The dimeric intermediate on the pathway of reconstitution of lactate dehydrogenase is enzymatically active

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1. INTRODUCTION

It has been shown by a variety of methods that lactate dehydrogenase from pig heart and skeletal muscle require the tetrameric quaternary structure in order to exhibit catalytic activity. Dissociation even under most gentle conditions, is accompanied by complete deactivation [1,2]. On the other hand, reactivation after dissociation and denaturation has been shown to parallel tetramer formation: using both 'structure monomers' and metastable dimers reconstitution starts from zero activity [2–4].

In order to investigate structural and functional properties of intermediates on the pathway of reconstitution a number of approaches have been applied. The particle distribution can be measured by chemical cross-linking with glutaraldehyde [3,5]. The properties of intermediates of association can be evaluated simply by comparison of the particle distribution with the resulting changes of characteristic properties of the enzyme.

Stable dimers formed by limited proteolysis with thermolysin during reconstitution have been shown to be essentially inactive under standard conditions [6]. Since they show specific binding to oxamate and dinucleotide affinity columns, their structure must be closely similar to the native enzyme; however, their affinity is markedly below that of the intact tetramer, due to increased structural flexibility of the proteolytically modified polypeptide chains [7]. Correspondingly, 'structure-making ions' induce partial activity of the proteolytic 'dimer' if applied to the assay mixture [7].

Reactivation experiments after dissociation and deactivation of native lactate dehydrogenase in 6 M guanidine–HCl or at acid pH show that the unmodified dimeric intermediate on the pathway of reconstitution exhibits high catalytic efficiency: in the presence of 1.5 M ammonium sulfate, 50% of the native (tetramer) activity returns at the level of the dimeric association intermediate.

2. MATERIALS AND METHODS

Porcine lactate dehydrogenase, thermolysin from *Bac. thermoproteolyticus*, trypsin, and NADH were obtained from Boehringer (Mannheim), buffer reagents (A-grade) from Roth (Karlsruhe) and Merck (Darmstadt). Quartz doubly distilled water was used throughout. Standard buffer was 0.1 M sodium phosphate (pH 7.6) containing 1 mM dithioerythritol. Enzyme solutions were prepared by dialysis at 4°C against standard buffer. Enzyme concentration was determined spectrophotometrically ($A^{0.1\%}_{280nm}$ = 1.40 $cm^2 . mg^{-1}$); enzyme activity was monitored at 366 nm (25°C) in the presence of varying concentrations of ammonium sulfate (standard buffer plus 0.84 mM pyruvate and 0.2 mM NADH).

Denaturation of the native enzyme by guanidine–HCl was achieved by 1:5 dilution with 7.5 M Gdn–HCl in standard buffer (20°C); incubation time 5 min. Acid dissociation was performed by 15 min incubation (20°C) in 1 M

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glycine–H_3PO_4, pH 2.3. Reactivation at 20°C was initiated by 1:500 dilution with standard buffer. For further details see [3,5].

3. RESULTS AND DISCUSSION

Native lactate dehydrogenase at high concentrations of 'structure-making ions' has been shown to undergo tightening of its structure, as indicated by increased stability and lowered enzymatic activity [7–9]. In its monomeric or dimeric state (either as kinetic intermediate [3,5], or as proteolytically modified entity [7]), the enzyme under standard conditions of the test does not exhibit catalytic function. On the other hand, proteolytically modified dimers in the presence of 2 M ammonium sulfate have been reported to be (partially) active [7].

Here the salt-induced activity of intact dimers which accumulate transiently during reassociation was determined. After maximum unfolding of the enzyme in 6 M guanidine–HCl, both slow folding of monomers (M) to a correctly folded intermediate state (M*), and slow association of dimers (D) to tetramers (T) are rate-limiting [5]. The following kinetic model was proposed on the basis of cross-linking experiments:

$$4\,M \xrightarrow{k_1} 4\,M^* \xrightarrow{\text{fast}} 2\,D \xrightarrow{k_2} T \qquad (1)$$

where: $k_1 = 8 \times 10^{-4}.s^{-1}$ and $k_2 = 3 \times 10^4\ M^{-1}.s^{-1}$ as first- and second-order rate constants, respectively. The rate of the fast monomer association ($2\,M^* \longrightarrow D$) is close to the value of a diffusion-controlled reaction [10].

The slow folding step of the monomers may be eliminated by dissociation conditions where some of the correct backbone structure is preserved in the dissociated state. After short-term acid dissociation [11] or acid dissociation in the presence of stabilizing salt [3,5], reconstitution starts from a structure close to M* (cf. eq.1), so that dimer association becomes the only rate-determining step (careful analysis of the association pattern reveals that monomers and dimers are in actual fact in a fast dissociation–association equilibrium which is shifted predominantly towards the dimers [3,10]):

$$4\,M^* \xrightarrow{\text{fast}} 2\,D \xrightarrow{k_2} T \qquad (2)$$

Table 1

Enzymatic activity of native porcine muscle lactate dehydrogenase and its dimeric association intermediate in the presence of increasing ammonium concentrations

$[(NH_4)_2SO_4]$ (M)	Spec. act. (IU/mg)	
	Native tetramer	Dimeric intermediate[a]
0	420	0
1	395	100
1.5	400	182
2	350	105
3	250	15
4	120	3

[a] Corrected for tetramer activity regained after 1 min reconstitution

Immediately after short-term acid dissociation, dimers are predominant in the reassociation mixture. The activity of dimeric intermediates as a function of the amount of ammonium sulfate present in the test mixture was therefore determined after 1 min reassociation (table 1). Intact dimers as intermediates of association show maximum activity in the presence of 1.5 M $(NH_4)_2SO_4$. About 7% native tetramers are formed within the first minute of association, as taken from the limiting activity at zero ammonium sulfate concentration. The activity of native enzyme is only slightly decreased by 1.5 M $(NH_4)_2SO_4$. At higher concentrations of the stabilizing salt both the activities of native tetramers and of dimeric intermediates decrease due to a decrease in protein flexibility (table 1).

In order to monitor the occurrence of enzymatically active dimeric intermediates on the pathway to reconstitution, reactivation after acid or guanidine–HCl denaturation was analyzed using the standard enzyme assay, and the assay with 1.5 M $(NH_4)_2SO_4$ in the test mixture.

After short-term acid dissociation, reassociation is mainly governed by the dimer $\longrightarrow$ tetramer association (eq.2). Since dimers are inactive in the absence of stabilizing salt, reactivation, as determined by standard assay, strictly parallels tetramer formation (fig.1; cf. [5]). Adding 1.5 M $(NH_4)_2SO_4$ to the test mixture leads to partial activity of the dimeric intermediates. As shown in

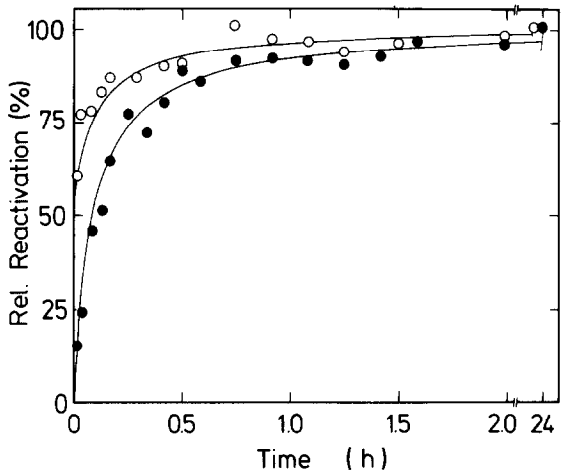

Fig.1. Reactivation of porcine muscle lactate dehydrogenase after acid denaturation (15 min, 1 M glycine–H_3PO_4, pH 2.3, 20°C). Reactivation in standard buffer; enzyme concentration 8.3 μg/ml; yield 91%. (●) Reactivation analyzed by standard assay. Curve calculated for the rate-determining dimer ⟶ tetramer association with $k_2 = 3 \times 10^4\ M^{-1}.s^{-1}$ (cf. [5]). (○) Activity determined in the presence of 1.5 M $(NH_4)_2SO_4$; curve calculated with the same rate constant k_2, assuming the dimers to possess 50% activity.

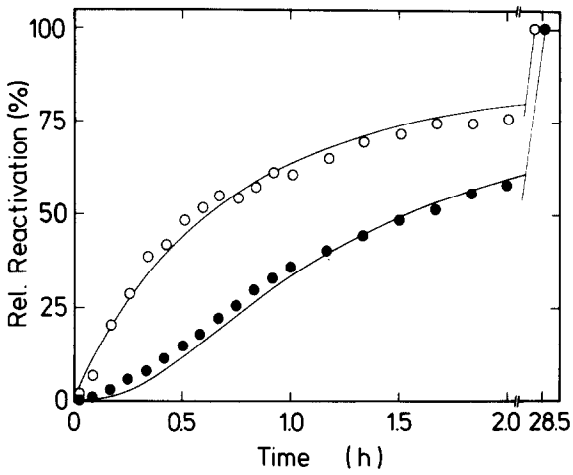

Fig.2. Reactivation of porcine muscle lactate dehydrogenase after denaturation in 6 M guanidine–HCl (5 min, 0.1 M sodium phosphate, pH 6.2, 1 mM dithioerythritol, 1 mM EDTA, 20°C). Reactivation in standard buffer, containing 12 mM residual guanidine–HCl; enzyme concentration 1.7 μg/ml; yield 40%. (●) Reactivation analyzed by standard assay. Curve calculated for a consecutive first-order/second-order mechanism with $k_1 = 8 \times 10^{-4}.s^{-1}$ and $k_2 = 3 \times 10^4\ M^{-1}.s^{-1}$ for the dimer ⟶ tetramer association, respectively (cf. [5]). (○) Activity determined in the presence of 1.5 M $(NH_4)_2SO_4$. Curve calculated with the same rate constants k_1 and k_2, assuming the dimers to possess 50% activity.

fig.1, the respective reactivation kinetics may be adequately described by the association data assuming that the dimers contain 50% of the native activity.

After dissociation in 6 M guanidine–HCl, reassociation is determined by slow monomer folding, in addition to the dimer ⟶ tetramer association step (eq.1). Correspondingly, reactivation under standard test conditions parallels the sigmoidal kinetics of tetramer formation (fig.2; cf. [5]). If 1.5 M $(NH_4)_2SO_4$ are present in the test mixture, the reactivation profiles become hyperbolic in accordance with the assumption that the dimers exhibit 50% activity (fig.2).

In order to separate the rate-limiting folding step (preceding the diffusion-controlled dimerization) from the second-order tetramer formation, reconstitution of proteolytically modified 'dimers' [6,7] may be applied. As predicted from the given kinetic model, the respective kinetics show no concentration dependence obeying the given first-order rate constant (Girg et al., unpublished).

Since no initial activity is detectable upon reconstitution after guanidine–HCl dissociation, it is obvious that the monomers (M, cf. eq.1) cannot be enzymatically active.

Analysis of the kinetics of refolding by circular dichroism reveals that most of the native backbone structure is recovered immediately after dilution of the denaturant [12]. As a consequence, M contains

Table 2

Structural and functional properties of the association intermediates of porcine muscle lactate dehydrogenase

	θ_{222} (% of native)	Activity (% of native)	
		$-(NH_4)_2SO_4$	$+1.5$ M $(NH_4)_2SO_4$
Monomer (μ)	0	0	0
Monomer (M)	≅75	0	0
Monomer (M*)	(100)	(0)	(0)
Dimer (D)	≅100	0	50

about 75% of the native helicity (table 2). The remaining structure is recovered in the M ⟶ D transition (governed by the slow M ⟶ M* folding step) which implies that the native backbone structure is fully established at the level of the dimer.

Since the folded monomers (M*) are only marginally populated during reassociation, only hypothetical values of the structural and enzymatical properties of this intermediate can be given (table 2).

4. CONCLUSIONS

Native tetrameric lactate dehydrogenase consists of functionally independent subunits each of which is capable of sequentially binding one coenzyme and one substrate molecule [13]. From these observations one might expect the monomer to represent the catalytically active entity. However, in the past a variety of experimental approaches proved the tetrameric quarternary structure to be indispensable for the function of the enzyme [3,5,12,14].

The instability of the dimer towards proteolysis suggested structural flexibility to be responsible for the lack of catalytic activity although preformation of the active centre is indicated by the binding of proteolytically modified 'dimers' to a dinucleotide affinity column. Correspondingly, in these experiments, stabilization of the backbone structure by adding 'structure-making ions' to the assay mixture of the enzymatic test is found to generate enzymatic activity at the level of the dimeric intermediates of association. The fact that the activation effect shows an optimum curve is easily explained by the assumption that beyond a certain limit, tightening of the structure is expected to interfere with catalytic function.

The given data clearly show that the unibimolecular mechanism of reconstitution is sufficient to describe the reactivation kinetics. Both rate-determining folding and association steps are observed in the monomer ⟶ tetramer transition after denaturation in 6 M guanidine–HCl, where the enzyme in its denatured state may be described as a random coil. Starting from 'structured monomers' after acid dissociation, folding is negligible, so that the dimer ⟶ tetramer transition remains as the only rate-limiting step. Reactivation after both acid or guanidine–HCl dissociation may be correlated with the association data if the intermediates of association are inactive under standard test conditions. In the presence of stabilizing salt, the dimeric intermediates of association possess 50% of the native activity of the tetramers.

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