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High pressure dissociation of lactate dehydrogenase from *Bacillus stearothermophilus* and reconstitution of the enzyme after denaturation in 6 M guanidine hydrochloride*

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Abstract. Tetrameric lactate dehydrogenase from *Bacillus stearothermophilus* exhibits unusual stability towards high hydrostatic pressure: In contrast to the mesophilic enzyme, incubation at pressures up to 2.8 kbar does not cause irreversible denaturation. Hybridization under these conditions suggests partial dissociation to the dimer, indicating that reassociation occurs within the dead-time after pressure release (< 20 s at $\leqq 40$ µg/ml, 20° C). Incubation at $P < 2.8$ kbar affects neither the native quaternary structure nor the catalytic function of the enzyme.

Reconstitution of the unfolded and dissociated subunits after denaturation, e.g., in 6 M guanidine · HCl, is characterized by fast association favouring the native assembled structure.

Evidence from spectroscopic measurements shows that reconstitution starts with a fast refolding reaction generating a native-like conformation. The subsequent rate-determining transconformation of the "structured monomers" governs the kinetics of reactivation and reassociation as one single first-order process. Chemical crosslinking with glutaraldehyde proves that the "structured monomers" undergo fast association to form the tetrameric final state of reconstitution, with significant amounts of dimeric intermediates being detectable. The renatured enzyme is indistinguishable from the native enzyme regarding its physicochemical and enzymological properties (e.g., activation by fructose-1,6-bisphosphate, and susceptibility towards proteolytic digestion).

Introduction

Studies on the in vitro reconstitution of proteins focus on the question how the nascent polypeptide chain reaches its native state. Taking into account the number of degrees of freedom of a polypeptide chain of average length, a defined pathway of folding must exist allowing the unstructured chain molecule to acquire its functional conformation within a biologically feasible time (Wetlaufer and Ristow 1973; Baldwin and Creighton 1980). The pathway of folding determines the kinetics of reconstitution. In the case of oligomeric enzymes, unimolecular (folding) and bimolecular (association) steps are expected to be involved in the mechanism of reconstitution (Jaenicke 1984).

In the present study we deal with the mechanism of reconstitution of lactate dehydrogenase from *Bacillus stearothermophilus*, a moderately thermophilic bacterium.

The primary aim of the project was a detailed thermodynamic investigation of the structural stability of this enzyme, applying reversible high pressure deactivation to monitor denaturation/renaturation (Müller et al. 1981a; Müller et al. 1982; Jaenicke 1983). So far, comparative data referred to the thermal stability using irreversible heat denaturation to estimate the thermodynamic quantities (Zuber 1981). As will be shown, the thermophilic enzyme exhibits unusual stability towards high hydrostatic pressure. Comparing its chemical properties, the enzyme closely resembles its mesophilic analogues. In its native state it is tetrameric showing a high sequence homology with e.g., the enzymes from chicken, dog fish and pig (Schär et al. 1982; Tratschin et al.1983). On the other hand, the enzyme from *Bacillus stearothermophilus* differs with respect to its allosteric activation by fructose-1,6-bisphosphate which is not observed for the mesophilic enzyme isolated from higher organisms. Apart from the thermodynamic characterization, the main purpose of this study was to compare the folding mechanisms of an enzyme obtained from different organisms that are far from each other regarding both ecology and

Abbreviations: DTE Dithioerythritol; Gu · HCl Guanidine hydrochloride; LDH-H_4, LDH-M_4 Lactate dehydrogenase from heart and skeletal, muscle, respectively; Tris Trishydroxymethyl amino methane

* Dedicated to Professor Fritz Lipmann on occasion of his 85th birthday

evolution. Lactate dehydrogenase serves as an example since its three-dimensional structure is known (Holbrook et al. 1975), and its reconstitution properties have been extensively studied (Jaenicke and Rudolph 1983).

Materials and methods

Materials

Lactate dehydrogenase from *Bacillus stearothermophilus* was a gift from Professor H. Zuber, ETH Zürich. Buffer substances (A grade purity), pyruvate, sodium borohydride and trypsin were purchased from Merck (Darmstadt); guanidine · HCl (ultra pure) from Schwarz-Mann (Orangeburg, NY), DTE from Roth (Karlsruhe), glutaraldehyde (25% solution in water) from Fluka (Basel), NADH (grade II) and fructose-1,6-bisphosphate from Boehringer (Mannheim). Solutions were prepared using quartz bi-distilled water throughout.

Methods

Enzyme: The enzyme was stored as an ammonium sulfate suspension at 4° C. In order to obtain stock solutions the suspension was centrifuged (20 min, 12,500 *g*) and the pellet dissolved in buffer; subsequently the protein was repeatedly dialyzed against buffer (0.2 M Tris/HCl, plus 2.5 mM DTE, pH 7.6 or 0.2 M imidazolium · HCl, plus 2.5 mM DTE, pH 7.2) at room temperature. Protein concentrations were calculated from absorption measurements at 280 nm using a value of $A_{1cm} = 1.4$ for 1 mg protein/ml. (K. Müller, unpublished). Molar concentrations of the enzyme refer to the subunit molecular weight ($M_r = 35{,}000$).

Denaturation was accomplished by 10 min incubation in 6 M guanidine · HCl, plus 2.5 mM DTE at 20° C. Experimental details and protein concentrations are given in the figure legends.

Renaturation was initiated by 1 : 40 dilution with 0.2 M imidazole buffer pH 7.2, plus 10 mM DTE at 35° C (Jaenicke and Rudolph 1984).

Enzymatic assay: Catalytic activity was determined by monitoring the decrease in NADH absorption at $\lambda = 366$ nm. The assay mixture was 0.2 M Tris/HCl pH 7.6, plus 2 mM DTE, containing 300 μM NADH and 5.5 mM pyruvate. Under these conditions the specific activity was 200 ± 20 IU/mg at 25° C. In the presence of 6 mM fructose-1,6-bisphosphate, the activity was found to be increased to about 350 IU/mg. Assays were performed using an Eppendorf spectrophotometer equipped with a recorder and a thermostated cuvette holder. In order to inhibit reconstitution of the enzyme during the enzymatic assay, in certain experiments trypsin was added to the test mixture (Jaenicke and Rudolph 1984).

Spectroscopic measurements: Circular dichrosism measurements made use of a JASCO I 500 A spectropolarimeter equipped with a data processor JASCO DP 500 N. Spectra were accumulated four times. Fluorescence emission was monitored using a Hitachi-Perkin Elmer MPF 44A spectrofluorimeter.

Chemical modification (crosslinking): Acetylation was performed according to Rajewsky (1966). In order to obtain sufficiently sharp bands on the native polyacrylamide gels, acetylation was performed in three steps using twice the amount of acetic anhydride and Na_2SO_3. Residual activity of the modified enzyme was ~ 10%.

Otimum conditions for the crosslinking of the protein were found to be the same as published previously for lactic dehydrogenase from pig skeletal muscle (Hermann et al. 1979; Hermann et al. 1981), except for the buffer. Since the protein is unstable in the presence of phosphate, imidazolium · HCl was used as buffer. Polyacrylamide gel-electrophoresis made use of standard procedures (Jaenicke and Rudolph 1984; Hermann et al. 1981).

High pressure experiments: Solutions of lactate dehydrogenase from *Bacillus stearothermophilus* were pressurized at 20° C making use of the high pressure cells described previously (Schade et al. 1980b). Residual catalytic activity was determined at ambient pressure after rapid decompression (< 20 s). Protein concentration in all high pressure experiments was 20 μg/ml (0.54 μM); the buffer was 0.2 M Tris/HCl, plus 10 mM DTE, pH 7.6.

Results

Stability of the native protein towards high hydrostatic pressure

It has been previously reported that lactate dehydrogenases from higher organisms are completely inactivated at pressures beyond 1,500 bar. As shown by various techniques, this loss of activity is accompanied by dissociation of native tetramers into inactive structured monomers (Müller et al. 1981a; Müller et al. 1982; Schade et al. 1980b). The kinetics of reconstitution after decompression to ambient pressure closely resemble the time course after acid

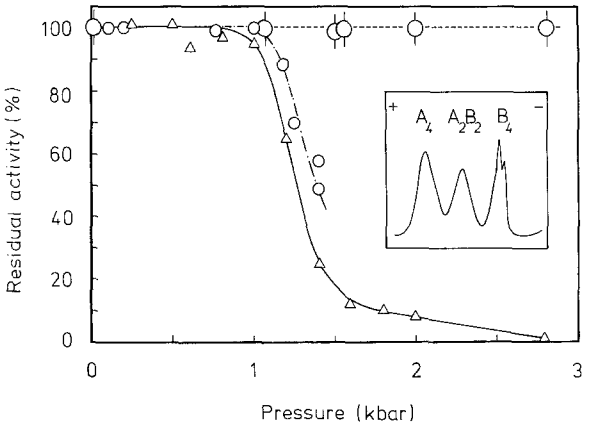

Fig. 1. Pressure dependent deactivation of mesophilic and thermophilic lactate dehydrogenase: Residual activity after pressure release (up to 24 h reconstitution at 1 bar; for the enzyme from *Bacillus stearothermophilus* complete reactivation is obtained after < 20 s. △, ○: porcine lactate dehydrogenase from heart and skeletal muscle, respectively; ⌀: lactate dehydrogenase from *Bacillus stearothermophilus*. For experimental details see (Müller et al. 1981a; Schade et al. 1980b) and Materials and methods. *Insert:* High pressure hybridization of native (B) and acetylated (A) lactate dehydrogenase from *Bacillus stearothermophilus*. Densitogram after electrophoretic separation. 20 µg/ml solution of B and A were incubated for 1 h at 2.6 kbar (20° C); polyacrylamide gel-electrophoresis under native conditions immediately after pressure release

dissociation and denaturation (Rudolph and Jaenicke 1976; Schade et al. 1980a; Müller et al. 1981b). This holds inspite of the fact that the respective initial states of denaturation differ significantly with respect to their conformation (Müller et al. 1982; Schade et al. 1980b; Rudolph and Jaenicke 1976).

No loss of catalytic activity of lactate dehydrogenase from *Bacillus stearothermophilus* is observed after incubation at $P < 2.8$ kbar for 1 h: enzymatic assays at ambient pressure and 20° C immediately after decompression (< 20 s) are in agreement with the activity of reference samples kept at atmospheric pressure. The result suggests an unusual stability of the enzyme (Fig. 1).

To find out whether the observed constant value of the specific activity reflects the intrinsic stability of the native quaternary structure, or fast reconstitution of the enzyme after pressure release, hybridization experiments were performed applying the native and acetylated enzyme. Acetylation of lysyl residues (Rajewsky 1966) yields a partially deactivated tetramer with significantly changed electrophoretic mobility. To achieve sufficiently sharp bands, acetylation was performed in three steps applying a total amount of 0.3 mmol acetic anhydride per milligram of enzyme. Joint incubation of native and chemically modified enzyme at atmospheric pressure causes only slight hybrid formation. On the other hand, incubation at 2.6 kbar (1 h, 20° C) leads to about 50% hybridization (Fig. 1, insert).

As indicated by the single hybrid band, high pressure may be assumed to cause partial dissociation to the dimer. However, solvent conditions may influence the relative rates of hybrid formation (Jaenicke and Koberstein 1971), so that monomerization cannot be excluded.

One has to keep in mind that the interpretation of the pressure-induced hybridization in terms of a shift of the thermodynamic equilibrium may not necessarily be valid. A kinetic interpretation, based on a pressure-induced decrease of the activation energy of dissociation, may be another explanation. The transition state of a reaction is determined by the properties of both the educt and product.

If a reaction parameter (like pressure) favours the properties of the educt, increased stabilization of the educt will be observed, resulting in a decreased rate of the reaction. On the other hand, stabilization of the properties of the product will lead to a decrease of its energy level relative to the initial state, thus increasing the rate, as well as the final equilibrium concentration.

A further possible mechanism of hybrid formation could be a pressure-induced increase in the rate of subunit exchange (without significant dissociation) according to a reaction sequence $A_4 + B_4 \rightarrow A_4 \cdot B_4 \rightarrow 2\,A_2B_2$. However, this mechanism appears highly unlikely because of the specific intersubunit interactions in the tetrahedral quaternary structure of the enzyme. Previous results of hybridization experiments with a number of oligomeric enzymes (includig mesophilic LDH) were confirmed by cross-linking (Müller et al. 1981a; Schade et al. 1980b) and reconstitution kinetics (Schade et al. 1980a; Müller et al. 1981b), providing unambiguous evidence for the involvement of subunit dissociation. Therefore it seems justified to assume the same mechanism in the present context.

Summarizing the results, lactate dehydrogenase from *Bacillus stearothermophilus* is found to exhibit higher stability towards hydrostatic pressure than the enzyme from pig heart and skeletal muscle. Obviously, the subunit interactions within the thermophilic enzyme exceed those stabilizing the homologous mesophilic enzyme.

Unfolding of the protein in 6 M Gu · HCl

Figure 2 shows the intrinsic fluorescence spectra of native and denatured dehydrogenase from *Bacillus stearothermophilus*. As indicated by the wavelength of maximum emission, $\lambda_{max} = 335$ nm, in the native state tryptophan residues are shielded from the solvent in a hydrophobic environment. In 6 M

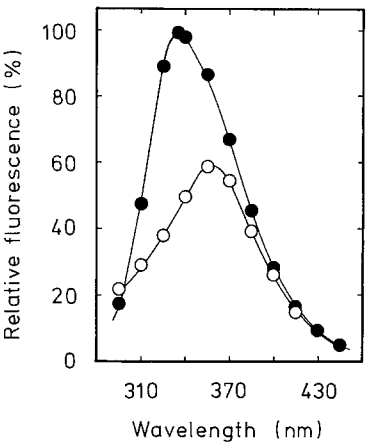

Fig. 2. Fluorescence emission spectra of native and unfolded lactate dehydrogenase from *Bacillus stearothermophilus*. (●) Native protein in 0.2 M Tris/HCl at pH 7.2. (○) Unfolded protein at 6 M Gu · HCl resolved in the same buffer. λ_{exc} = 280 nm; enzyme concentration c = 10 µg/ml; 35° C

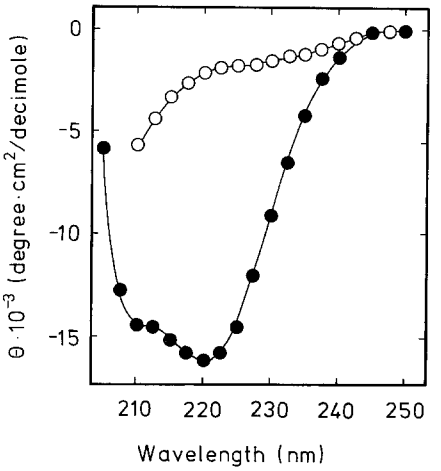

Fig. 3. Circular dichroism of lactate dehydrogenase from *Bacillus stearothermophilus* in the far-ultraviolet region. Enzyme concentration: c = 200 µg/ml; optical path d = 1 mm. All other specifications as given in Fig. 2

Gu · HCl solution, the emission spectrum of the enzyme is typical for a protein in its random conformation (λ_{max} = 355 nm). Circular dichroism measurements corroborate this result. Figure 3 indicates that the native enzyme contains a high amount of α-helices and β-structures. No detectable secondary structure remains in 6 M Gu · HCl. At pH < 2 an intermediate state is formed: the enzyme loses its catalytic function in a slow process preserving part of its secondary structure. It is concluded that the native as well as the acid denatured (and dissociated) protein represent structural globular entities, whereas in 6 M Gu · HCl the completely randomized polypeptide chain is obtained.

Refolding of the protein

Fluorescence emission and circular dichroism of lactate dehydrogenase from *Bacillus stearothermophilus* change markedly upon refolding. Both show biphasic kinetics. Within the dead-time of mixing (~ 10 s), the maximum fluorescence intensity shifts back to λ_{max} = 335 nm, and the emission intensity increases from 45 to 85% of the value observed for the native protein. The effects may be considered to reflect the shielding of tryptophan residues from the aqueous solution, accompanied by a significant regain of secondary and tertiary structure. Circular dichroism measurements corroborate this explanation: After 20 s of renaturation, > 70% of the $\theta_{222\,nm}$ value of the native enzyme is recovered.

Both fluorescence and circular dichroism prove that upon reconstitution the protein reaches a native-like conformation within the time range of seconds. After the fast phase ($\tau_{1/2}$ < 4 s) only slight further spectral changes occur. Obviously, "reshuffling" subsequent to the rapid refolding steps alters the conformation of the polypeptide chain only marginally. Since enzyme concentration must be kept low in order to avoid side reactions in the process of reconstitution (Jaenicke 1984), the kinetics of the slow phase cannot be resolved quantitatively.

Yield and kinetics of reactivation

Evidence from the spectroscopic measurements shows that incubation of the enzyme in 6 M Gu · HCl generates inactive monomers devoid of significant residual structure. Reactivation may be initiated by rapid dilution to 0.1 M Gu · HCl. In order to study the kinetics of reactivation in a quantitative way, the experimental conditions had to be optimized. *1.* In order to achieve a high time resolution, the half-time of reactivation had to be long enough to allow a sufficient number of assays to be performed during the regain of catalytic activity. *2.* To reduce perturbations from side reactions, the yield of reactivation had to be as high as possible. Both conditions were found to be fulfilled if reactivation was performed at 25–40° C in the concentration range from 0.4 to 7.5 µg/ml. Under these conditions about 65% of the initial catalytic activity of the native enzyme were recovered with a half-time of 16 to 4 min (see below). Figure 4A shows reactivation profiles at 35° C. The kinetics of reactivation are found to be independent of protein concentration and may be linearized

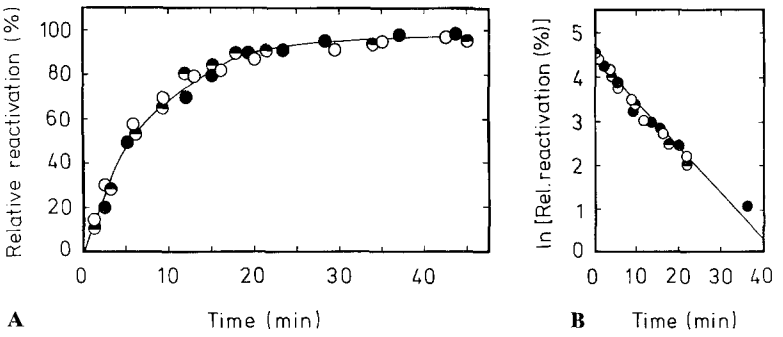

Fig. 4. Reactivation kinetics of lactate dehydrogenase from *Bacillus stearothermophilus*. **A.** Kinetic profile at varying enzyme concentration. Denaturation was achieved by incubation in 6 M Gu · HCl for 10 min at room temperature. Renaturation was initiated by dilution of the unfolded enzyme with 0.2 M imidazolium · HCl pH 7.2 plus 10 mM DTE, 35° C; the final concentration of Gu · HCl in the reactivation mixture was 0.1 M. At defined time intervals of reactivation aliquots were taken from the renaturation solution and assayed for catalytic activity. Final protein concentrations during reactivation were (○) 7.4 μg/ml, (◓) 0.8 μg/ml, and (●) 0.4 μg/ml, respectively. **B.** Semi-logarithmic linearization of the data given in **A.** The slope of the straight line corresponds to a unimolecular rate constant of $k_1 = 1.8 \cdot 10^{-3}\ s^{-1}$

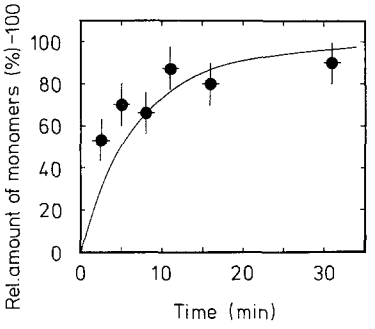

Fig. 5. Reassociation kinetics of monomeric lactate dehydrogenase from *Bacillus stearothermophilus* as measured by crosslinking with glutaraldehyde. The concentration of the protein was 5 μg/ml (143 nM). Conditions of denaturation and renaturation as given in Fig. 3. Experimental errors were estimated from the duration of the crosslinking reaction (time-axis) on one hand, and the precision of the determination of peak areas on gel scans (concentration-axis), on the other. The profile is taken from Fig. 4A

according to first order (Fig. 4B). This finding demonstrates that a unimolecular folding reaction limits the kinetics of reactivation. The rate-determining step in the process of reactivation is preceded by the above mentioned fast spectral changes ($\tau_{1/2} < 10$ s), accompanying the initial phases of the folding of the polypeptide backbone.

Kinetics of reassociation of monomers

As shown by Hermann et al. (Hermann et al. 1979; Hermann et al.1981; Hermann et al.1983), glutaraldehyde cross-linking may be applied to study the reassociation of oligomeric enzymes. Crosslinking of native lactate dehydrogenase from *Bacillus stearothermophilus* under optimum conditions (enzyme concentration 5 μg/ml (135 nM), (1% w/v glutaraldehyde, 2 min) leads to the quantitative fixation of dimers. On the other hand, gel-filtration and ultracentrifugation (R. Jaenicke, unpublished results) clearly prove the enzyme to be tetrameric in its native state. There may be two explanations for the discrepancy: Either the dimer (in the presence of the crosslinking reagent) is the stable entity or the tetramer is a stable "dimer of dimers" with unfavourable crosslinking sites, such that the fixation does not go to completion (Jaenicke and Rudolph 1983). At present the two alternatives cannot be resolved. Although the crosslinking reaction does not allow us to monitor the reassociation of tetramers, we may nonetheless measure the reassociation of monomers to form the dimer. The kinetics of this process are illustrated in Fig. 5. The traces in Fig. 4 (reactivation) and in Fig. 5 (association of monomers) parallel each other within the range of experimental error. This suggests that conformational changes within the monomers limit the kinetics of reactivation; subsequent reassociation appears to be fast.

Final product of reconstitution

As mentioned, tetramerization of lactate dehydrogenase from *Bacillus stearothermophilus* cannot be measured directly by crosslinking with glutaraldehyde. However, there are two findings which suggest that reconstitution proceeds fully to tetramers: *1.* Fructose-1,6-bisphosphate as an allosteric effector of the native enzyme enhances the catalytic activity regained upon reconstitution to the same extent as the activity of the native tetramer; *2.* following the reactivation kinetics by monitoring the enzymatic activity in the absence and in the presence of trypsin (Jaenicke and Rudolph 1984), identical reactivation

Table 1. Unimolecular rate constants describing the kinetics of reactivation as a function of temperature

Temperature (K)	rate constant k_1 (s^{-1})
298	$0.72 \cdot 10^{-3}$
303	1.2
308	1.8
313	2.9

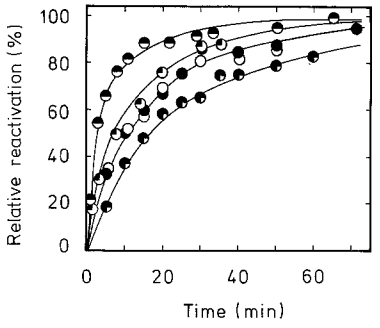

Fig. 6. Dependence on the temperature and the presence of specific ligands of the reactivation kinetics of lactate dehydrogenase from *Bacillus stearothermophilus*. Enzyme concentration: 5 µg/ml. (◓) 40° C, (◐) 35° C in the presence of 6 mM fructose-1,6-bisphosphate, (●) 30° C in the absence of ligand, (○) 30° C in the presence of 250 µM NADH, (◐) 25° C. In all experiments, the final pH at the temperature given was 7.2. All other specifications as given in Fig. 3

profiles are obtained: Catalytic tests were performed at a protease concentration at which the native protein loses 50% of its activity within 4 min (0.4 mg/ml under standard conditions). Since intermediates of reconstitution are known to exhibit significantly higher susceptibility towards proteolytic digestion than the native enzyme (Girg et al. 1981; Girg 1983), reconstitution products other than the native tetramer should show a faster loss of activity. This is not observed. Therefore, we conclude that such intermediates do not become transiently accumulated during reconstitution. It is then most likely that the reactivation measured pertains to the regeneration of tetramers as the final product of reconstitution.

Influence of temperature and effectors on reactivation

Figure 5 shows that increased temperature accelerates reactivation. Plotting the corresponding rate constants (Table 1) in an Arrhenius diagram, an activation energy of 17 kcal/mol (~ 71 kJ/mol) is obtained. Figure 5 also shows that neither the coenzyme, NADH, nor the allosteric effector, fructose-1,6-bisphosphate alter the kinetics of reactivation. This suggests that species involved in the rate-limiting step of reactivation do not bind either of the two ligands. As suggested before, the kinetics of reactivation are limited by the refolding of the monomer. This assignment is compatible with the observation that the presence of ligands does not affect the kinetics of reactivation. Experimental evidence available so far clearly indicates that monomeric intermediates of reconstitution of lactate dehydrogenase do not possess ligand binding sites.

Discussion

High pressure stability

Tetrameric lactate dehydrogenase from *Bacillus stearothermophilus* exhibits unusual stability towards high hydrostatic pressure: contrary to the enzyme from mesophilic organisms, incubation at pressures up to 2.8 kbar does not cause irreversible denaturation. Instead, hybridization experiments suggest that dissociation of the native tetramer (at least to the dimer) can occur. Reassembly from the intermediate dissociated state is observed within the dead-time of pressure release experiments so that apparent high pressure stability is observed. Obviously the subunit interactions within the thermophilic lactate dehydrogenase are stronger that those stabilizing the mesophilic enzyme (Zuber 1981; Jaenicke 1981). As suggested by the kinetics of reconstitution after denaturation in 6 M guanidine · HCl, the increased stability may be related to the difference in the rate constants describing the reassociation of the various proteins. Assuming the rate of dissociation to be essentially the same for the different proteins, an increase by three orders of magnitude in the rate of association of the thermophilic enzyme would be sufficient to explain the observed stabilization. A detailed study concerning the stability of the thermophilic enzyme towards temperature and pressure is in progress.

Mechanism of reconstitution

The present results reveal that renaturation of lactate dehydrogenase from *Bacillus stearothermophilus* is a multi-step process. The sequence of events during the self-assembly includes folding and association reactions: (*i*) fast folding of unstructured monomers to form a native-like conformation; (*ii*) slow "reshuffling" of "structured monomers" as the rate-determining step in the overall process of reconstitution;

(*iii*) fast association reactions (dimerization of monomers and dimers) leading to the renatured tetramer.

Tetramer formation is responsible for the recovery of enzymatic activity in the process of reconstitution. According to the proposed mechanism, "structured monomers" and dimers (generated in step (*ii*) and (*iii*) do not become transiently populated. Therefore, no information can be given regarding the functional properties of the two species. What is clear is that the native-like subunits accumulated in step (*i*) do not exhibit biological function: they precede the occurrence of catalytic activity.

Comparison with other proteins

Fast formation of secondary structure is the first event detectable on the reconstitution pathway. It is followed by conformational rearrangements generating the "structured monomer". This exhibits a native-like topology. A biphasic reaction of this kind has been reported for single-chain proteins, like octopine dehydrogenase (Zettlmeißl et al.1984), as well as for a number of oligomers (lactate dehydrogenase (Jaenicke and Rudolph 1983), malate dehydrogenase (Jaenicke and Rudolph 1983), phosphoglycerate mutase (Hermann et al. 1983) and the bifunctional enzyme aspartokinase-homoserine-dehydrogenase I from *Escherichia coli* (Müller and Garel 1984a, b). It appears as if rapid refolding with subsequent slow "reshuffling" of folded polypeptide chains were a general pattern characteristic of the reconstitution mechanism of proteins. In the case of oligomeric enzymes subsequent association to form the native quaternary structure may add one more rate-determining step. The corresponding second-order rate constants vary over a wide range. Even considering a single enzyme, like lactate dehydrogenase, the bimolecular rate constants are found to differ by three orders of magnitude: In the case of LDH-M_4 and LDH-H_4 from pig, reassociation is slow [$k_2 \sim 10^3\ M^{-1} \cdot s^{-1}$ (Jaenicke and Rudolph 1983)] while for the protein from *Bacillus stearothermophilus* the reaction is essentially diffusion controlled ($k_2 > 10^6\ M^{-1} \cdot s^{-1}$).

For the given reason, generalizations with respect to the mechanism of reconstitution have to be made with care.

Reconstitution in vitro vs folding in vivo

The reconstitution kinetics of lactate dehydrogenase from *Bacillus stearothermophilus* are governed by conformational changes within folded monomers. The rate constant which quantitatively decribes this step amounts to $1.1 \cdot 10^{-2}\ s^{-1}$ at 55° C, the average temperature in the biotope of the bacterium. This value (obtained from an extrapolation of rate constants determined at 25° C–40° C) corresponds to a half-time of less than 1 min for the assembly of the protein under physiological conditons. It is likely that this estimate also holds for in vivo conditions. The time interval given is short compared to the generation time of *Bacillus stearothermophilus*. As a consequence folding of lactate dehydrogenase does not seem to be crucial in the regulation of biological functions of this organism. One would expect this conclusion to be true for the whole enzyme inventory of the cell.

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