition does not affect the ratio of reactivated enzyme to aggregates [cf. Table 1 in Zettlmeissl et al. (1979)].

Under renaturing conditions only native oligomers and aggregates are stable, so that eq 4 degenerates to

$$N \leftarrow \vartheta_i \rightarrow \frac{1}{x}A \quad (5)$$

By reconsideration of the fact that the first step in the $\vartheta_i \rightarrow$ N transition is the fast formation of a structured monomeric intermediate D, [cf. Rudolph et al. (1977)], eq 5 may be split into two parallel irreversible reactions

$$\vartheta_i \xrightarrow{\tau} D \quad (6a)$$

$$\vartheta_i \xrightarrow{\tau'} \frac{1}{x}A \quad (6b)$$

In the concentration range under concern, the subsequent transition, D → N, is limited by a slow second-order reaction (Rudolph & Jaenicke, 1976). Equation 6a may be correlated with the fast transitions observed for the process of renaturation by using optical rotary dispersion (Teipel & Koshland, 1971) or fluorescence (Rudolph & Jaenicke, 1976). The competing reaction represented by eq 6b includes association steps; therefore, its rate should depend on the concentration of the reactivating enzyme: at low enzyme concentration it should be slow and thus not interfere with the first-order transition (eq 6a). This is verified in the concentration range from 2 to 400 nM (Rudolph & Jaenicke, 1976). At higher concentrations, the rate of aggregation should increase, causing the yield of reactivation to decrease. As shown in Figure 1, this is indeed the case; the rate of the aggregation is significantly concentration dependent (Figure 2), with an apparent reaction order of $n > 2$ (Figure 3).

At the specific concentration where the yield of reactivation is 50% of the maximum yield, both the rate of reactivation and the rate of aggregation should be equal. At this concentration, a rough estimate of the rate of the first folding reaction (eq 6a) may be deduced from the rate of the aggregation reaction. As a result, the relaxation time is estimated to be $\tau \approx 10^2$ s at 20 °C. Obviously, the aggregation reaction (eq 6b), which becomes predominant at $c > 400$ nM (cf. Figure 1), includes a certain degree of structure formation, as taken from the pronounced minimum of ellipticity at 222 nm (Table I and Figure 4).

Since both τ and τ' may be influenced by the residual structure in the various denatured states (ϑ_i) [Figure 4; cf. Rudolph et al. (1977)], the limiting concentration range for the predominant formation of aggregates is expected to depend on the denaturant. The given explanation of the present results may be complicated by the fact that during refolding a whole spectrum of transconformation reactions may occur. In fact, refolding studies with a number of monomeric enzymes [cf. Baldwin (1975)] (which may be compared with the present $\vartheta_i \rightarrow$ D transition) have shown that there exist slow and fast refolding reactions, which may be attributed to an equilibrium of several denatured forms (Garel & Baldwin, 1973). Including the corresponding additional parameters into the present model may provide a more adequate picture of the complex process of reconstitution. However, at this stage it is not possible to quantitatively determine the respective kinetic parameters. Therefore, the given minimum scheme is considered sufficient to at least qualitatively describe the observed data. The highly stable aggregates represent a heterogeneous population of weakly bound particles. Their stability is manifested by their insolubility under normal solvent conditions which led previous investigators to believe them to be "irreversibly denatured". Experiments reported in the following paper make use of the noncovalent character of the intermolecular interactions. As will be shown, redissolution of aggregates, i.e., reequilibration according to eq 3, can be easily achieved by strong denaturants which then allow partial reconstitution of the native enzyme. It seems promising to use this approach in connection with the isolation and purification of enzymes on a preparative scale.

Acknowledgments

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