Reconstitution of Lactic Dehydrogenase. Noncovalent Aggregation vs. Reactivation. 2. Reactivation of Irreversibly Denatured Aggregates†

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ABSTRACT: Noncovalent aggregation is a side reaction in the process of reconstitution of oligomeric enzymes (e.g., lactic dehydrogenase) after preceding dissociation, denaturation, and deactivation. The aggregation product is of high molecular weight and composed of monomers which are trapped in a minium of conformational energy different from the one characterizing the native enzyme. This energy minimum is protected by a high activation energy of dissociation such that the aggregates are perfectly stable under nondenaturing conditions, and their degradation is provided only by applying strong denaturants, e.g., 6 M guanidine hydrochloride at neutral or acidic pH. The product of the slow redissolution process is the monomeric enzyme in its random configuration, which may be reactivated by diluting the denaturant under optimum conditions of reconstitution. The yield and the kinetics of reactivation of lactic dehydrogenase from pig skeletal muscle are not affected by the preceding aggregation–degradation cycle and are independent of different modes of aggregate formation (e.g., by renaturation at high enzyme concentration or heat aggregation). The kinetics of reactivation may be described by one single rate-determining bimolecular step with $k_2 = 3.9 \times 10^4$ M^{-1} s^{-1} at zero guanidine concentration. The reactivated enzyme consists of the native tetramer, characterized by enzymatic and physical properties identical with those observed for the enzyme in its initial native state.

As summarized in the preceding paper (Zettlmeissl et al., 1979b), the formation of noncovalent aggregates represents a side reaction competing with the reconstitution of the native enzyme from the denatured and dissociated state.

In the case of oligomeric enzymes, the mechanism of reactivation comprises consecutive transconformation and association steps (Jaenicke, 1979). At high enzyme concentrations, the aggregation to high molecular weight particles outruns the folding to structured monomers, which are essential in the acquisition of the native quaternary structure. Consistent with this mechanism, the aggregates are found to closely resemble the denatured monomers regarding their internal structure. As shown by the conditions which have been applied for their preparation, they are highly stable. Therefore, they are conventionally referred to as "irreversibly denatured aggregates", this term being used to distinguish the inactive high molecular weight material from the reactivated native enzyme.

In the present study, attempts have been made to regenerate catalytic activity from noncovalent aggregates of lactic dehydrogenase (LDH).[1] This enzyme may be considered a representative example for an oligomeric enzyme with noncooperative subunits (Holbrook et al., 1975) which are inactive in their isolated form (Jaenicke & Rudolph, 1977).

The final goal of the approach is threefold: (1) it is intended to examine experimental conditions which are suitable to restore enzyme activity from the aggregated state; (2) if significant reactivation is accomplished, characterization of the reconstituted enzyme is required to prove or disprove the native three-dimensional structure of the final product; (3) the optimization of the procedure may then be applied, on a preparative scale, to separate enzymes with different aggregation characteristics.

Materials and Methods

LDH-M_4 from pig skeletal muscle (Boehringer) was used. All reagents were the same as those reported in the preceding paper (Zettlmeissl et al., 1979b). The preparation of the stock solutions of the enzyme and the determination of enzyme concentration and enzyme activity have also been previously described (Zettlmeissl et al., 1979b).

Aggregation was achieved by two different methods.

(1) Native LDH (c = 5 mg/mL) was denatured by dilution (1:5) in 0.1 M H_3PO_4, pH 2.3, in the presence of 1 mM EDTA and 1 mM DTE (15 min at 20 °C); subsequent dilution (2:5) in 0.1 M phosphate buffer (final pH 6.8) plus 1 mM EDTA and 1 mM DTE (30 min at 20 °C) causes aggregates to be formed which can be separated by centrifugation (20 min at 48000g). After the aggregates were washed with 0.1 M phosphate buffer plus 10 mM EDTA and 1 mM DTE and repeatedly centrifuged, the collected aggregates were resuspended in the same buffer (final concentration ~3 mg/mL). No enzyme activity could be detected in the suspension; the yield of aggregates was >90% relative to the initial enzyme concentration. In some experiments, aggregates obtained from the given procedure were resuspended and washed in water and subsequently lyophilized at room temperature in order to provide aggregates in their unsolvated form.

(2) Aggregates produced by heat denaturation (10 min at 80 °C) in 0.1 M phosphate buffer, pH 7.6, plus 10 mM EDTA and 1 mM DTE could be used without further treatment.

Deaggregation (degradation) was provided either by incubation of the aggregates for 20 min (20 °C) in 0.1 M H_3PO_4, pH 2.3, in the presence of 6 M guanidine hydrochloride plus 1 mM EDTA and 1 mM DTE or by incubation in 0.1 M phosphate buffer, pH 7.6, plus 6 M guanidine hydrochloride, 1 mM EDTA, and 1 mM DTE.

Reactivation of the redissolved material at varying enzyme and guanidine concentrations (20 °C) made use of dilution with 0.1 M phosphate buffer, pH 7.6, plus 10 mM EDTA and 1–10 mM DTE. For analysis of the kinetics of reactivation,

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[1] Abbreviations used: LDH-M_4, porcine lactic dehydrogenase from skeletal muscle; DTE, dithioerythritol; c and c_G, concentration of enzyme and guanidine hydrochloride, respectively.

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Table I: Comparison of Native and Reconstituted LDH-M_4. Reconstitution after Degradation of Aggregates in 6 M Guanidine Hydrochloride (cf. Materials and Methods)[a]

state of the enzyme[b]	$s^0_{20,w}$	M_r	F_{rel} λ_{max}	F_{rel} %	$[\Theta]_\lambda$ (deg cm² dmol⁻¹) 222 nm	$[\Theta]_\lambda$ (deg cm² dmol⁻¹) 208 nm	$\Delta\epsilon$ (M^{-1} cm^{-1}), 282 nm	specific activity (IU/mg)	$K_{m,pyruvate}$ (μM)
native enzyme	7.2 ± 0.1	140 000	339	100	−15.3	−11.4	57.3	640	163 ± 30
reactivated enzyme	7.2 ± 0.1	140 000	338	103	−15.6	−12.3	58.2	650	169 ± 30

[a] Sedimentation analysis (Beckman Model E), sedimentation velocity at 40 000 rpm, and high-speed meniscus depletion sedimentation equilibrium at 20 000 rpm; relative fluorescence (Hitachi Perkin-Elmer MPF 44A), λ_{exc} = 280 nm; circular dichroism (Jouan-Roussel Dichrographe II), dichroic absorption $\Delta\epsilon$ and ellipticity $[\Theta]_\lambda$ referring to M_r 140 000 and MRW 113. [b] 0.1 M phosphate buffer, pH 7.6, plus 1–10 mM EDTA and 1 mM DTE, 20 °C.

aliquots were taken at defined times; the optical tests were performed as indicated (Zettlmeissl et al., 1979b). Final values of reactivation were measured after up to 120 h. The relative reactivation and the yield of reactivation were calculated according to

$$\% \text{ reactivation} = \frac{\text{reactivation at time } t}{\text{final reactivation}} \times 100$$

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

The final reactivation was calculated relative to the native enzyme incubated under identical conditions of the experiment. In general, the stability of the enzyme was high so that no correction for time-dependent deactivation of the native reference was required during the whole time course of reactivation.

Results

Degradation of Aggregates. Earlier investigations in connection with the heat aggregation of globular proteins (Jaenicke, 1967) have shown that the degradation of aggregates in mixed solvents may regenerate the initial particle weight of a given protein. If this approach is applied to oligomeric enzymes like LDH after aggregation under various conditions, it is obvious that treatment with strong denaturants in the presence of SH-protecting agents leads back to the enzyme in its monomeric state as shown by direct light-scattering measurements (Jaenicke, 1967). In the present study, we made use of the reconstitution of monomers to active tetramers to monitor the degradation of aggregates. This method is based on the observation that the catalytic activity of the redissolved subunits may be restored.

By use of this approach, the kinetics of degradation may be easily followed by incubating the aggregates under dissociating conditions for defined times and subsequent reactivation.

Contrary to the exothermic depolymerization reactions observed in the case of cold inactivated enzymes (Nagradova et al., 1975) or tobacco mosaic virus protein (Lauffer, 1975), temperature changes do not affect the state of aggregation. This is shown by the fact that at neutral pH in the absence of denaturants no dissociation of aggregates is observed in the temperature range from 0 to 50 °C. Similarly, no degradation of aggregates is detectable in the presence of moderate denaturants such as acidic pH (1 M glycine–H_3PO_4, pH 2.3) or 6 M urea. Only guanidine hydrochloride in the presence or absence of 0.1 M H_3PO_4 (pH 2.3) is sufficiently potent to dissociate the aggregates of LDH. The kinetics of dissociation of aggregates formed by method 1 is found to be a slow first-order process with a time constant of the order of k_1 = 1.7 × 10^{-3} s^{-1} (Figure 1).

Reactivation and Renaturation. As mentioned before, the redissolved subunits may be reactivated by diluting the guanidine to concentrations below 0.5 M.

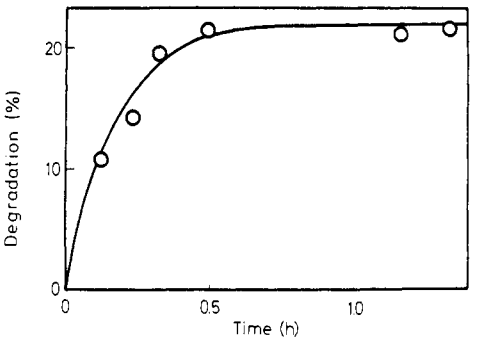

FIGURE 1: Kinetics of degradation of aggregates of lactic dehydrogenase (LDH-M_4) in 6 M guanidine hydrochloride–0.1 M H_3PO_4, pH 2.3, in the presence of 1 mM EDTA and 1 mM DTE at c = 0.4 mg/mL (20 °C). Aggregates were prepared as described under Materials and Methods without lyophilization (method 1). Degradation was measured as the reactivation yield after 120 h. The solid line was calculated according to a first-order reaction with k_1 = 1.7 × 10^{-3} s^{-1}.

The recovery of the activity in the course of reassociation and renaturation was followed by sampling aliquots at certain time intervals under varying enzyme and guanidine concentrations. The latter parameter is of crucial importance since it has been shown (Zettlmeissl et al., 1979a) that the kinetics of reactivation of LDH-M_4 are strongly affected by the presence of low concentrations of guanidine hydrochloride. Under optimum conditions, reactivation amounts to 25 ± 5%. After separation from "irreversibly denatured aggregates" the reactivated tetramer is indistinguishable from the native enzyme with regard to a variety of physicochemical and enzymological criteria (Table I).

The kinetics of reactivation of redissolved material at varying final concentrations of guanidine hydrochloride are illustrated in Figures 2 and 3.

By neglection of the contribution of unimolecular reactions to the rate-limiting process of the reconstitution reaction, the time course of reactivation may be approximated by second-order rate constants k_2; at low guanidine concentration (~0.01 M) k_2 is of the order of 10^4 M^{-1} s^{-1} [Table II; cf. Zettlmeissl et al. (1979a)]. This limiting value is in agreement with previous determinations (Rudolph & Jaenicke, 1976).

At a given constant guanidine concentration, the rate-limiting step of the reactivation process depends on the enzyme concentration according to second order (n = 2.0 ± 0.1) (Figure 4). By comparison of the reactivation of the enzyme after acid denaturation–renaturation and heat aggregation, no differences are observed concerning the yield and kinetics, as well as the rate-decreasing effect of guanidine hydrochloride; the same holds for the lyophilized aggregates. Taking the second-order rate constants of the reactivation reaction as a quantitative measure for the rate of reconstitution under the different conditions of denaturation and renaturation, it is obvious that under comparable conditions closely similar rates are observed (Table II).

Table II: Second-Order Rate Constants, k_2, for the Reactivation of Lactic Dehydrogenase after Denaturation and Aggregation-Degradation in Various Denaturants [cf. Rudolph & Jaenicke (1976)]

degradation conditions	k_2 (M^{-1} s^{-1}) at the following Gdn·HCl concn (M)[a] in the reactivation medium					
	0	0.01	0.03	0.05	0.10	0.12
nonaggregated, after denaturation in 1 M glycine-H_3PO_4, pH 2.3	3.9×10^4	1.18×10^4	4.9×10^3			8.3×10^2
nonaggregated, after denaturation in 6 M Gdn·HCl-0.1 M H_3PO_4, pH 2.3		1.14×10^4	4.3×10^3			9.2×10^2
aggregated after acid denaturation (method 1), degraded in 6 M Gdn·HCl-H_3PO_4, pH 2.3		1.16×10^4	4.7×10^3			8.1×10^2
aggregated after acid denaturation (method 1 and lyophilization), degraded in 6 M Gdn·HCl-H_3PO_4, pH 2.3		1.16×10^4	4.9×10^3			6.7×10^2
aggregated by heat (method 2), degraded in 6 M Gdn·HCl-phosphate buffer, pH 7.6		1.16×10^4		2.3×10^3	7.2×10^2	

[a] With regard to the effect of the guanidine concentration in the reactivation buffer cf. Zettlmeissl et al. (1979a).

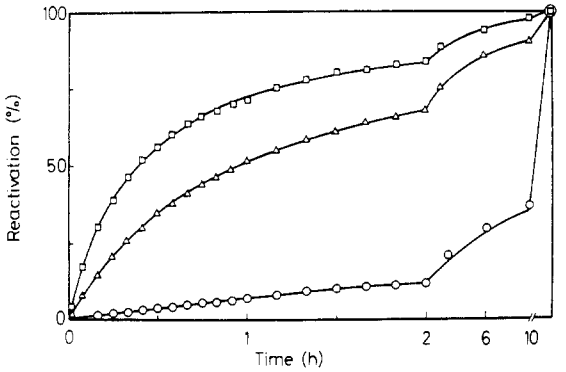

FIGURE 2: Kinetics of reactivation of LDH-M_4 after aggregation (by method 1 under Materials and Methods) and degradation by 20-min incubation in 6 M guanidine hydrochloride–0.1 M H_3PO_4, pH 2.3, in the presence of 1 mM EDTA and 1 mM DTE (20 °C). Reactivation was in 0.1 M phosphate buffer, pH 7.6, in the presence of 1 mM EDTA and 1 mM DTE. c_{LDH} = 62 ± 2 nM. Guanidine hydrochloride concentration: 0.01 (□); 0.03 (△); 0.12 M (○). The solid lines were calculated according to an irreversible bimolecular mechanism with $k_2 = 1.16 \times 10^4$ M^{-1} s^{-1}, $k_2 = 4.7 \times 10^3$ M^{-1} s^{-1}, and $k_2 = 8.1 \times 10^2$ M^{-1} s^{-1} for 0.01, 0.03, and 0.12 M guanidine hydrochloride, respectively.

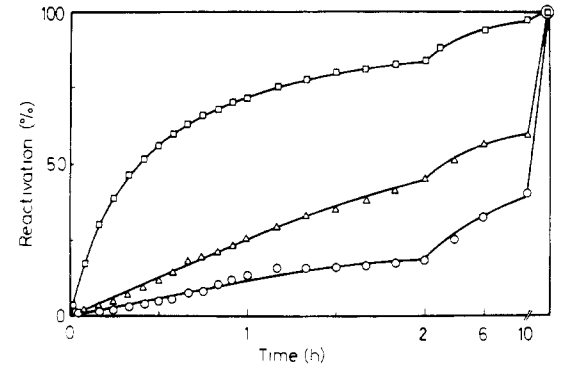

FIGURE 3: Kinetics of reactivation of lactic dehydrogenase (LDH-M_4) after heat aggregation (method 2) and degradation in 6 M guanidine hydrochloride–0.1 M H_3PO_4, pH 2.3 (20 min at 20 °C), in the presence of 1 mM EDTA and 1 mM DTE. Reactivation was in 0.1 M phosphate buffer, pH 7.6, in the presence of 1 mM EDTA and 1 mM DTE (20 °C) at c_{LDH} = 62 ± 2 nM. Guanidine hydrochloride concentration: 0.01 (□); 0.05 (△); 0.10 M (○). The solid lines were calculated according to an irreversible bimolecular mechanism with $k_2 = 1.16 \times 10^4$ M^{-1} s^{-1}, $k_2 = 2.3 \times 10^3$ M^{-1} s^{-1}, and $k_2 = 7.2 \times 10^2$ M^{-1} s^{-1} for 0.01, 0.05, and 0.10 M guanidine hydrochloride, respectively.

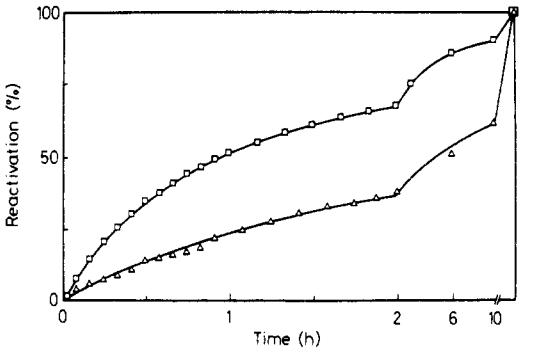

FIGURE 4: Kinetics of reactivation of lactic dehydrogenase (LDH-M_4) after aggregation and degradation. Effects of enzyme concentration at constant guanidine concentration (c_G = 0.03 M). Experimental conditions were as described in Figure 2. Enzyme concentration: 62 (□); 16 nM (△). The solid lines were calculated according to a second-order reaction with $k_2 = 4.7 \times 10^3$ M^{-1} s^{-1}.

The fact that the kinetics of reactivation of the nonaggregated fraction (Rudolph & Jaenicke, 1976), as well as the reactivation of the various types of aggregates, obey a bimolecular mechanism with comparable rate constants and a similar dependence on the guanidine concentration suggests an identical pathway of reactivation. Aggregation and degradation experiments with rabbit muscle aldolase (Zettlmeissl, 1978) show that the present findings may be generalized for other oligomeric enzymes. Recent reactivation studies with "irreversibly inactivated" aspartase (Tokushige & Eguchi, 1978) or trypsin (Klibanov & Mozhaev, 1978) point in the same direction.

Discussion

The foregoing results provide evidence that "irreversibly denatured aggregates" may be reconstituted to the native state, provided that stabilization of aggregates by covalent bonds is excluded. The noncovalent aggregates consist of monomers with partially restored secondary structure. From the irregular aggregates observed by electron microscopy, the conclusion may be drawn that specific interactions dominating the native quaternary structure are not decisive for their stabilization.[2] Nevertheless, they are found to be highly stable under solvent

[2] This does not imply that mixed aggregates are formed if reconstitution is performed in the presence of other proteins (see below). In this regard, the present reconstitution experiments confirm earlier results, e.g., with aldolase (Gerschitz et al., 1977) or tryptophanase (London et al., 1974).

conditions providing optimum stability of the native state, despite the fact that the tetrameric native enzyme is expected to be favored with respect to its free enthalpy. When the loss of translational and rotational entropy upon aggregate formation is considered, the predominant formation of aggregates at moderate denaturant concentrations, or at high concentrations of the renaturing enzyme, needs explanation.

As discussed previously in connection with the stabilization of native molecular assemblies (Chothia & Janin, 1975), a possible mechanism which is most likely to balance the decrease of entropy would be hydrophobic interactions stabilizing the noncovalent aggregates. Other weak interactions, like electrostatic forces between polar groups forming hydrogen bonds or ion pairs, may also participate in the compensatory entropic effects in the solvent (Kauzmann, 1959). By consideration of the stability of the aggregates at low temperatures where hydrophobic interactions are weakened, the latter contribution seems to be established. The aggregates are stable in the presence of moderate denaturants, e.g., low pH or urea. Obviously, within the incubation time applied, the denaturing potential of these agents is insufficient to compensate or to overcome the forces responsible for the stabilization of the aggregates. Only strong denaturants such as guanidine hydrochloride at neutral or acidic pH are suitable to disrupt the interactions. However, this dissociation is much slower than the respective reaction of native tetramers (Wassarman & Burgner, 1972). This difference may be equally well ascribed to a high energy of activation for the dissociation reaction of aggregates or to the restricted accessibility of the denaturant to the high molecular weight particles.

The reactivated fraction consists of the native tetrameric enzyme. This is established by its structural and enzymological properties, as well as by the kinetics of reactivation (Tables I and II). The reaction order and the reaction rate of the redissolved enzyme are found to be identical with the respective data for the reactivation after dissociation of the native enzyme at acidic pH or by 6 M guanidine hydrochloride. The various modes of aggregate formation have no effect on the kinetics of reactivation.

It seems suggestive to apply the given results to recover aggregation products occurring during the purification of enzymes. On the other hand, enzyme fractionation may make use of the approach by selectively reactivating one specific enzyme in a mixture of enzymes, especially in cases where similar electrophoretic and solubility properties do not allow a satisfactory separation. In this context, it is worthwhile mentioning that the specificity of association is considerable. It has been demonstrated that dissociation and regeneration of mixtures of different oligomeric enzymes do not show interference from foreign monomers in the reactivation of a given enzyme [Heider, Rudolph, and Jaenicke, unpublished results; cf. Cook & Koshland (1969)].

Acknowledgments

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