THE ASSEMBLY PATHWAY OF LACTIC DEHYDROGENASE ISOZYMES FROM THEIR UNFOLDED SUBUNITS

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1. Introduction

The folding and assembly of polypeptide chains to form the native architecture of an oligomeric protein must proceed via a specific pathway to insure the selective formation of the biologically active molecules. Although the amino acid sequence of the polypeptide chains determines the spectrum of the three-dimensional structures, it is quite clear that environmental conditions (pH, ionic strength, temperature) will select certain refolding and assembly pathways over others [1].

Lactic dehydrogenases offer a unique opportunity for the investigator to study assembly since many isozyme variants are available and the three-dimensional structure of the enzyme tetramer is known in great detail [2].

2. Materials and methods

Pig muscle LDH* (M_4), pig heart LDH (H_4), NAD, NADH and sodium pyruvate were all purchased from Boehringer. The conventional LDH assay was used [3].

Experimental conditions were used to insure both the complete dissociation of the LDH tetramer to subunits and to achieve 95–100% regain of activity upon renaturation. The enzyme was incubated in 0.2 M glycine-H_3PO_4, pH 2.5 containing 1×10^{-3} M DTT and 1×10^{-3} M EDTA in the cold (4°) or for not more than 4 min at 25°. To achieve renaturation the enzyme was diluted into 0.2 M sodium phosphate buffer pH 7.4 containing 1×10^{-3} M DTT and

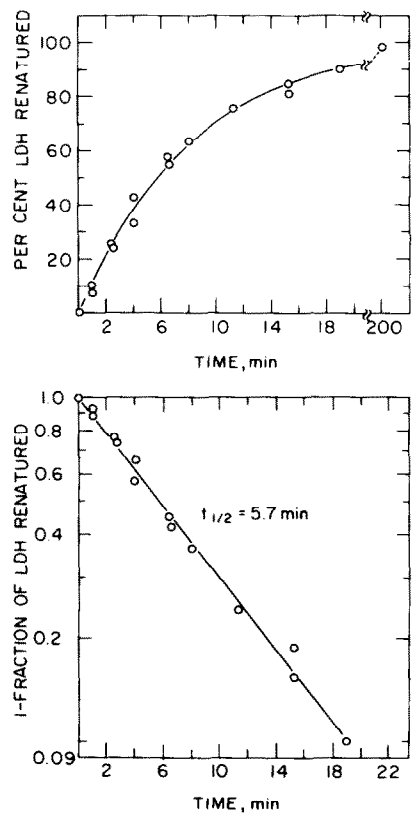

Fig. 1. The renaturation kinetics of pig M_4. Top: activity *vs.* time plot. Bottom: Semilog plot of the same data.

* Abbreviations: LDH, lactic dehydrogenase; DTT, 5,5′-dithio-bis(2-nitrobenzoic acid).

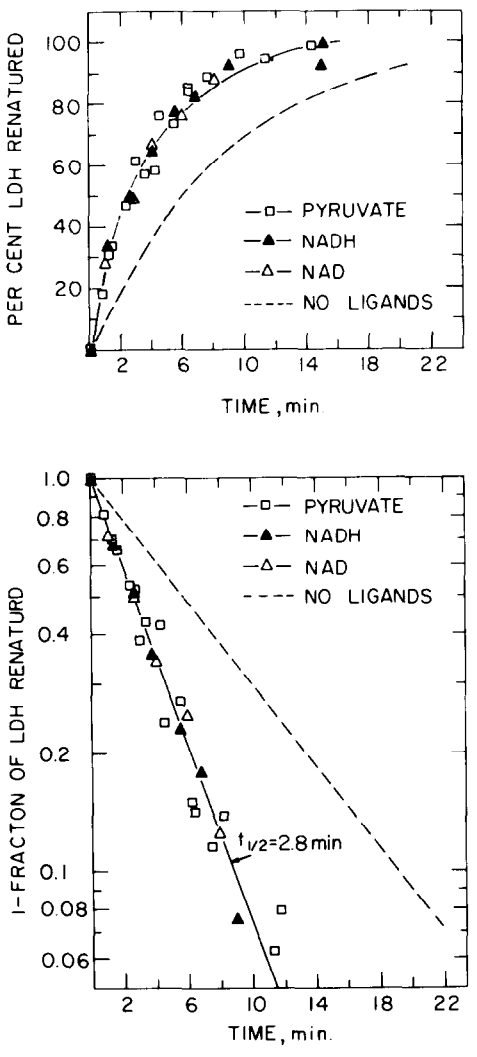

Fig. 2. The effect of specific ligands on LDH renaturation. The ligands sodium pyruvate, NAD^+ and NADH were incorporated in the renaturation mixture each at a concentration of 2×10^{-3} M.

1×10^{-2} M EDTA. Further experimental details are given in the figure legends.

3. Results

3.1. *Renaturation of pig M_4 and pig H_4*

The kinetics of activity regain of pig muscle LDH is shown in fig. 1. The process follows strict first-order kinetics. Ligands such as NAD^+, NADH and pyruvate affected markedly the rate of renaturation but surprisingly do not affect the order of the reaction (fig. 2). A similar behavior was found for the pig heart (H_4) isozyme; the process, however, being much slower ($t_{1/2}$ = 11 min).

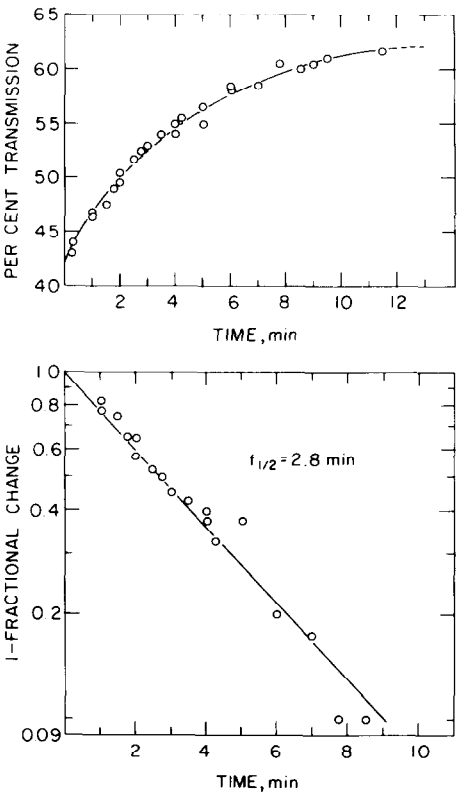

Fig. 3. The regain of protein fluorescence upon renaturation. The experimental conditions are the ones described in Materials and methods. $\lambda_{excitation}$ = 290 nm; $\lambda_{emission}$ = 350 nm.

3.2. *Protein fluorescence regain upon renaturation of pig M_4*

When the protein fluorescence was followed as a probe to study the transformation from unfolded subunits to the renaturated tetramer the results shown in fig. 3 were obtained. The rate of fluorescence regain was first-order in protein concentration and was not affected by the presence of the pyruvate ligand. It is interesting to note that the rate of fluorescence regain is identical to the rate of activity regain *in the*

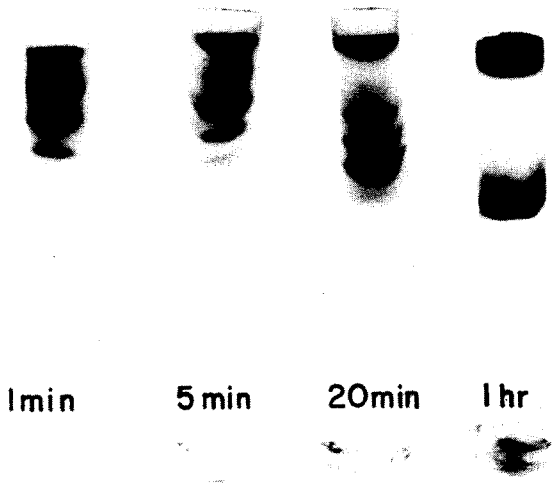

Fig. 4. The hybridization capacity of renaturing M_4 LDH with H-subunits as a function of time. H_4 and M_4 pig isozomes were denatured and renatured separately as described in Materials and methods. At different times from the start of renaturation samples of M and H were mixed and allowed to continue to assemble and hybridize for 1 hr before electrophoresis was begun. Electrophoresis was conducted at pH 8.6 [4].

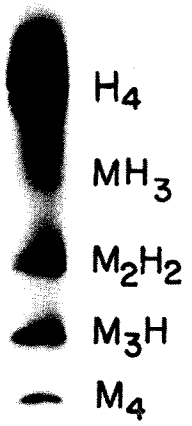

Fig. 5. The H_2M_2 doublet band. Upon prolonged electrophoresis of the reassembled mixture of H–M hybrids the middle H_2M_2 band splits into two bands.

presence of ligands (fig. 2) and thus may reflect the same structural transition. The rate limiting first-order processes could either reflect a structural change at the subunit level followed by rapid assembly or a slow annealing process at the tetramer level following the assembly steps.

In order to determine which of the two processes is the one observed, the following experiment was performed. Pig M_4 and H_4 isozymes were dissociated separately at pH 2.5 (see Materials and methods and fig. 1) and then refolded separately at pH 7.4. Samples of the refolding M_4 and H_4 were mixed at different times and allowed to hybridize in the mixture. Then the mixtures were subjected to disc-gel electrophoresis to determine the spectrum of LDH isozymes formed (fig. 4). From fig. 4 it is apparent that the assembly process is slow compared to the regain of activity (fig. 1). It seems therefore that the assembly process *follows* the first-order processes monitored by the activity regain (figs. 1 and 2) and the fluorescence change (fig. 3).

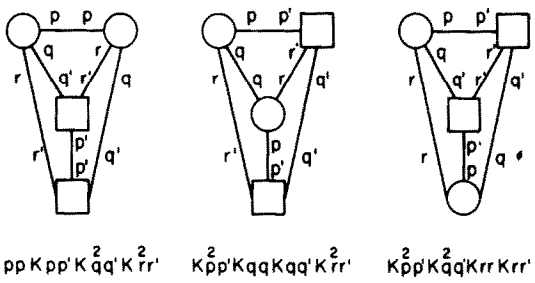

Fig. 6. Possible geometrical isomers of H_2M_2. In drawing the structures it is assumed that the isologous mode of binding remains thus for example the binding domain p can only interact with p′ to form a peudoisologous pair pp′.

3.3. *Possible geometrical isomers of M_2H_2*

When a hybrid set of isozymes prepared by the reassembly experiment discussed above is electrophoresed for a longer time, the band corresponding to M_2H_2 is split into two sub-bands (fig. 5). The two M_2H_2 are probably two of the three possible geometrical isomers of M_2H_2 (fig. 6).

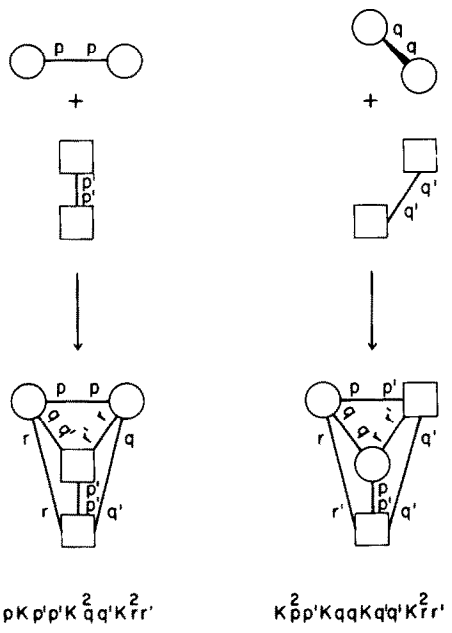

Fig. 7. The assembly of pp-type dimers and qq-type dimers. The assembly of pp dimers with p'p' ones and qq dimers with q'q' ones yield different geometrical isomers.

4. Discussion

The rate limiting step in the attainment of the native structure of lactic dehydrogenase differs in various isozymes (Levitzki, unpublished). In the case of pig M_4 and pig H_4 the rate limiting step is a first-order process (fig. 1) probably involving a structural change within the subunit, whereas in the case of beef H_4, for example, the rate limiting step is a bimolecular step (Levitzki, unpublished). The ligands pyruvate, NAD^+ and NADH facilitate this structural transformation presumably by binding to a *partially refolded subunit species.* The first-order process observed in the presence of ligands (fig. 2) reflects a faster structural change within the subunit, preceding the step affected by the ligands. This conformational change could be the one monitored by the fluorescence change because of the similarity in the rate constants (figs. 2 and 3).

The assembly of the M_4 isozyme *follows* the refolding of the M subunit and therefore after 5 min of renaturation the assembly mixture still contains monomers, dimers and trimers, since the species H_3M, H_2M_2 and HM_3 are still observed (fig. 4). At 20 min the assembly mixture of the muscle isozyme is already depleted of monomers since no H_3M are observed (fig. 4).

The splitting of the H_2M_2 band into two subbands upon longer electrophoresis (fig. 5) may reflect the existence of two of the three possible geometrical isomers of H_2M_2 (fig. 6). The three species differ only slightly in their electrophoretic mobility and are resolved only upon long electrophoresis (1–1¼ hr). The absence of a third band may indicate that only pp dimers and qq dimers (fig. 7) are viable intermediates in the assembly process whereas rr dimers are unstable and therefore are absent from the assembly mixture. One can speculate further and take the latter observation as an indication of the weaker nature of the rr interactions compared to either pp or qq interactions.

Acknowledgement

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References

[1] A. Wachman and A. Levitzki, J. Theor. Biol. 34 (1972) 277.
[2] M.G. Rossmann, M.J. Adams, M. Buehner, G.C. Ford, M.L. Hackert, P.J. Lentz, Jr., A. McPherson, Jr., R.W. Schevitz and I.E. Smiley, Cold Spring Harbor Symp. Quant. Biol. 36 (1971) 179.
[3] H.U. Bergmeyer, E. Bernt and B. Hess, in: Methods in Enzymatic Analysis, ed. H.E. Bergmeyer (Academic Press, 1963) p. 736.
[4] B.J. Davis, Ann. N.Y. Acad. Sci. 121 (1964) 404.