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Effect of Coenzymes and Temperature on the Process of in Vitro Refolding and Reassociation of Lactic Dehydrogenase Isoenzymes†

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ABSTRACT: Dissociation and deactivation of the H_4 and M_4 isoenzymes of lactic dehydrogenase in strong denaturants may be reversed with a yield of reactivation up to 100%. The products of reconstitution are indistinguishable from the native enzymes as far as the Michaelis constants and the dissociation constants for substrate and coenzyme as well as spectral and hydrodynamic properties are concerned. The presence of NAD^+ and NADH does not affect either the conformational state of the product of reconstitution, or the kinetics of reactivation, using the pure apoenzymes as a reference. At 20 °C the kinetics of reactivation for LDH-M_4 in the presence and absence of coenzyme may be quantitatively described by a second-order rate equation ($k_2 = 23.4 \pm 2.6\ mM^{-1}\ s^{-1}$) while LDH-$H_4$ is characterized by a uni-bimolecular reaction sequence ($k_1 = 1.45 \pm 0.45 \times 10^{-3}\ s^{-1}$, $k_2 = 5 \pm 1\ mM^{-1}\ s^{-1}$), in agreement with earlier observations (Rudolph, R., et al. (1977), *Biochemistry 16,* 3384–3390). Regarding the influence of temperature on the rate of reactivation no significant anomalies are detectable within the range of 0–25 °C. The (apparent) activation energies, taken from the linear Arrhenius plots, are 58 kcal/mol for the association reaction of LDH-M_4, and 41 kcal/mol for the transconformation reaction of LDH-H_4.

The role of the amino acid sequence in the attainment of the three-dimensional structure of proteins is an established fact (Sela et al., 1957; Anfinsen, 1973). On the other hand, solvent parameters, like temperature and salts, or specific ligands (e.g., substrates or coenzymes) were shown to play an important role in the acquisition of the native structure of enzymes (Wetlaufer and Ristow, 1973). A number of mechanisms were proposed to define possible kinetic constraints in the thermodynamically determined process of reconstitution (divergency of stable structures, nucleation, ligand binding to unfolded polypeptide chains, stabilization of the native state, etc.).

In the case of NAD-dependent dehydrogenases, reactivation

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studies seem to demonstrate that the rate and extent of refolding are substantially influenced by specific ligands which would indicate "metabolic control" on the level of the folding and assembly of the nascent polypeptide chains into active enzymes or other specific protein structures (Deal, 1969). For glyceraldehyde-3-phosphate dehydrogenase, lactic dehydrogenase, and malate dehydrogenase, NAD^+ and/or NADH were claimed to have a significant effect on the time course of reactivation (Chilson et al., 1966; Deal, 1969; Teipel and Koshland, 1971; Levi and Kaplan, 1971; Jaenicke, 1974; Tenenbaum-Bayer and Levitzki, 1976). For the first enzyme it has been reported that the presence of NAD^+ in the reassociation medium is essential (Deal, 1969). On the other hand, anomalous temperature effects, e.g., in the reactivation kinetics of LDH,[1] have been supposed to prove hydrophobic clusters as nucleation centers in the process of refolding (Jaenicke, 1974; Gutfreund, 1975). Elimination of the given "nucleation parameters" has been reported to cause folding to different enzyme conformations which, in certain cases, may be transformed to the native structure by addition of the nucleating agent.

Recent denaturation-renaturation experiments have clearly shown that the recovery of enzymatic activity is paralleled by full reconstitution of the native quaternary structure (Jaenicke et al., 1975). Reactivation and renaturation of the apoenzyme are characterized by identical rate constants, no matter what the mode of denaturation used to provide dissociation and deactivation (Gerschitz et al., 1977; Jaenicke and Rudolph, 1977). Separation of irreversibly denatured aggregates leads to a final product of reactivation indistinguishable from native enzyme by all available biochemical and physicochemical criteria (Rudolph et al., 1976, 1977a,b; Jaenicke and Rudolph, 1977). The given results make it necessary to reevaluate the effects of coenzymes and temperature on the reactivation of oligomeric enzymes. The present data show that, in the case of lactic dehydrogenase, NAD^+ and NADH do not accelerate the process of reactivation, nor does the temperature dependence of the respective rate constants indicate deviations from normal Arrhenius behavior.

Materials and Methods

Lactic dehydrogenase (LDH)-H_4 from pig heart and M_4 from pig skeletal muscle, trypsin, NADH, and NAD^+ were obtained from Boehringer (Mannheim); dithiothreitol and dithioerythritol were from Calbiochem (Luzern); ultra-pure guanidine hydrochloride was from Schwarz/Mann (New York). Trypsin was treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Serva, Heidelberg) (Carpenter, 1967). All other reagents were of A-grade purity (Merck, Darmstadt). Quartz twice distilled water was used throughout.

Stock solutions of the enzyme (~4 mg/mL) were prepared by dialysis at 4 °C against 0.2 M phosphate buffer (pH 7.6) containing 1 mM EDTA and 0.1 mM dithiothreitol.

Enzyme concentration was calculated from $A_{280\,nm}^{0.1\%} = 1.4$ cm²/mg (Jaenicke and Knof, 1968); this figure holds for both the native and renatured enzyme. Molar concentrations refer to the subunit molecular weight of 35 000.

Enzyme activity was measured in phosphate buffer (pH 7.0), $I = 0.1$ M, or in 0.2 M phosphate buffer (pH 7.6) containing 1 or 10 mM EDTA and 0.1-10 mM dithiothreitol (plus 0.74 mM pyruvate and 0.2 mM NADH) using a thermostated recording Eppendorf spectrophotometer. The specific activities of the native and renatured H_4 and M_4 isoenzymes were 400 ± 20 and 640 ± 30 IU/mg, respectively.

Deactivation and dissociation of the native enzymes at 0-25 °C were achieved by 3-15-min incubation after dilution (1:5) with 1 M glycine/H_3PO_4, pH 2.3 (plus 1 mM EDTA and 1 mM dithiothreitol).

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

To characterize the reactivated enzyme, solutions were concentrated in an Amicon Diaflo with PM 10 filters and dialyzed against 0.2 M phosphate buffer (pH 7.6), containing 1 mM EDTA + 0.1 mM dithioerythritol. Irreversibly denatured aggregates were removed by centrifugation and subsequent Millipore filtration. Equilibrium constants for the binary complexes of LDH and NADH were determined by measuring the increase in coenzyme fluorescence upon complex formation (Winer et al., 1959): Hitachi Perkin-Elmer MPF 44A; λ_{exc} = 320 nm, λ_{em} = 435 nm; 0.2 M phosphate buffer (pH 7.6) + 1 mM EDTA + 0.1 mM dithioerythritol, 25 °C. Michaelis constants were derived from Eadie-Hofstee plots measuring the initial velocities of the enzymatic reaction at saturating concentrations of NADH and varying amounts of pyruvate. The degree of saturation was calculated on the basis of the following equilibrium constants (25 °C): (LDH-H_4) $K_{D,NADH}$ = 3.9 μM; K_{D,NAD^+} = 0.32 mM (Schmid et al., 1976); (LDH-M_4) $K_{D,NADH}$ = 8.6 μM; K_{D,NAD^+} = 0.50 mM (Hinz et al., 1977).

The kinetics of reactivation were analyzed by taking aliquots at defined times. In the case of the temperature dependent measurements samples were withdrawn using thermostated pipets, and tested in the presence of 20 μg/mL trypsin (Chan et al., 1973), in order to avoid reactivation in the enzymatic test.

Results

Maximum reactivation of lactic dehydrogenase depends on three requirements which may be realized by choosing the right experimental conditions: (1) elimination of chemical modification of the polypeptide chain during the denaturation-renaturation cycle (D ⇌ N) (1-10 mM EDTA, 0.1-10 mM dithiothreitol, short incubation under extreme denaturing conditions); (2) inhibition of incorrect aggregation by applying essentially irreversible conditions during reactivation; rapid transition D → N and low enzyme concentrations ($c < 0.1$ mg/mL); (3) sufficiently long time of reactivation to provide the true final value of reactivation which is essential for the kinetic analysis (at low concentrations and temperature, up to 500 h).

For both isoenzymes the reactivation temperature does not influence the extent of reactivation (Table I). The reactivation of LDH-M_4 is limited by a second-order reaction (Rudolph and Jaenicke, 1976). For this association process strict linearity in the Arrhenius plot is observed even at low temperature (0 °C); at 20 °C only slight deviations are detectable. The activation energy estimated from the Arrhenius plot is 58 kcal/mol (Figure 1). In the case of LDH-H_4 the reaction order of the reactivation reaction is a function of the concentration of the enzyme; the kinetics of reactivation can be described by a consecutive uni-bimolecular reaction (Rudolph et al., 1977b). At the given enzyme concentration and 20 °C, the overall reaction is mainly governed by the unimolecular reaction. Neglecting the sigmoidicity, and assuming that the first-order reaction is rate determining over the whole temperature range,

[1] Abbreviations used: LDH, lactic dehydrogenase; H_4 and M_4 denote the homologous isoenzymes from heart and skeletal muscle; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotides; c, enzyme concentration; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Temperature Dependence of the Rate and Yield of Reactivation for LDH-M_4 and LDH-H_4 (cf. Figure 1).

	LDH-M_4			LDH-H_4		
Temp (°C)	Yield (%)	Half-time (h)	k_2 (mM^{-1} s^{-1})	Yield (%)	Half-time (h)	k_1 (s^{-1})
25.0	90.1			24.4	0.15	1.16
20.0	99.0	0.06	15.5	52.7	0.4	0.40
15.0	90.1	0.15	6.4	47.7	1.1	0.17
11.0	85.5	0.60	1.4	52.2	7	0.05
7.0	86.1	3.0	0.33	56.5	13	0.02
3.0	79.7	15	0.08	47.3	43	0.006
0.0	53.2	60	0.03	15.5[a]		

[a] Reaction too slow for a proper evaluation of the final value of reactivation.

TABLE II: Dissociation Constants (K_D) for the Binary Complex LDH-NADH and Michaelis Constant (K_M) for Pyruvate for the Native and Reactivated Isoenzymes of Lactic Dehydrogenase.

		$K_{D,NADH}$ (μM)		$K_{M,pyruvate}$ (μM)[b]	
Isoenzyme	c^a (μM)	Native	Reactivated	Native	Reactivated
LDH-H_4	0.002			92 ± 13	92 ± 13
	1.8	1.6	1.5		
	3.0	1.9	1.7		
	4.5	3.1	2.8		
LDH-M_4	0.002			240 ± 50	243 ± 50
	10.2	6.4	7.3		
	14.2	7.4	8.0		

[a] Enzyme concentration. [b] Ranges of error indicate maximum deviation.

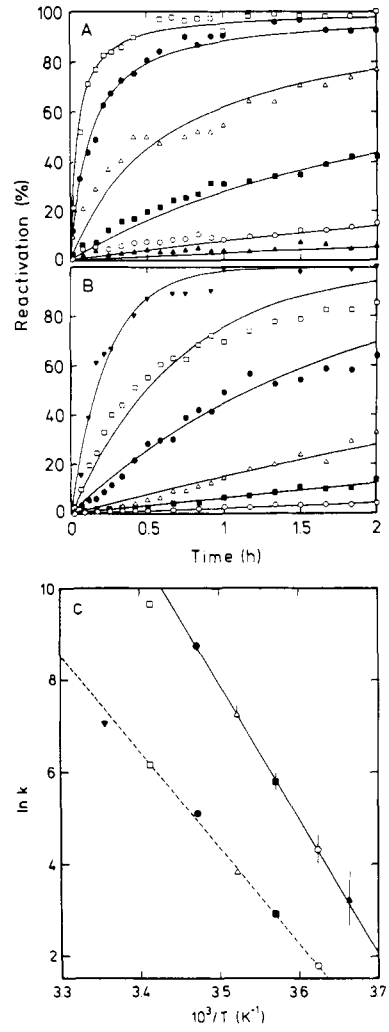

FIGURE 1: Temperature dependence of the reactivation kinetics of LDH-M_4 and LDH-H_4 after deactivation at 11.4 μM (M_4) and 16.7 μM (H_4), respectively. Reactivation by 1:30 dilution: 25 (▼), 20 (□), 15 (●), 11 (△), 7 (■), 3 (○), 0 °C (▲). Reactivation (%) was calculated relative to the final yield determined after up to 360 h (cf. Table I). (A) Reactivation of LDH-M_4: full lines calculated for a bimolecular reaction with rate constants given in Table I. (B) Reactivation of LDH-H_4: full lines calculated for a unimolecular reaction with rate constants given in Table I. (C) Arrhenius diagram for the reactivation of LDH-M_4 and LDH-H_4. Activation energy for the bimolecular reaction of LDH-M_4 (full line): E = 58 kcal/mol. Activation energy for the unimolecular reaction of LDH-H_4 (broken line): E = 41 kcal/mol.

as a first approximation the reactivation relaxations were treated as first-order transconformation processes. The respective activation energy of 41 kcal/mol represents an upper limit because the association reaction is neglected in the present approximation.

According to the specific activity and all available physicochemical criteria the reactivated enzymes, after separation from inactive high aggregates, turn out to be indistinguishable from the native isoenzymes (Rudolph and Jaenicke, 1976; Rudolph et al., 1977b). As shown in Table II this holds also for the Michaelis constants for pyruvate. Since one of the objectives of this study has been the analysis of the influence of the coenzymes, NAD^+ and NADH, on the yield and rate of reactivation, dissociation constants for the binary complexes LDH-NADH for the native and reactivated isoenzymes were determined. As taken from fluorescence titration data, $K_{D,NADH}$ turns out to be identical for the native and reactivated enzymes (Table II). As a consequence the degree of saturation of the renatured or reactivated isoenzymes can be calculated on the basis of the equilibrium constants for NAD^+ and NADH reported for native LDH. The dissociated and denatured enzymes do not show coenzyme binding (McKay and Kaplan, 1961; Anderson and Weber, 1966). In order to compare the reactivation kinetics in the presence of increasing amounts of NAD^+ or NADH, the yield of reactivation and the stability of the reactivated enzymes had to be analyzed over the whole range of coenzyme concentrations. Because of the difference in the dissociation constants, one has to apply much higher concentrations of NAD^+ as compared with NADH in order to provide full saturation of the isoenzymes. In Figure 2 the yield of reactivation of LDH-H_4 in the presence of increasing amounts of coenzyme is shown. NADH has no effect on the yield after 3 h of reactivation while in the presence of 0.5–5 mM NAD^+ a slight increase is observed; incubation over a longer period of time (5–6 days) causes a considerable loss of regained activity at NADH concentrations > 0.02 mM, while in the presence of NAD^+ only slight deactivation occurs. These deactivation effects are observed for both native and reconstituted LDH. They are slowed down significantly by adding dithioerythritol to the reactivation mixture. Since the proper kinetic analysis of the regain of enzymatic activity requires the correct final value of the yield, deactivation of the enzymes during the course of reactivation was taken into account by calculating the yield of reactivation relative to the specific activity of the native enzymes incubated under comparable conditions. Figure 3 shows the deactivation of native LDH-H_4 in the presence of increasing amounts of NAD^+ after long incubation.

Allowing for the aforementioned effects of the time of in-

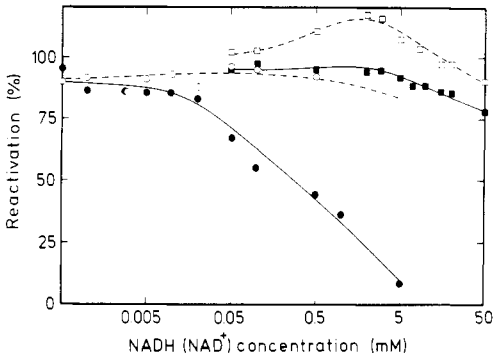

FIGURE 2: Effect of NAD^+ and NADH on the yield of reactivation of LDH-H_4. c = 13.2 μg/mL, 0.2 M phosphate buffer (pH 7.6) plus 10 mM EDTA plus 10 mM dithiothreitol, 20 °C. Reactivation in the presence of NAD^+ (□, ■) and NADH (○, ●) was calculated relative to the final yield of reactivation with no coenzyme present (~36%) and determined after ~3 h (open symbols) and 5-6 days (closed symbols) of reactivation.

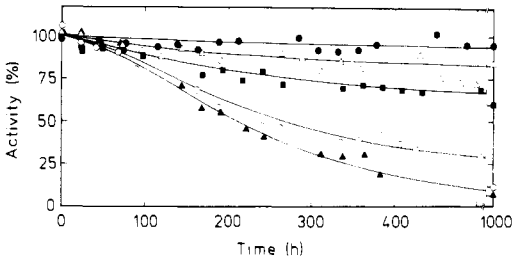

FIGURE 3: Deactivation of native LDH-H_4 in the presence of increasing amounts of NAD^+. c = 10.8 μg/mL, 0.2 M phosphate buffer (pH 7.6) plus 10 mM EDTA plus 10 mM dithiothreitol, 20 °C. (●) Apoenzyme; (△) 0.1 mM NAD^+ (24% saturation); (■) 1 mM NAD^+ (76%). (○) 5 mM NAD^+ (94%); (▲) 10 mM NAD^+ (97%).

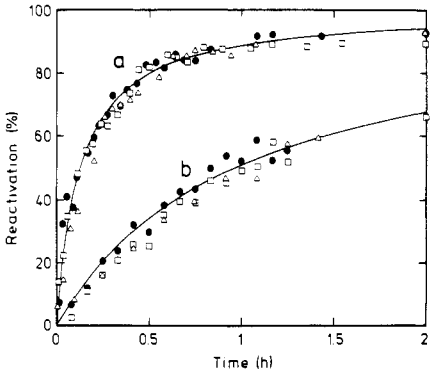

FIGURE 4: Kinetics of reactivation of LDH-M_4 in the presence and absence of NAD^+ and NADH, with 0.2 M phosphate buffer (pH 7.6) plus 1 mM EDTA plus 0.1 or 1 mM dithioerythritol, 20 °C. (a) c = 89 ± 2 nM; (●) apoenzyme; (△) 1.14 mM NAD^+ (70% saturation); (□) 1.14 mM NADH (99%). (b) c = 11 ± 1 nM; (●) apoenzyme; (△) 1.0 mM NAD^+ (67% saturation); (□) 1.0 mM NADH (99%). Solid lines were calculated according to a bimolecular mechanism (k = 23.4 mM^{-1} s^{-1}) [cf. Rudolph and Jaenicke (1976)].

cubation and the concentration of the coenzyme, the profiles of reactivation for the apo- and holoenzyme of LDH-M_4 turn out to be identical within the limits of error (Figure 4). Evidently all the experimental data may be fitted by a single second-order rate constant. The full line corresponds to the bimolecular reactivation and refolding mechanism with k_2 = 23.4 mM^{-1} s^{-1} which was reported earlier for this enzyme in the absence of coenzyme (Rudolph and Jaenicke, 1976).

The respective data for LDH-H_4 are summarized in Figure 5. This time the apoenzyme was titrated with increasing

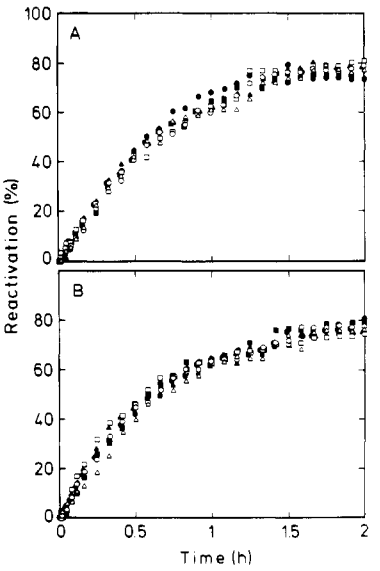

FIGURE 5: Kinetics of reactivation of LDH-H_4 in the presence and absence of NAD^+ and NADH, with 0.2 M phosphate buffer (pH 7.6) plus 10 mM EDTA plus 10 mM dithiothreitol, 20 °C. The final reactivation was determined after up to 47 days and refers to the native enzyme under identical experimental conditions. The final yield of reactivation (60 ± 5%) is taken as 100%; the concentration of the renatured enzyme was 204 ± 15 nM. (A) Reactivation in the presence of NAD^+: (●) apoenzyme; (△) 0.5 μM (11% saturation); (■) 1 μM (20%); (○) 5 μM (66%); (▲) 10 μM (72%); (□) 50 μM (93%). (B) Reactivation in the presence of NADH: (●) apoenzyme; (△) 0.1 mM (24% saturation); (■) 0.5 mM (61%); (○) 1 mM (76%); (▲) 5 mM (94%); (□) 10 mM (97%).

amounts of NAD^+ and NADH. Again the kinetics of reactivation turn out to be unaffected by the coenzyme. The half-times for the given profiles correspond precisely to the half-time calculated for the uni-bimolecular mechanism which has been found to be sufficient to describe the sigmoidal reactivation kinetics for this isoenzyme (Table III), cf. Rudolph et al. (1977b).

Discussion

Dissociation and deactivation of the H_4 and M_4 isoenzymes of lactic dehydrogenase in strong denaturants may be reversed with a yield up to 100%. The products of reactivation are indistinguishable from the native enzymes. Quantitative data proving this statement include Michaelis constants and dissociation constants for substrate and coenzyme, as well as spectral and hydrodynamic properties (cf. Jaenicke et al., 1975). The presence of coenzyme has no effect on the conformational state which forms upon renaturation (Chilson et al., 1966). The kinetics of reactivation are described by a second-order reaction in the case of the M_4 isoenzyme (Rudolph and Jaenicke, 1976), and by a uni-bimolecular reaction sequence for LDH-H_4 (Rudolph et al., 1977b). Regarding the influence of temperature and coenzymes on the rate and/or final extent of reactivation, contradicting results have been reported. The crucial point in this context seems to be the problem of determining the true final value of reactivation. At low temperatures and low enzyme concentrations, it may take considerable time to reach this final value. During this time, side reactions may compete with the refolding process. Correcting for these side reactions makes the anomalous temperature dependence (Gutfreund, 1975) disappear in the case of the second-order reaction governing the reactivation of LDH-M_4. For LDH-H_4 a proper analysis according to the Arrhenius relationship is not feasible because of the complex reactivation mechanism. Describing the reaction by a first-

TABLE III: Half-Times of the Reactivation of LDH-H_4 in the Presence of Various Amounts of NAD^+ or NADH. Deactivation and Reactivation Conditions as in Figure 5.[a]

Coenzyme Concn			Half-time
$[NAD^+]$ (mM)	[NADH] (μM)	Saturation (%)	$\tau_{1/2}$ (min)
0	0	0	35.5
0.1	0	24	38.0
0.5	0	61	37.5
1.0	0	76	39.5
5.0	0	94	36.5
10.0	0	97	40.0
0	0.5	11	42.0
0	1.0	20	45.5
0	5.0	56	36.5
0	10.0	72	34.0
0	50.0	93	33.5
Calcd			34.6 ± 8.0[b]

[a] The degree of saturation was calculated for the final concentration of reactivated enzyme (204 nM). [b] Calculated for the given temperature and concentration according to a uni-bimolecular reaction mechanism with the rate constants $k_1 = 1.45 \pm 0.45 \times 10^{-3}$ (s^{-1}) and $k_2 = 5 \pm 1$ ($mM^{-1}\ s^{-1}$) (Rudolph et al., 1977b).

order or second-order mechanism may therefore lead to deviations from linearity in the Arrhenius diagram. Nonlinearity like this (which may be detected for LDH-M_4 too) reflects the complexity of the process of reconstitution and cannot be interpreted in terms of a defined mechanism of activation. The calculation of the activation energy for the reactivation of LDH-H_4 on the basis of a first-order reaction which predominates under the given experimental conditions is expected to provide a first approximation for this rate-limiting step. The influence of the coenzyme on the rate and yield of reactivation has been extensively studied by a number of investigators. Although the discrimination between rate and yield is vague in many of these studies, a pronounced effect of the coenzyme has been proposed (Chilson et al., 1965; Teipel and Koshland, 1971; Levi and Kaplan, 1971; Levitzki and Tenenbaum, 1974; Tenenbaum-Bayer and Levitzki, 1976). Several authors claimed the effect of the coenzyme on the reactivation of LDH to be restricted to the H_4 isoenzyme (Chilson et al., 1966; Anderson and Weber, 1966; Lindy, 1974). Probably this apparent difference of the two isoenzymes is caused by the different rates of reactivation which allow a more reliable determination of the final extent of reactivation in the case of the faster reactivating M_4 isoenzyme.

A possible explanation for the discrepancies between these reports and the present results may be the two antagonistic effects of the coenzyme: (a) increase of the yield of reactivation; and (b) deactivation of reconstituted or native tetramers. In the presence of coenzyme concentrations high enough to provide maximum saturation and minimum deactivation, no "nucleation effect" of reduced or oxidized coenzyme on the reactivation of LDH-M_4 and LDH-H_4 can be observed.

The effect of the coenzyme has been discussed in greater detail in connection with the refolding of glyceraldehyde-3-phosphate dehydrogenase from yeast where more drastic changes in the yield of reactivation occur, again without significant changes of the rate of reactivation (Rudolph et al., 1977a). Obviously the ligand stabilizes the reactivated entities such that aggregation of refolding monomers with the tightened tetrameric holoenzyme is reduced. Chilson et al. (1965) gave a similar explanation as one possible interpretation of their data, while more recent results suggested a nucleation mechanism (Teipel and Koshland, 1971) or a rapid equilibrium of semistructured intermediates (Levi and Kaplan, 1971). Concerning the enzymes investigated in the present study there is no direct experimental evidence for either of the two mechanisms. Therefore, further reaching consequences based on these mechanisms regarding the regulatory function of metabolites in the process of protein folding should be considered with care.

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