Biochimica et Biophysica Acta, 445 (1976) 261—279
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67909

THE REFOLDING OF LACTATE DEHYDROGENASE SUBUNITS AND THEIR ASSEMBLY TO THE FUNCTIONAL TETRAMER

HANA TENENBAUM-BAYER * and ALEXANDER LEVITZKI **

Department of Biophysics, The Weizmann Institute of Science, Rehovot (Israel)

(Received January 22, 1976)
(Revised manuscript received May 17th, 1976)

Summary

The renaturation process of different lactate dehydrogenase isozymes (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) from their unfolded subunits was investigated using a number of techniques, (a) kinetics of activity regain, (b) the kinetics of fluorescence change of the protein tryptophans, (c) kinetics of regain of the fluorescence properties of a covalently attached fluorescence probe (fluorescein) and (d) the kinetics of assembly, by following the intermediate oligomeric species appearing in the assembly pathway from monomers to tetramers. The results indicate that the unfolded polypeptide is converted to the active oligomeric species by the following scheme:

Denatured subunit $\xrightarrow{I}$ partially refolded subunit $\xrightarrow{II}$ folded subunit

$\xrightarrow{III}$ dimer $\xrightarrow{IV}$ tetramer.

Step I and step II are first-order where step II is rate limiting. The ligands NAD^+ and NADH accelerate step II, thus converting step I to the rate-limiting process. The fact that partially folded lactate dehydrogenase subunits are capable of coenzyme binding may indicate the possible role of these ligands in the assembly of lactate dehydrogenase in vivo. Steps III and IV were found to be fast. The intermediate formation of an enzyme dimer which then dimerizes to the tetrameric species is found to be the major assembly pathway. Only a small portion of the lactate dehydrogenase tetramer is formed through the intermediate formation of a trimer intermediate.

---

* In partial fullfilment for an M.Sc. thesis, submitted to the Feinberg Graduate School at the Weizmann Institute, Rehovot, Israel.

** To whom correspondence should be addressed at the Department of Biological Chemistry, The Hebrew University of Jerusalem, Israel.

Abbreviations: NAD^+: nicotinamide-adenine dinucleotide; NADH: reduced nicotinamide-adenine dinucleotide; LDH: lactate dehydrogenase; Nbs_2: 5′,5″,dithio-bis-(2-nitrobenzoic) acid.

Introduction

The process by which a protein obtains its final structure has been a subject of intense study in the last decade. Crick stated in 1958 [1] that "Folding is simply a function of the order of amino acids", which suggests that the primary structure of the protein determines the final three-dimensional configuration which is the most stable thermodynamically.

Anfinsen has indeed proposed a detailed theory [2], proposing the capacity of amino acid sequences to determine tertiary conformations. Anfinsen's hypothesis was first proven to be correct from studies on the renaturation of monomeric enzymes. Studies of Epstein et al. [3] revealed that lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) can be reversibly denatured, demonstrating that both the tertiary and the quaternary conformation are uniquely determined by the amino acid sequence. These results indicate that the amino acid sequence is the only genetic information required to obtain an active enzyme. However, it is known that some enzymes need metal ions, cofactors or other template molecules in order to be active and, therefore, may be essential in the refolding process. It was shown by Saxena and Wetlaufer [4] in circular dichroism studies of lysozyme that a very rapid chain folding occurs before the formation of disulfide bonds. Refolding is, therefore, faster than renaturation and full regain of activity. For alkaline-phosphatase of *Escherichia coli*, it was found that the refolding of the subunit takes place prior to reassociation [5]. Stellwagen and Schachman [6] showed that renaturation of aldolase is first-order with respect to protein concentration, indicating that the subunit association process is not the rate-limiting step.

Cofactors seem to have a dominant effect on the rate of renaturation. NADH has been found to prevent dissociation of lactate dehydrogenase in the presence of sodium dodecyl sulphate [7], in dilute solutions [8] and under other denaturing conditions [9]. The presence of NADH during reassociation was found to stabilize the quaternary structure [10]. In addition, it plays a prominent role in forming the most active and stable structure of the lactate dehydrogenase molecule [11].

Although other workers have investigated the problem of renaturation of various enzymes [5,6,12,13,29] little is known about the detailed mechanism of refolding and the process of assembly of subunits into the active oligomer. Lactate dehydrogenase seems to be an ideal model enzyme for studies on the reassembly of multisubunit proteins. It is a stable tetrameric enzyme composed of four identical subunits, possessing no S-S bonds; its various isozyme forms are easily prepared in large quantities and are available commercially, and both its structure and function are extremely well characterized [14,15,16]. The different lactate dehydrogenase isozymes differ in their amino acid sequence, but the degree of homology is extremely high and thus hybrids are formed in vivo and can be demonstrated in vitro. The phenomenon of hybridization between isozyme forms offers a unique opportunity as a tool for the investigation of the assembly of the tetrameric structure [17]. In this investigation we report on mechanism of lactate dehydrogenase assembly from its acid denatured subunits.

Materials and Methods

The different lactate dehydrogenase isozymes were obtained from Boehringer (Mannheim, W. Germany), NAD^+, NADH and sodium pyruvate were also obtained from Boehringer, L-lactic acid was obtained from Calbiochem. All other chemicals were of the highest analytical grade available. All solutions were prepared in Corning double distilled water.

Assay of lactate dehydrogenase activity

The assay was performed on a Gilford 2400-S spectrophotometer according to the Bergmeyer method [18,30]. The assay mixture was composed of 0.67 mM NADH and 3.3 mM pyruvate in 0.02 mM phosphate buffer (pH 7.4), containing 1 mM EDTA and 1 mM dithiothreitol. (The mixture was freshly prepared prior to use). To 3 ml of assay mixture in a spectrophotometric cuvette, an aliquot of the enzyme was added. The change in absorbance at 340 nm versus time was recorded. The activity was determined from the initial slope. The extinction coefficient for NADH at 340 nm is 6220 $M^{-1} \cdot cm^{-1}$.

Denaturation of lactate dehydrogenase isozymes and their renaturation

In order to determine the optimal condition of denaturation, various sets of experimental conditions were tried for pig isozyme systems. 5 min at 0°C (pH 2.5) were necessary to obtain complete dissociation to subunits and denaturation of the enzyme [17,19]. Longer periods of denaturation or higher temperatures prolonged the half life of renaturation and caused incomplete renaturation.

Ten minutes of incubation in the denaturation medium results in 35—45% renaturation whereas 90—100% renaturation was achieved under the conditions used. Similar low renaturation values are obtained if denaturation is carried out at 25°C instead of 0°C. The cause for the irreversible damage to the lactate dehydrogenase molecule under these conditions is not known. The percent renaturation was defined as the activity of the totally renaturated sample compared to the specific activity of the initial sample prior to denaturation, multiplied by 100.

Experimental conditions were used to obtain 90—100% regain of activity upon renaturation. Denaturation was, therefore, conducted at 0°C for 5 min in 0.2 M glycine/phosphate buffer pH 2.5, containing 1 mM EDTA and 1 mM dithiothreitol in polypropylene or polycarbonate test tubes. It was found that glass should be avoided whenever lactate dehydrogenases are exposed to low pH. The exposure of lactate dehydrogenase to low pH in the presence of glass causes irreversible denaturation. This finding was also observed in the acid denaturation of beef isozymes [20]. Renaturation was achieved by dilution of the acid denatured enzyme into 0.06 M phosphate buffer pH 7.6 to obtain a final pH of 7.4. The process of renaturation was followed by (a) regain of enzyme activity, measured by taking aliquots for lactate dehydrogenase assay (10—50 μl); (b) by the change in the protein fluorescence measured on a Hitachi Perkin-Elmer MPF III fluorescence spectrophotometer (excitation wavelength 289 nm, emission wavelength 340 nm) and (c) following the fluorescence change of the covalently attached reporter group fluoresceinmercury-

(λ_{ex} = 504 nm, λ_{em} = 526 nm) upon renaturation. In (a) and (b) the enzyme concentration was 1 mg/ml in the denaturation mixture and 0.033 mg/ml in renaturation mixture. In (c) the concentration was 0.74 mg/ml in the denaturation mixture and 0.05 mg/ml in the renaturation mixture. The remaining experimental details are described in the legends to Figs. 1—4. Details on the preparation and properties of fluorosceinmercury-lactate dehydrogenase are given below. In all graphs 100% renaturation was defined as the maximal regain of activity or the total change in fluorescence of the renatured sample. In all cases full renaturation measured in either way corresponded to 90—100% of the theoretical absolute value.

Gel electrophoresis

Disc gel electrophoresis was performed according to the method of Davis [21] in the cold. The gel buffer was 0.38 M Tris · HCl, pH 8.8, containing 5% glycerol. The running buffer was 0.025 M Tris/0.192 M glycine, pH 8.4. The gels were fixed with 12.5% trichloroacetic acid for about 30 min, and then stained with a solution of G-250, Coomassie Blue (0.012%) in 12.5% trichloroacetic acid for at least another 30 min. The gels were destained using 7% acetic acid overnight.

Determination of the molecular nature of the assembly intermediates

In order to analyze the pathway of the assembly of pig lactate dehydrogenase subunits, the following experiment was performed. One of the isozymes (H or M) was dissociated and denatured at pH 2.5 as described above. Its subunits were then diluted into the renaturation mixture as described in the experimental section. At various specified times a 5-fold excess of subunits of the other isozyme was added to aliquots of the reassembly mixture of the first isozyme. In this way most of the species formed subsequent to this addition are hybrids of the form H_n M_{4-n}. The mixtures were then allowed to complete renaturation for 4 h. They were then concentrated by vacuum dialysis, using the collodium membranes (Sartorius vacuum dialysis concentrating device, Cat. No. SM 16304), to a concentration of about 2 mg/ml. Disc gel electrophoresis was then performed on the concentrated samples as described in the previous section.

Determination of thiols

SH groups were determined using the Ellman reagent, 5',5',dithiobis(2-nitrobenzoic)acid (Nbs_2) [22]. The reaction with the enzyme was performed in 8 M urea in phosphate buffer pH 8.0 with a 25 molar excess of Nbs_2 over enzyme.

The modification of lactate dehydrogenase SH with fluoresceinmercury acetate

The reagent was prepared according to the method of Karush et al. [23]. Fluorosceinmercury acetate was allowed to react with enzyme in 5 : 1 ratio in 0.02 M PO_4 buffer pH 7.4. The modification process was judged complete when 99% of the enzymatic activity was lost. The modified enzyme was separated from excess reagent on a Biogel P-6 column (2 × 22 cm) equilibrated with 0.02 M phosphate buffer pH 7.4. The enzyme was concentrated to 7.4 mg/ml by ultrafiltration, on collodium membranes, using a Sartorius vacuum dialysis

concentrating device. The change of fluorescence, under renaturation condition, as described before, was measured (λ_{ex} = 504, λ_{em} = 526). Determination of SH groups revealed that the isolated fluoresceinmercury-lactate dehydrogenase derivative possesses two fluoresceinmercury moieties bound per subunit. One of the two fluoresceinmercury groups is probably bound to the active site SH group since the derivative is devoid of enzyme activity (see below). The fluoresceinmercury-lactate dehydrogenase retains its tetrameric structure both before denaturation and following renaturation.

The denaturation of lactate dehydrogenase

The procedure used for lactate dehydrogenase denaturation was shown previously [20] to cause complete dissociation of the enzyme to subunits, accompanied with total loss of enzyme activity.

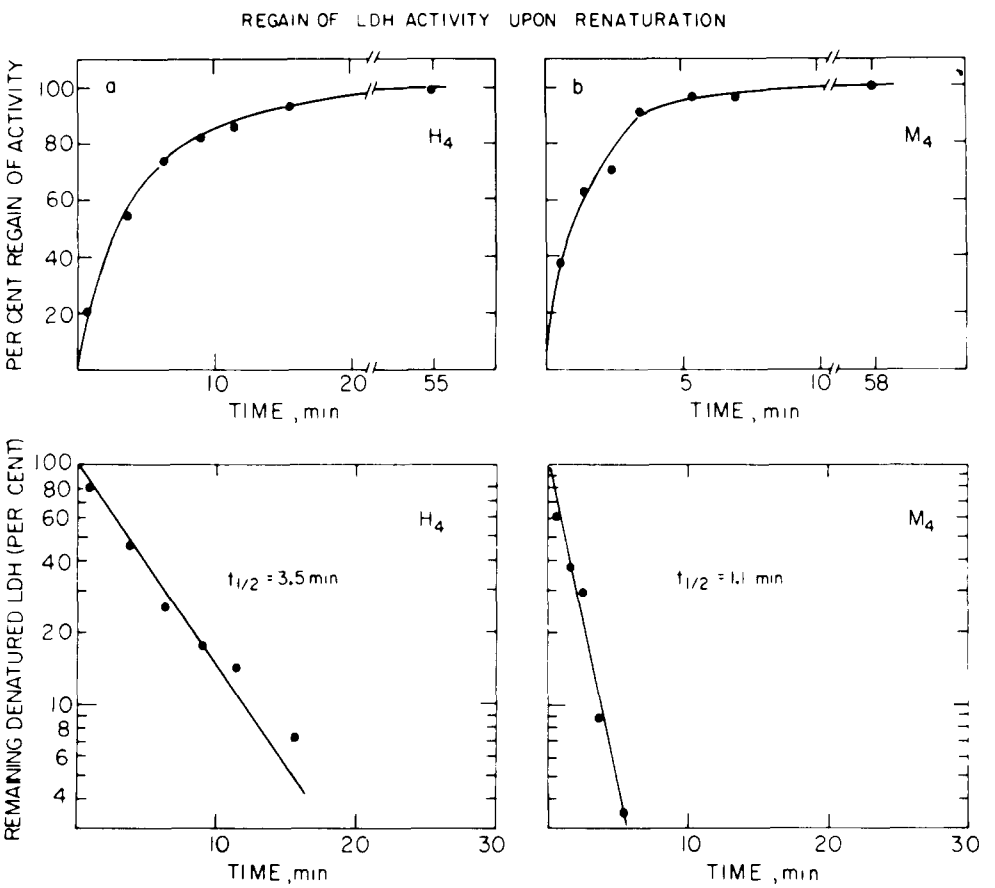

Fig. 1. The regain of protein activity upon renaturation of (a) H_4 and (b) M_4. 1 mg/ml protein was incubated at pH 2.5, 0.2 M glycine/H_3PO_4. 1 mM dithiothreitol 1 mM EDTA for 5 min at 0°C and then diluted 30-fold into 0.06 mM inorganic phosphate buffer pH 7.4 containing 1 mM dithiothreitol 1 mM EDTA. Top: activity vs. time. Bottom: semilog plot of the same data. LDH, lactate dehydrogenase.

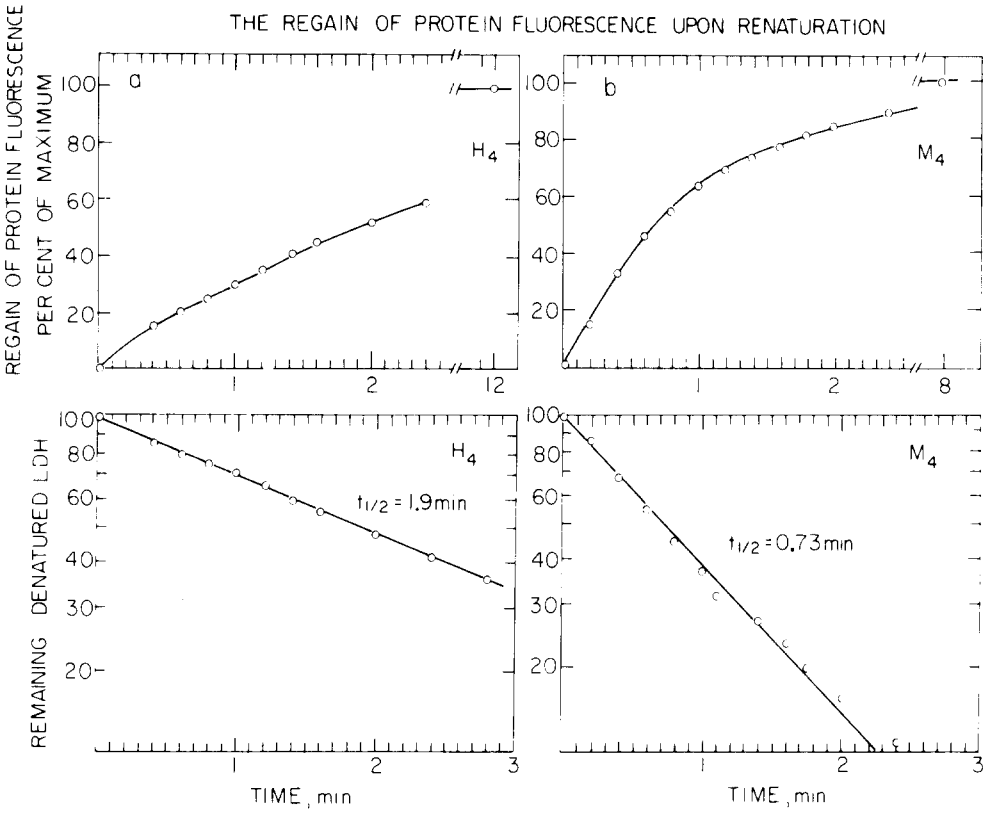

Fig. 2. The regain of protein fluorescence upon renaturation. (a) H_4 and (b) M_4 (λ_{ex} = 289 nm, λ_{em} = 350 nm) experimental conditions as in Fig. 1. LDH, lactate dehydrogenase.

Results

The order of renaturation reaction

The kinetics of renaturation of pig lactate dehydrogenase isozymes (Fig. 1) was found to follow strict first-order kinetics. The rate of renaturation was found to be higher for pig muscle (M_4) than for the pig heart isozyme (H_4). It was also observed that the regain in protein fluorescence (Fig. 2) occurs at a faster rate than the regain of enzyme activity, but is still a first-order process. The data could not be fitted to a second-order process. The zero points were obtained by diluting the denatured enzyme directly into the assay mixture (Fig. 1) or into the fluorescence cuvette (Fig. 2) respectively.

Changes in protein concentration in the range of 10—50 μg/ml in the renaturation solution did not affect the order of the renaturation reaction.

Changes in the conditions of denaturation or renaturation (e.g. temperature, time or pH) did not affect the order of the reaction but affected both the renaturation half-life and the final yield of renatured material (Table I).

The effect of glycerol on renaturation

Glycerol slows markedly the renaturation process of lactate dehydrogenase

TABLE I

EXPERIMENTAL CONDITIONS OF DENATURATION AND RENATURATION FOR LACTATE DEHYDROGENASE ISOZYMES

Isozyme	Denaturation			Renaturation		
	Time	Temperature °C	Temperature °C	Half-life (min)	% *	k (min^{-1})
Pig H_4	5 min	0	25	3.5	90	$1.98 \cdot 10^{-1}$
Pig H_4	1 h	0	18.5	57	42	$1.22 \cdot 10^{-2}$
Pig H_4	5 min	25	25	12.5	67	$5.54 \cdot 10^{-2}$
Pig H_4	5 min	0	25(30% glycerol)	10.5	91	$6.60 \cdot 10^{-2}$
Pig M_4	5 min	0	25	1.1	97	$6.30 \cdot 10^{-1}$
Pig M_4	5 min	0	25(30% glycerol)	6	91	$1.16 \cdot 10^{-1}$
Rabbit M_4	5 min	0	25	2.3	67	$3.01 \cdot 10^{-1}$
Rabbit M_4	5 min	0	25(30% glycerol)	11	66	$6.30 \cdot 10^{-2}$

* percent renaturation of maximal value expected.

isozymes (Fig. 3). Increasing glycerol concentrations led to longer half-lives of renaturation in all three lactate dehydrogenase isozymes tested although the first-order nature of the renaturation process remained unchanged (Tables I and II, Fig. 3).

Effect of ligands on the rate of renaturation

The coenzymes NAD^+ and NADH were found to accelerate the rate of renaturation. These results are summarized in Fig. 4 and also in Table III. The effect of the substrates L-lactate and pyruvate is rather small when alone, whereas the effect of the cofactors NAD^+ and NADH is very significant. The renaturation reaction is substantially accelerated in the presence of either coenzyme. The accelerating effect is further amplified when the renaturation is performed in the presence of 30% glycerol (Fig. 5). Higher concentrations of ligands did not accelerate further the process of renaturation.

The combination of NADH and L-lactate in the renaturation medium is more effective than NADH alone in accelarating the renaturation rate and the effect of the two ligands seems to be additive (Table III). The combination of NAD^+ and pyruvate, on the other hand, results in no regain of activity. This effect has

TABLE II

THE EFFECT OF GLYCEROL ON LACTATE DEHYDROGENASE RENATURATION

For experimental details see Fig. 5, and for % renaturation see Table I.

Lactate dehydrogenate isozyme	Renaturation half-life (min)		
	No glycerol	30% glycerol	50% glycerol
Pig heart H_4	3.5	10.5	37.0
Pig muscle M_4	1.1	6.0	36.0
Rabbit muscle RM_4	2.3	11.0	23.5

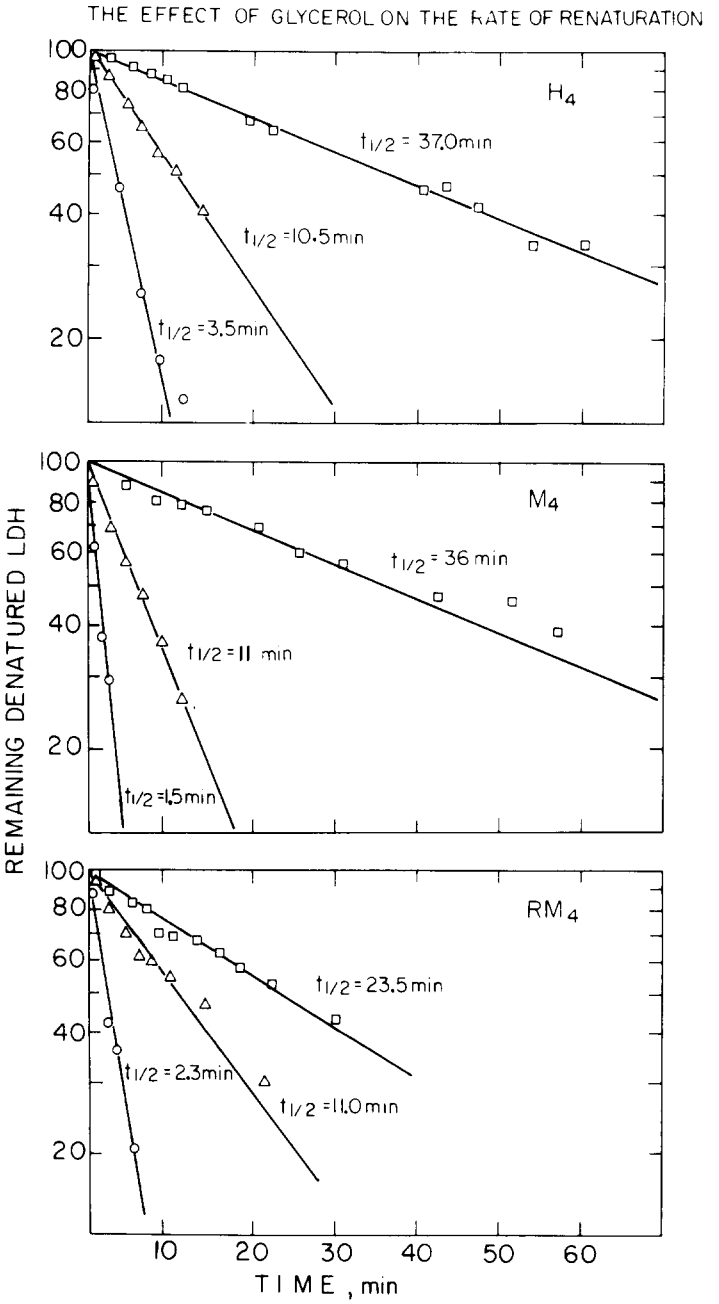

Fig. 3. The effect of glycerol on the rate of lactate dehydrogenase (LDH) renaturation ○———○, no glycerol present; △———△ 30% glycerol; □———□, 50% glycerol. 1 mg/ml of protein was dissociated at pH 2.5, 0° C for 5 min and then diluted 30-fold into the renaturation mixture pH 7.4.

been observed previously [24] and is probably due to the formation of an abortive ternary complex which inhibits the enzyme. NAD^+ and NADH did not have any effect on the rate of protein fluorescence regain upon renaturation.

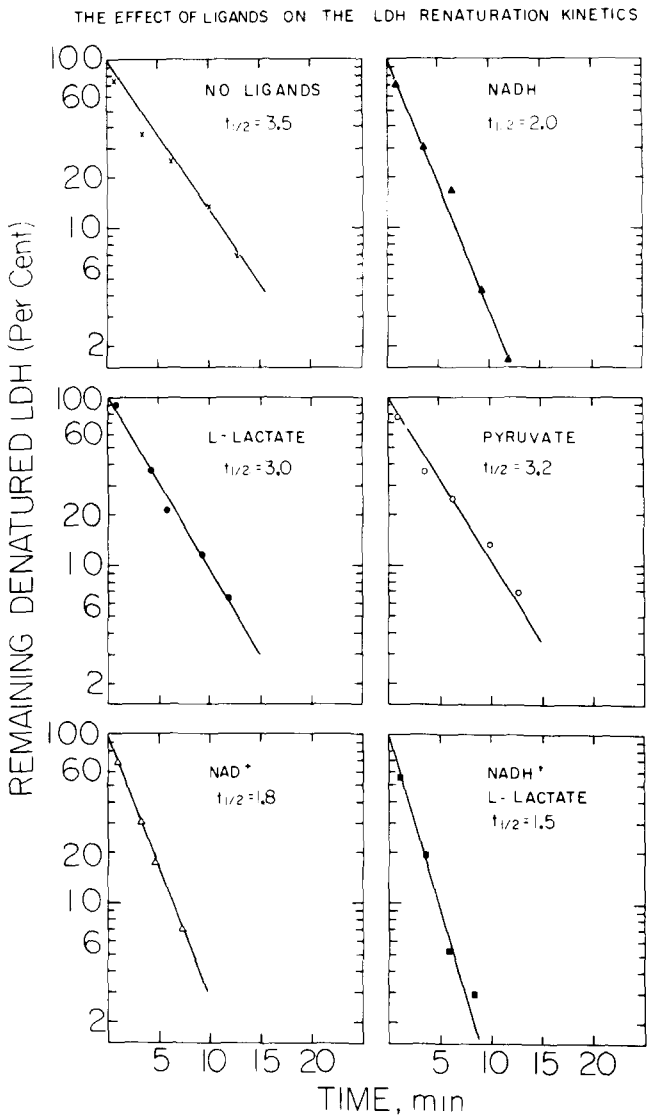

Fig. 4. The effect of ligands on the renaturation kinetics of pig heart, (semilog plot). The denaturation and renaturation of the enzyme was conducted as described in Fig. 3. All ligands were incorporated in the renaturation mixture, each at a concentration of 10 mM. LDH, lactate dehydrogenase.

The renaturation of fluoresceinmercury-lactate dehydrogenase

Incubation of H_4-lactate dehydrogenase with a 5-fold molar excess of fluoresceinmercury acetate over enzyme subunit, results in the formation of an inactive lactate dehydrogenase derivative. This derivative possesses 2 mol of fluoresceinmercury acetate per enzyme subunit. The fluoresceinmercury is bound to 2 of the 4 SH groups per subunits, since after the fluoresceinmercury reaction only 2 out of 4 SH groups [25] can be identified by the reaction with Nbs_2 in 8 M urea. The absorption spectrum of the fluoresceinmercury-H_4-

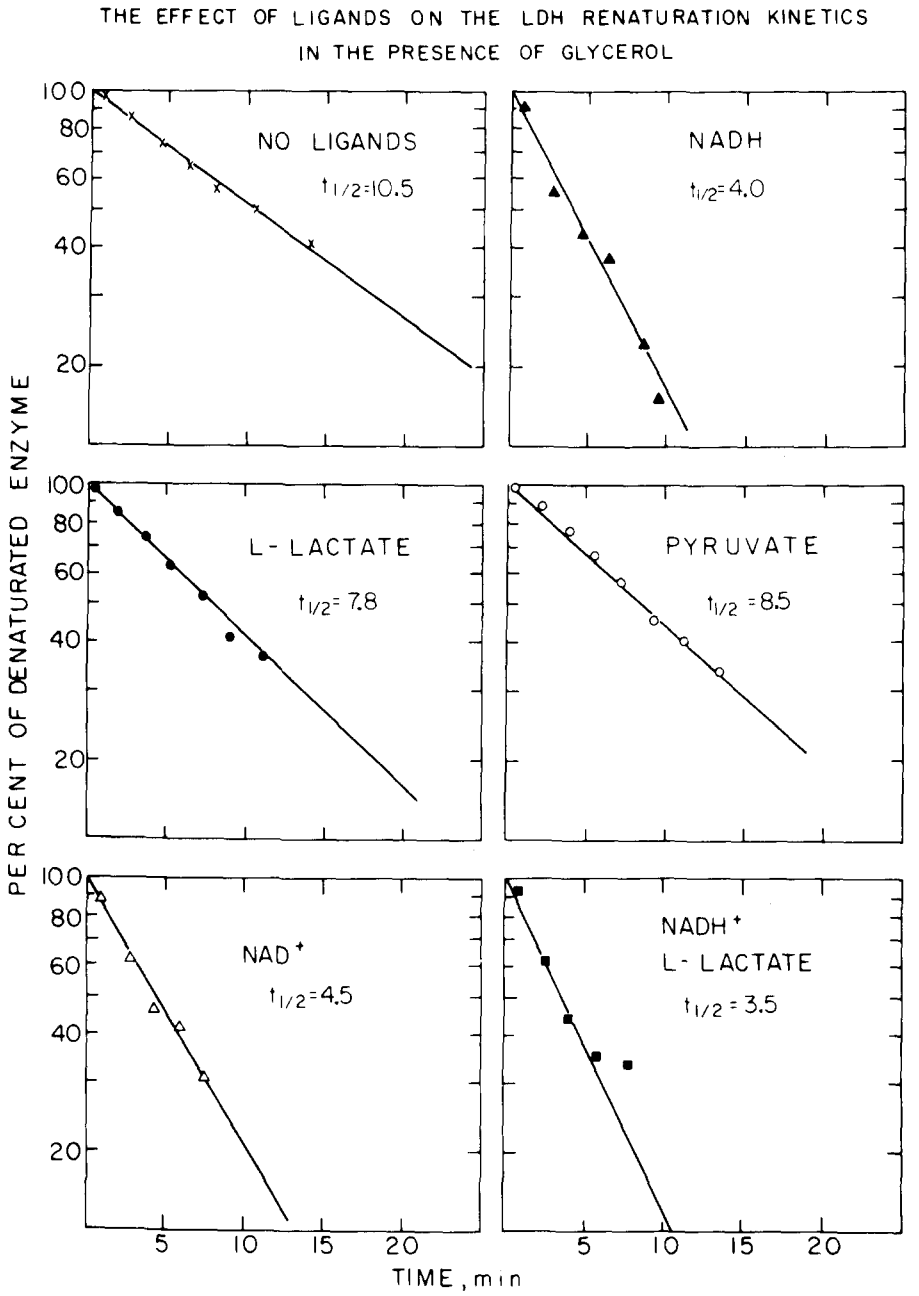

Fig. 5. The effect of ligands on the renaturation kinetics of pig H_4 in 30% glycerol. LDH, lactate dehydrogenase.

lactate dehydrogenase derivative is shown in Fig. 6 and the corrected emission spectrum is shown in Fig. 7.

Denaturation-renaturation experiments were performed on the purified fluoresceinmercury-conjugated H_4 isoenzyme, under the same conditions as used

TABLE III

THE EFFECT OF LIGAND AND GLYCEROL ON LACTATE DEHYDROGENASE-H_4 RENATURATION

Denaturation and renaturation of pig heart was conducted as described in the Materials and Methods section. The ligands were incorporated in the renaturation mixture each at a concentration of 10 mM.

Ligands	Half-life (min) of renaturation	
	No glycerol	30% glycerol
None	3.5	10.5
Pyruvate	3.2	8.5
L-Lactate	3.0	7.8
NAD^+	1.8	4.5
NADH	2.0	4.0
NADH + L-lactate	1.5	3.5
NAD^+ + pyruvate	No regain of activity	No regain of activity

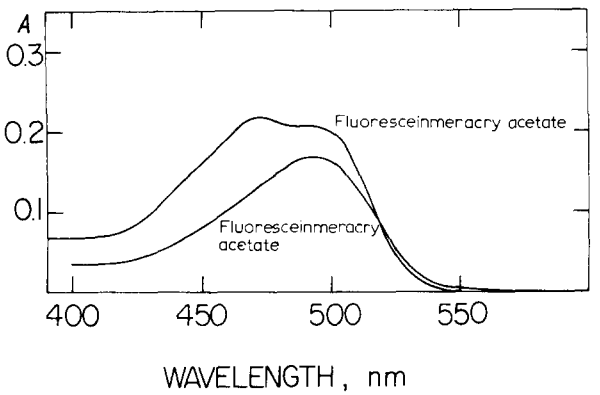

Fig. 6. Absorbance spectra of fluoresceinmercury acetate and fluoresceinmercury acetate-conjugated H_4. (10^{-5} M in fluoresceinmercury acetate) in 0.02 M phosphate buffer pH 7.4 on Cary 14.

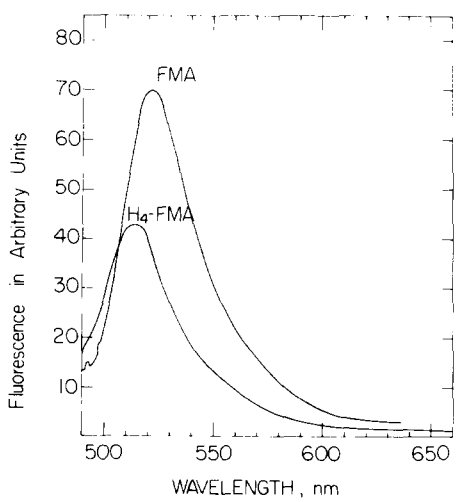

Fig. 7. Fluorescence spectra of fluoresceinmercury acetate and fluoresceinmercury acetate-conjugated H_4 (10^{-6} M in fluoresceinmercury acetate) in 0.02 M phosphate buffer pH 7.4 (λ_{ex} = 490). FMA, fluoresceinmercury acetate.

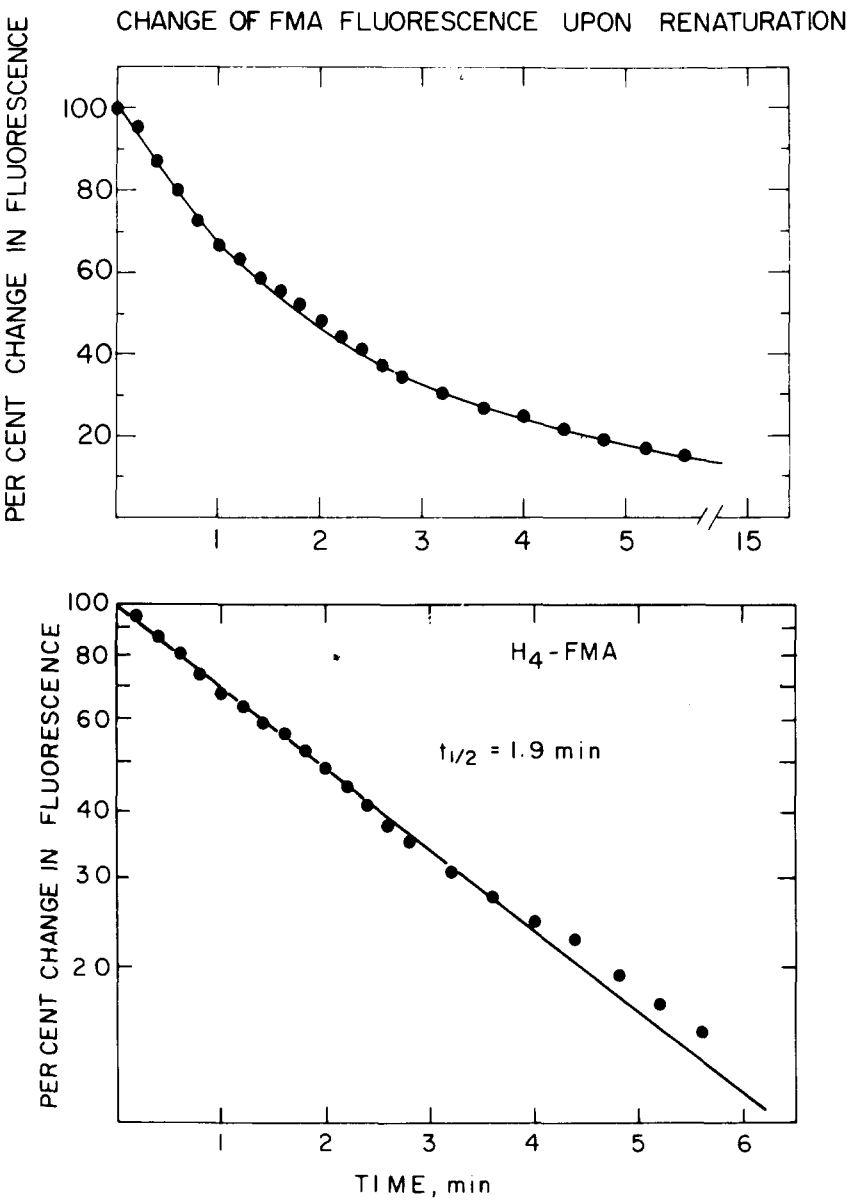

Fig. 8. The change in fluorescence due to renaturation of fluoresceinmercury acetate-conjugated pig heart isozyme. (λ_{ex} = 504, λ_{em} = 526), see Fig. 4 for experimental details. FMA, fluoresceinmercury acetate.

for the unmodified enzyme. The decrease in fluorescence upon renaturation was followed (Fig. 8). The renaturation process was found to be first-order with a half-life of 1.9 min.

The molecular nature of the assembly intermediates

When equal amounts of H_4 and M_4 are allowed to renature together after separate denaturation as described in the experimental section, five bands are

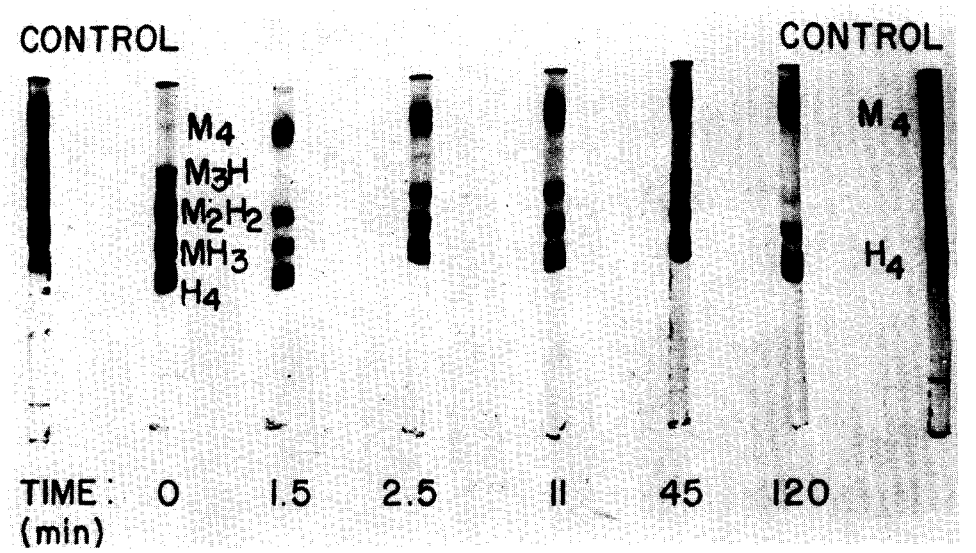

Fig. 9. The reaction of assembly of M_4 with excess of H subunits. M_4 is dissociated to M subunits at pH 0.2 M glycine/H_3PO_4 containing 1 mM dithiothreitol and 1 mM EDTA in a final concentration of 1 mg/ml at 0° C for 5 min. Then the sample is diluted 30-fold into 0.05 M sodium phosphate buffer pH 7.4 containing 1 mM dithiothreitol and 1 mM EDTA at room temperature. At different times a sample of 5-fold excess of H subunits is added to a sample of the renaturing M species. The assembly is then allowed to continue for another 2 h. Then the samples are concentrated by vacuum dialysis using a Sartorius vacuum dialysis device. The concentrate was centrifuged and then electrophoresed on polyacrylamide gels.

observed upon electrophoresis. A modification of the technique can be used to follow the pathway of assembly of H_4 and M_4 isozymes. A denatured preparation of one of the isozymes was allowed to renature and at various times samples of the renaturing subunits were challenged with a five-fold excess of denatured subunits of the alternative isozyme. Thus the species which were present in the renaturing sample would preferentially react with the subunit added in excess. After addition of the quenching subunit, the process of renaturation is allowed to proceed to completion (see Materials and Methods) and the samples concentrated and analyzed electrophoretically.

The pathway of assembly of M_4 can be followed in Fig. 9. Five isozymes (M_4, M_3H, M_2H_2, MH_3 and H_4) can be detected upon hybridization of the M_4 assembly mixture with H_4 subunits, in excess. MH_3 represents the monomer intermediate (M); M_2H_2 the dimer intermediate (M_2); M_3H the trimer intermediate (M_3); and M_4 represents the assembled muscle isozyme. Almost no M_3H is formed during the assembly indicating that M_3 does not appear at significant concentrations as an intermediate in the renaturation pathway of the muscle isozyme. On the other hand both MH_3 and M_2H_2 are present throughout the renaturation process. There is a gradual decrease in the concentration of these two isozymes and a corresponding increase in the concentration of the M_4 homoisoenzyme.

The assembly pathway of H_4 is illustrated in Fig. 10. In a similar manner as before M_3H stands for H; M_2H_2 for H_2; MH_3 for H_3; and H_4 for the reassembled heart isozyme. The results are basically similar to those obtained for the assem-

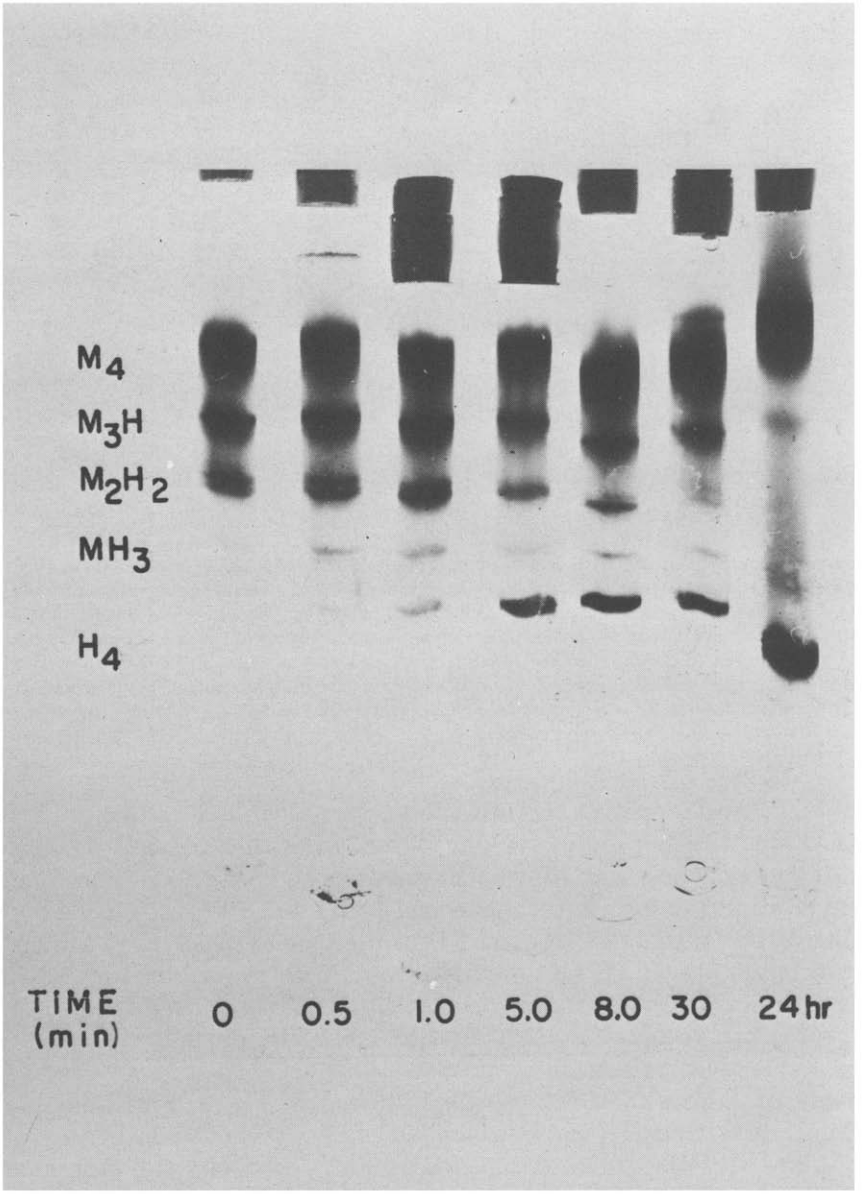

Fig. 10. The reaction of assembly H_4 with excess of M subunits (see Fig. 9).

bly of muscle isozyme. There is a continuous increase, with time, in the formation of H_4, parallel to a gradual decrease in the concentration of M_2H_2 and M_3H. A very light band of MH_3 is present throughout the entire renaturation process. This band could represent either the rapid formation of H_3 followed by rapid dissociation because of high trimer instability, thus yielding a low but relatively constant concentration of H_3. MH_3 could result only to a small extent from the interaction of MH with H_2. However, this reaction cannot con-

tribute more than a negligible fraction of MH_3 since most MH dimers would react preferentially with M_2 dimers present in a five-fold molar excess over the H_2 dimers.

Discussion

The rate-limiting step in the assembly of lactate dehydrogenase isozymes

The renaturation process of two types of pig lactate dehydrogenase isozymes, heart (H_4) and muscle (M_4) occurs via refolding and reassembly from their unfolded subunits and follow first order kinetics. The first-order kinetics implies a unimolecular rate-limiting step which controls the kinetics of the overall renaturation of the lactate dehydrogenase tetramer form its unfolded subunits. Essentially two mechanisms could account for such behaviour (Fig. 11): (a) A slow refolding of the denatured subunit representing a rate-limiting step, followed by rapid assembly of the refolded subunits, and (b) rapid refolding and assembly into an inactive tetramer followed by a rate-limiting annealing process within the tetramer to form an active enzyme.

Situation (a) would imply that if H subunits or M subunits are first allowed to renature and reassemble separately for several half times, and then mixed with subunits of other isozymes in excess, hybrids will form as was indeed found (Figs. 9 and 10) [17]. Situation (b) would imply that if H and M subunits are allowed to renature and reassemble separately for a short period and then mixed no $H_n\ M_{4-n}$ hybrids will be formed. From our present findings and previous results [17,19] we can eliminate the second alternative (b).

The possibility that the rate-limiting step occurs during the refolding of the isolated subunit followed by rapid assembly, is further supported by the fact that monomers still exist in the assembly mixture after a few half-lives of regain activity (Figs. 9 and 10). Furthermore, the results suggest that species such as H_2 may have enzymatic activity, since then the activity is almost fully regained, significant quantities of H and H_2 can still be observed.

The effects of ligands and glycerol on lactate dehydrogenase renaturation

Glycerol slows down the regain activity (Tables II and III, Fig. 3), but does

A. Sequential Pathway

$$M + M \longrightarrow M_2$$
$$M_2 + M \longrightarrow M_3$$
$$M_3 + M \longrightarrow M_4$$

B. The Dimer Pathway

$$M + M \longrightarrow M_2$$
$$M_2 + M_2 \longrightarrow M_4$$

Fig. 11. Possible assembly pathways of H_4.

not change the order of reaction. This fact indicates that even in a viscous medium the rate-limiting step is still at the subunit level. The presence of glycerol in the renaturation mixture can serve as a tool in amplifying the effect of ligands upon renaturation. The effect of ligands is summarized in Table III. The cofactor in either the oxidized (NAD^+) or in the reduced form (NADH), has a significant effect on the renaturation rate, whereas the substrates L-lactate and pyruvate have only a minor effect. This observation confirms previous findings by Novoa and Schwert [26] that the substrate can combine only with the binary enzyme-coenzyme complex. When the substrate L-lactate and cofactor NADH were present together in the renaturation mixture their accelerating effect was found to be additive. On the other hand, the presence of NAD^+ and pyruvate in the renaturation mixture completely inhibited regain activity. This is apparently the result of the formation of an abortive ternary complex of lactate dehydrogenase · NAD^+ · pyruvate [24]. It was shown previously by Jaenicke et al. [9] and by Cho and Swaisgood [8], that the presence of the cofactor alone, under various dissociation conditions, inhibits tetramer dissociation. This observation indicates that the cofactor has a profound influence on the tertiary and the quaternary structure of the enzyme, as was indeed proved directly by the crystallographic studies of Rossmann and his group [14,16]. The regain of protein fluorescence occurs faster than the regain of enzymatic activity (Figs. 1 and 2). Thus the monomer presumably refolds into a native tertiary structure at the subunit level in at least two consecutive slow steps, one slightly faster than the other. Only then the subunit is 'ripe' for assembly which probably occurs much faster, as was shown for alkaline phosphatase [5]. Subsequent association into the tetramer does not affect the fluorescence but is necessary for the regain of activity.

In a recent publication Jaenicke [27] has reported that the rate-limiting step in the reactivation of lactate dehydrogenase is a second-order process. The difference between the experiments described here and in previous communications [17,19] and those described by Jaenicke [27] is, that his renaturation experiments were conducted at a protein concentration of 0.1 to 1 μg/ml, whereas our experiments were conducted in the 20 to 50 μg/ml concentration range. At low concentrations (0.1 μg/ml) it has, indeed, been shown [10] that the native lactate dehydrogenase tetramer dissociates to dimers. It seems, therefore, that the formation of the active oligomeric form of the enzyme becomes rate-limiting at very low protein concentration. It is interesting to observe that similar results were found in the refolding kinetics of the dimeric triose phosphate isomerose [28]. Waley finds that the renaturation half-life depends markedly on protein concentration at low concentrations (0.1 μg/ml), but at higher concentrations the half-life becomes independent of concentration. These findings indicate that the oligomeric form of the enzyme is the active species and its formation becomes rate-limiting at low protein concentrations. In analogy, the findings of Jaenicke and our findings taken together, indicate that the refolded lactate dehydrogenase subunit is inactive and that only the oligomeric form of the enzyme is catalytically active. At low protein concentration the renaturation rate is controlled by the process of subunit assembly, whereas at high protein concentration the rate-limiting step is the first order process of subunit refolding. The refolding subunit, however, is capable of binding the coenzyme

NAD^+ (or NADH), since the latter increases markedly the rate of refolding. The overall scheme for LDH refolding and assembly is summarized in Fig. 12.

The ligands NAD^+, NADH have virtually no effect upon regain of enzyme fluorescence [17,19], in contrast to the marked effect of coenzyme upon the regain in activity. Thus, there are apparently at least two first-order processes (Fig. 12). One, which can be followed by protein fluorescence and a second, which can be monitored by the regain in enzymatic activity. The presence of coenzyme converts the second rate-limiting step to a fast reaction relative to the step detected by the change in protein fluorescence. The half-life of regain in activity of the enzyme-coenzyme complex converges to approximately that of regain in fluorescence of the enzyme protein itself and to that of a fluorescent probe fluoresceinmercury attached convalently to the enzyme. No change in the half-life of the regain of protein fluorescence (Fig. 2) or in the half-life for the regain of fluoresceinmercury fluorescence properties (Fig. 9) was observed in the presence of ligand. Therefore, these results indicate that the fluorescence regain of tryptophans (Fig. 2) and fluorescence quenching of fluoresceinmercury (Figs. 7 and 8) monitor the same first-order refolding process. The regain of activity in the absence of ligands monitors probably subsequent, slower first-order steps at the subunit level. Fig. 12 summarizes the postulated mechanism of lactate dehydrogenase assembly and renaturation, where the rate-limiting step is the regain of activity of the fatty assembled tetramer.

The assembly intermediates occurring in lactate dehydrogenase renaturation

Two possible mechanisms exist for the assembly of protein tetramers from its subunits, as illustrated in Fig. 11. The sequential pathway consists of the assembly of two subunits into a dimer, followed by the formation of a trimer for the dimer formed and another subunit, and finally the association of the trimer with a fourth subunit to form the native tetramer. The dimer pathway involves prior assembly of a dimer from two subunits and direct formation of the tetramer from two dimers. In the mixed reassembly experiments of M_4 (Fig. 9) almost no M_3H hybrid is evident during the reaction except for time zero. This small amount of M_3H observed can be explained by formation of M_3H from MH and M_2 that could be present in large enough quantities at the beginning of the reaction. The lack of significant trimer formation lends support to the

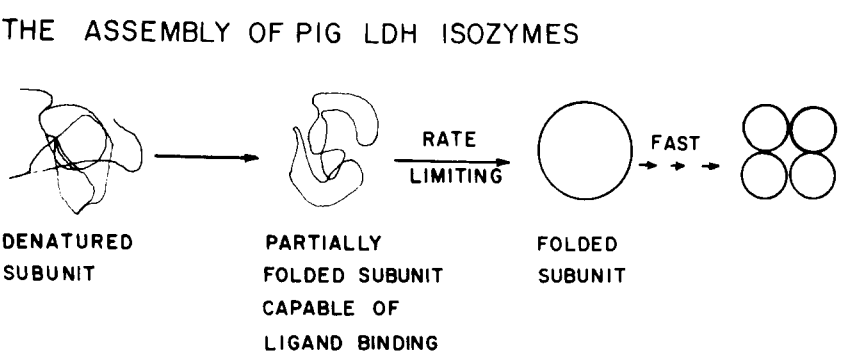

Fig. 12. The renaturation pathway for lactate dehydrogenase. The figure is drawn for the M isozyme, but the same mechanism applies to the H-isozyme.

dimer hypothesis, i.e. the pathway of renaturation proceeds via dimer formation from two monomers followed by tetramer formation from the association of two dimers.

The same conclusions apparently apply for the assembly of pig-heart isozyme. Although MH_3 is formed during the process, it is not formed in any significant concentrations to play a role in the overall process. It might be formed in a manner similar to that proposed for the small amount of M_3H during the assembly of the muscle isozyme, i.e. from H_2 + MH dimers. An alternative explanation would be that the trimer species, though unstable, may form fast but also dissociate fast because of lack of stability. Such circumstances would also lead to the formation of low quantities of M_3H or H_3M, respectively, in the mixed-assembly experiments described above (Figs. 9 and 10). It is, however, possible that subunit exchange may occur between the intermediate species, thus complicating the interpretation. The formation of two M_2H_2 sub-bands (Figs. 9 and 10) may indicate the formation of at least two of the three possible geometrical isomers for that species [17].

Physiological significance

The dramatic effects of NAD^+ and NADH on the rate of lactate dehydrogenase renaturation may indicate that these ligands may play a role in the assembly of lactate dehydrogenase from its subunits in vivo.

References

1 Crick, F.H.C. (1958) in On protein synthesis, Symposia of the Society for Experimental Biology Vol. 12, pp. 138—163
2 Anfinsen, C.B. (1962) Brookhaven Symp. Quant. Biol. 15, 184—198
3 Epstein, C.J., Carter, M.M. and Goldberger, R.F. (1964) Biochim. Biophys. Acta 92, 391—394
4 Saxena, V.P. and Weltaufer, D.B. (1970) Biochemistry 9, 5015—5023
5 Sela, M. and Lifson, S. (1959) Biochim. Biophys. Acta 36, 471—478
6 Stellwagen, E. and Schachman, H.K. (1962) Biochemistry 1, 1056—1069
7 Di Sabato, G. and Kaplan, N.O. (1964) J. Biol. Chem. 239, 438—443
8 Cho, I.C. and Swaisgood, H. (1973) Biochemistry 12, 1572—1577
9 Jaenicke, R. (1974) Eur. J. Biochem. 46, 149—153
10 Bartholmes, P., Durschlag, H. and Jaenicke, R. (1973) Eur. J. Biochem. 39, 101—108
11 Levi, A.S. and Kaplan, N.O. (1971) Biochem. Biophys. Res. Comm. 45, 1615—1621
12 Cook, R.A. and Koshland, Jr., D.E. (1969) Proc. Natl. Acad. Sci. U.S. 64, 247—254
13 Teipel, J.W. and Koshland, Jr., D.E. (1971) Biochemistry 10, 792—798
14 Rossmann, M.G., Adams, M.J., Buehner, M., Ford, G.C., Hackert, M.L., Lentz, Jr., P.J., McPherson, Jr., A., Schevitz, R.W. and Smiley, I.E. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 179—191
15 Everse, J. and Kaplan, N.O. (1973) Ad. Enzymol. 37, 61—133
16 Rossmann, M.G., Jeffery, B.A., Main, P. and Warren, S. (1967) Proc. Natl. Acad. Sci. U.S. 57, 515—524
17 Levitzki, A. (1972) FEBS Lett. 24, 301—304
18 Bergmeyer, H.U., Bernett, E. and Hess, B. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 736—740, Academic Press, New York
19 Levitzki, A. and Tenenbaum, H. (1974) Isr. J. Chem. 12, 327—337
20 Anderson, S. and Weber, G. (1966) Arch. Biochem. Biophys. 116, 207—223
21 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—440
22 Ellman, G.L. (1954) Arch. Biochem. Biophys. 82, 70—75
23 Karush, F. Klinman, N.R. and Marks, S.R. (1964) Anal. Biochem. 9, 100—114
24 Gutfreund, H., Cantwell, R. and McMurray, C.H. (1968) Biochem. J. 106, 683—687
25 Fondy, T.P., Everse, J., Driscoll, G.A., Castillo, F., Stolzenbach, F.E. and Kaplan, N.O. (1965) J. Biol. Chem. 240, 4219—4227
26 Novoa, W.B. and Schwert, G.W. (1964) J. Biol. Chem. 236, 2150—2153

27 Jaenicke, R., Koberstein, R. and Teusher, B. (1971) Eur. J. Biochem. 23, 150—159
28 Waley, S.G. (1973) Biochem. J. 135, 165—172
29 Teipel, J.W. and Koshland, Jr., D.E. (1971) Biochemistry 10, 798—805
30 Bergmeyer, H.U., Bernett, E. and Hess, B. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., Bernett, E. and Hess, B. (1963) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 1011—1012, Academic Press, New York