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PRESSURE-INDUCED STRUCTURAL CHANGES OF PIG HEART LACTIC DEHYDROGENASE *

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Lactic dehydrogenase from pig heart can be reversibly dissociated at hydrostatic pressures above 1000 bar. The breakdown of the native quaternary structure occurs at lower pressures compared to the isoenzyme from pig skeletal muscle. As shown by hybridization experiments of the two isoenzymes the final product of dissociation is the homogeneous monomer. Fluorescence emission spectra of the monomeric enzyme at elevated pressure are characterized by a decrease in fluorescence intensity without any red shift, indicating that no significant unfolding occurs upon high-pressure dissociation. The spectral changes are comparable to those observed after acid dissociation. The amount and rate of deactivation depend on pressure and on the conditions of the solvent. The presence of various anions (Cl^-, SO_4^{2-}, HPO_4^{2-}) has no effect on the stability of the enzyme towards pressure. High-pressure denaturation (as monitored by intrinsic protein fluorescence), and deactivation (measured immediately after decompression) run parallel; the pressure dependence of their first-order rate constants is characterized by an activation volume $\Delta V_{De}^{\neq} = -140 \pm 10$ cm^3/mol. As taken from the yield of reconstitution, dissociation, denaturation and deactivation are found to be fully reversible provided the pressure does not exceed a limiting value ($p = 1000$ bar in Tris, pH 7.6; 24 h incubation at 20°C). After extended incubation beyond the limiting pressure of 1000 bar, "irreversible high-pressure denaturation" occurs which is accompanied by partial aggregation after decompression. The coenzyme, NAD^-, stabilizes the native tetramer shifting the dissociation equilibrium to higher pressures. The overall dissociation-association reaction can be quantitatively described by a consecutive dissociation/unfolding mechanism $N \rightleftharpoons 4\,M' \rightleftharpoons 4\,M^*$ (where N is the native tetramer, and M′ and M* two different conformations of the monomer). The reaction volume of the dissociation reaction $N \rightleftharpoons 4\,M'$ is found to be $\Delta V_{Diss} = -360 \pm 30$ cm^3/mol; as indicated by the pressure dependence of the yield of reconstitution, the reaction volume of the equilibrium $M' \rightleftharpoons M^*$ is also negative.

1. Introduction

In previous studies from this laboratory high-pressure investigations on the structure and function of glycolytic enzymes were reported, applying turbidity and activity measurements at high pressure [1,2], as well as activity measurements after decompression [3,4]. Using these techniques both slow deactivation and rapid reconstitution may escape detection. Therefore, in the present study a high-pressure fluorescence cell has been designed allowing the conformational analysis of enzymes under equilibrium conditions at high pressure. Making use of the intrinsic protein fluorescence in this way true high-pressure data may be obtained, providing direct information with respect to pressure-induced structural changes in proteins.

As taken from earlier experiments [3,5,6] the action of high pressure on lactic dehydrogenase from pig muscle (LDH-M_4) exhibits remarkable differences compared to common denaturants like guanidine · HCl, urea, and extremes of pH. In the present study the isoenzyme from pig heart (LDH-H_4) was chosen in order to find out whether the results obtained so far may be generalized to describe equally the high-pressure characteristics of the two mechanistically differing systems: In contrast to the enzyme from skeletal muscle, LDH-H_4 exhibits different enzymological properties [7,8] and a more complex pathway of reconstitution

* This work is dedicated to Professor Hans Herloff Inhoffen on the occasion of his 75th birthday.

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after acid deactivation comprising unimolecular and bimolecular rate-determining steps [9–12].

2. Materials and methods

Lactic dehydrogenase (LDH) H_4 from pig heart and M_4 from pig skeletal muscle, NADH and NAD^+ were purchased from Boehringer (Mannheim); dithioerythritol (DTE) was obtained from Roth (Karlsruhe), acrylamide, bisacrylamide and TEMED from Serva (Heidelberg). All other reagents were of A-grade purity (Merck, Darmstadt). Quartz bidistilled water was used throughout.

Stock solutions of the enzymes (≈ 5 mg/cm^3) were prepared by dialysis at 4°C against oxygen-free Tris buffer, pH 7.6 (20°C), containing 1 mM EDTA and 10 mM DTE.

Enzyme concentrations were calculated from $A^{0.1\%}_{280\,nm} = 1.4$ cm^2/mg [13]. Molar concentrations refer to the subunit molecular weight of 35000.

Enzyme activity was measured in potassium phosphate buffer (0.2 M, pH 7.6) containing 1 mM EDTA and 2.5 mM DTE in the presence of 0.74 mM pyruvate and 0.2 mM NADH. Recording Eppendorf or Bausch and Lomb spectrophotometers thermostatically maintained at 25°C were used. The specific activities of the native H_4 and M_4 isoenzymes were 350 ± 30 and 480 ± 20 IU/mg, respectively.

Gel electrophoresis was performed at pH 8.5 according to ref. [14]. After staining and destaining with the Coomassie R system [15] the gels were scanned at 590 nm using a Gilford 2400 S single-beam spectrophotometer.

2.1. High-pressure techniques

Quench experiments were performed as described previously [3]. The design of the high-pressure fluorescence cell used in the present study is given in fig. 1. The cell body and all metal parts are machined from maraging steel (SV-RHF 32, Röchling, Völklingen (F.R.G.)). All metal parts in the interior of the optical cell are lined with Teflon. During operation a silicon hose (sealed with a glass stopper) serves as reservoir for the enzyme solution separating this solution from the liquid (ethylene glycol) used in the pressure-generating system. The compression of the enzyme solution under pressure is compensated by partial collapse of the silicon hose, thus avoiding a change of the position of the Teflon liner with pressure. The cylindrical sapphire windows (diameter 12 mm, height 6 mm) are glued with epoxy resin (Eccobond 104, Emerson and Cuming, Köln) into the support rings. To seal the high-pressure system O-rings are used; for details, cf. insert fig. 1. The temperature in the interior of the autoclave is monitored with a miniature thermocouple (Philips, Industrie Elektronik, Hamburg) to an accuracy of ± 0.2 K. The whole autoclave is kept at constant temperature by pumping thermostatically controlled petrol ether (b.p. 100–140°C) through the brass jacket given in fig. 1. Because of the high heat capacity of the autoclave, and the low compressibility of both the enzyme solution and the ethylene glycol, thermal effects of compression and decompression are small (< 1°C); the initial temperature is reestablished within 30 s. Pressure valves, capillaries, and the pressure-generating system are commercial products (HIP High Pressure Equipment, Erie, Pennsylvania, U.S.A.). Pressure was measured to an accuracy of ± 1 MPa with a Bourdon gauge (A. Wiegand, Klingenberg, F.R.G.).

In the case of fluorescence studies at high pressure, the light from a Hitachi-Perkin Elmer MPF 44A spectrofluorimeter was guided into the pressure cell using a 500 mm UV light guide (Volpi, München, F.R.G.); a second light guide was applied to collect the fluorescence intensity at 90° relative to the transmission beam, and to lead it back to the entrance slit of the spectrofluorimeter. In order to achieve high light intensity a biconvex quartz lens was placed in front of the excitation slit of the spectrograph.

3. Results

3.1. Kinetics of deactivation and denaturation by high pressure

Applying the previously mentioned buffer conditions, LDH-H_4 at concentrations $c_p \geq 0.01$ μg/cm^3 and normal atmospheric pressure retains

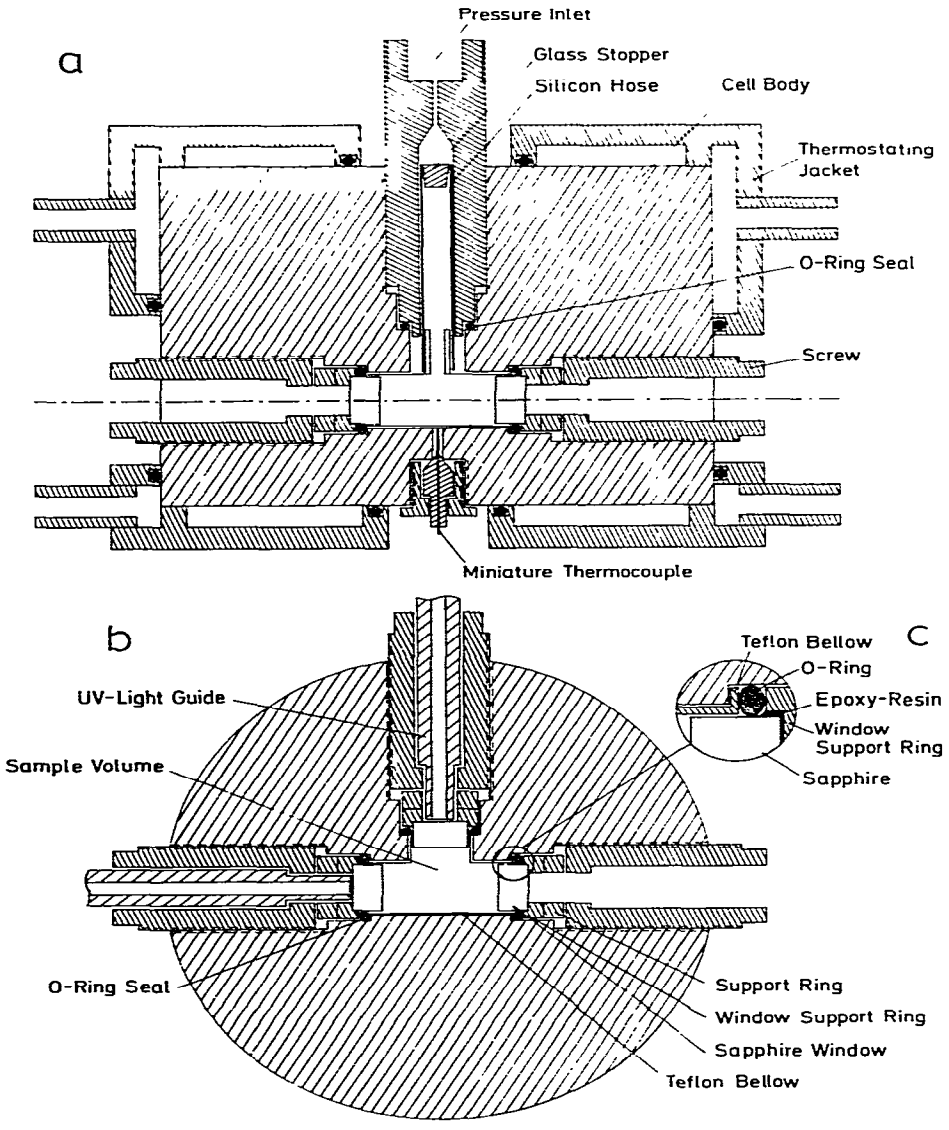

Fig. 1. High-pressure fluorescence cell. (a) Schematic view; (b) cross-section at the line ·–·–· given in (a); (c) exploded view of the details of the window seal.

more than 80% of its original enzymatic activity during incubation times up to 300 h, indicating stability of the enzyme towards slow irreversible deactivation at high dilution. Since in the case of oligomeric enzymes the high-pressure equilibrium states are reached only after long incubation, the choice of appropriate buffer conditions is of prime importance [3].

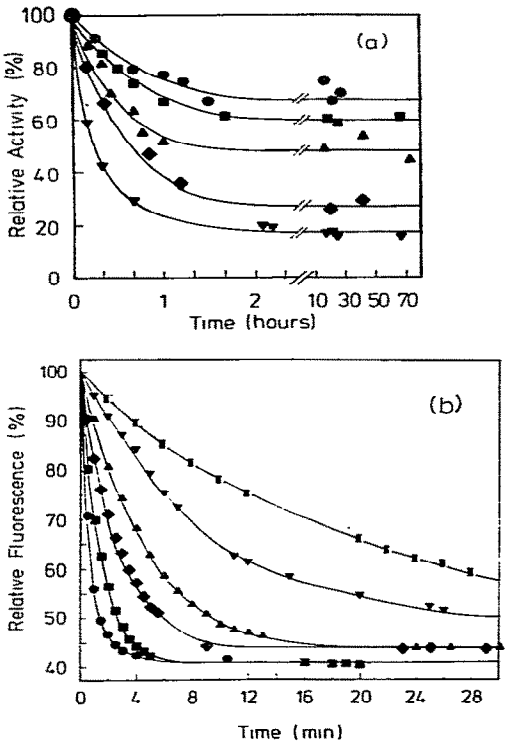

Fig. 2. Kinetics of the pressure-induced deactivation and denaturation of LDH-H_4 (apoenzyme). Incubation in Tris-HCl buffer, pH 7.6 (I=0.16 M) in the presence of 1 mM EDTA and 10 mM DTE; c_{LDH}=25 μg/cm³ (0.72 μM). T=20°C. Activity measurements were started 30 s after decompression. (a) Kinetics of deactivation at 300 (●), 400 (■), 500 (▲), 600 (◆) and 730 bar (▼). (b) Kinetics of the decrease in fluorescence emission at 700 (■), 820 (▼), 900 (▲), 1000 (◆), 1100 (■) and 1200 bar (●). λ_{exc}=285 nm, λ_{em}=345 nm. Final values of fluorescence emission intensity at 700 and 820 bar are 54 and 51%, respectively.

As shown in fig. 2a, slow high-pressure deactivation of LDH-H_4 is observed at $300 \leq p \leq 730$ bar. Depending on the pressure applied, the final value of specific activity is reached after an incubation time of 2 h at 300 bar, and 1 h at 730 bar. Comparing this behaviour to the changes in fluorescence emission, a similar time course is obtained at $700 \leq p \leq 1200$ bar (fig. 2b). The residual enzymatic activity is constant over a period of 72 h. Recovery of native fluorescence after decompression is found to be exceedingly slow (half-time $\tau_{1/2} \approx 12$ min [16]). Therefore, reconstitution of the enzyme during the unavoidable time lapse of 20–30 s between decompression and start of the enzyme assay does not cause a significant systematic error in the kinetics of deactivation, as measured by quench experiments.

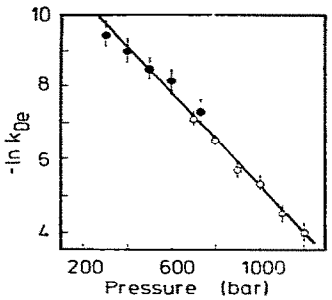

Fig. 3. Determination of the activation volume of denaturation and deactivation. (●) Activity, (○) fluorescence emission. First-order rate constants k_{De} were calculated as described previously [3,16]. $\Delta V^{\neq}_{De} = -140 \pm 10$ cm³/mol.

As illustrated in fig. 3, the kinetics of deactivation and the decrease in fluorescence intensity are described by identical first-order rate constants using the linearization procedure described previously [3,17]. Applying the pressure dependence of the respective rate constants, the activation volume $\Delta V^{\neq}$ for pressure deactivation and pressure denaturation may be calculated according to

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT}$$

The result, $\Delta V^{\neq}_{De} = -140 \pm 10$ cm³/mol, is similar to the value obtained for the isoenzyme from pig muscle, $\Delta V^{\neq}_{De} = -190 \pm 15$ cm³/mol [3].

3.2. Deactivation-reactivation equilibrium of LDH-H_4 at high pressure

It has been pointed out before that the pressure-induced processes in solutions of LDH-H_4 at pressures up to 730 bar lead to well defined final values of enzymatic activity (cf. fig. 2). As shown in fig. 4, the reactivation at atmospheric pressure after long-term deactivation at $p \leq 1000$ bar leads

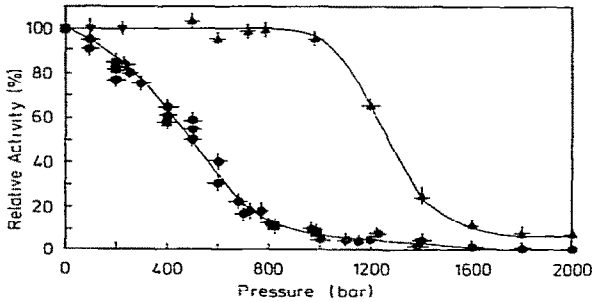

Fig. 4. Pressure-dependent deactivation and reactivation of apo LDH-H_4; conditions of incubation as given in fig. 2. (●) Deactivation for ⩾15 h; (▲) reactivation yield of (●) after 24 h reconstitution at 1 bar; (■) deactivation for 20 min at 1200 bar, reconstitution at the given pressures for 24 h; (◆) deactivation for 24 h at 750 bar, reconstitution at the given pressures for 24 h; (▼) reconstitution of (◆) at 1 bar for ⩾24 h.

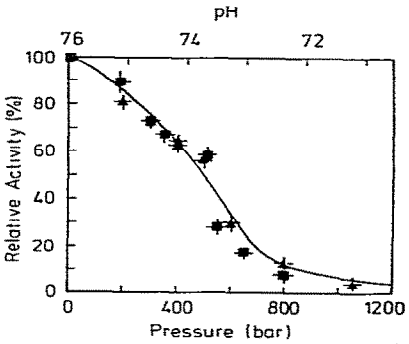

Fig. 5. Pressure-dependent deactivation of apo LDH-H_4 in Tris-H_3PO_4 and Tris-H_2SO_4 buffer, pH 7.6 (I=0.12 M) in the presence of 1 mM EDTA and 10 mM DTE; c_{LDH}=25 μg/cm³ (0.72 μM), T=20°C. Incubation at the given pressures for ⩾15 h. (▲) Tris-H_3PO_4, (■) Tris-H_2SO_4. The trace is taken from fig. 4. The upper abscissa gives the pH shift connected with pressure application.

to complete recovery of the initial enzymatic activity; this supports the idea that high-pressure deactivation at $p \leqslant 1000$ bar represents an equilibrium. In the given pressure range no hysteresis in the deactivation-reactivation transition is observed (cf. refs. [6,11]). This is shown by incubating the enzyme at 750 and 200 bar (or 100 bar) for ⩾15 h in subsequent steps. Comparing the residual activity immediately after decompression to 1 bar reveals no difference of the specific activity of the enzyme independent of the treatment at high or low pressure. The results of these experiments suggest that equilibria of active and inactive species of LDH-H_4 exist at elevated pressure. Variation of pressure in the range up to 1000 bar causes fully reversible shifts of this equilibrium. The observed absence of any hysteresis effects represents a remarkable difference comparing high-pressure deactivation with other procedures causing dissociation, denaturation, and deactivation of oligomeric enzymes (e.g., guanidine · HCl, urea, acid or alkaline pH [6,12]).

As in the case of LDH-M_4, the pressure-induced deactivation of LDH-H_4 is monophasic within the range of experimental error (cf. fig. 4). The pressure-induced deactivation of LDH-H_4 occurs at lower pressure ($p_{1/2} \approx 500$ bar) than that of LDH-M_4 ($p_{1/2} \approx 800$ bar [3]).

As illustrated in fig. 5, the equilibrium of deactivation is identical for buffers containing HPO_4^{2-} or SO_4^{2-} instead of Cl^-. This is in contrast to the behaviour observed for LDH-M_4 [3], where stabilization in the presence of bivalent anions is observed. Preliminary experiments indicate that high ionic strength has a stabilizing effect towards pressure deactivation of the enzyme. This holds true to an even greater extent for the coenzyme, NAD^+ (fig. 6), while NADH causes slow deactivation [18]. Addition of 10 mM NAD^+ to

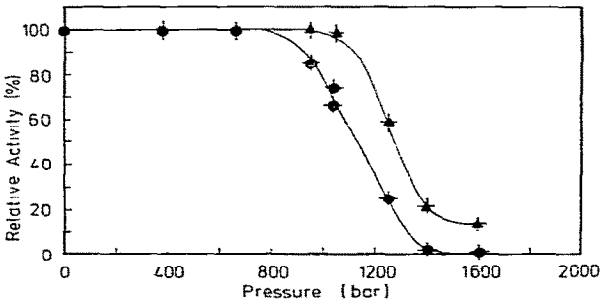

Fig. 6. Pressure-dependent deactivation and reactivation of holo LDH-H_4. Conditions of incubation as given in fig. 2, plus 10 mM NAD^+ (corresponding to 94% saturation at 1 bar). (●) Deactivation for 24 h at the given pressures; (▲) yield of reactivation 24 h after decompression.

the 720 nM enzyme solution at 1 bar yields a saturation of 97%. No deactivation of the holoenzyme is observed at 800 bar; on the other hand, the apoenzyme shows only 13% residual activity. One may thus conclude that the binding of NAD^+ to LDH-H_4 is enhanced by high pressure. This is clearly established by the negative reaction volume (decrease in partial specific volume) and the "contraction" of the enzyme upon coenzyme binding [19]. A similar pressure dependence has been observed for the "soft binding sites" involved in the binding of ANS to polycyclodextrin, and the binding of ethidium bromide to tRNA [20].

Fig. 4 proves that the long-term deactivation of LDH-H_4 is not completely reversible at pressures above 1000 bar. Addition of NAD^+ stabilizes the enzyme against denaturation without influencing the yield of reactivation.

As shown in fig. 7, the yield of reactivation depends on the pressure and the duration of deactivation. In general, loss of reversibility is enhanced at increased pressure of denaturation; at a given pressure, a limiting value of reversibility is reached which decreases with increasing pressure, thus indicating that in the transition range at $p \geq 1000$ bar the denatured state is in equilibrium with the active enzyme.

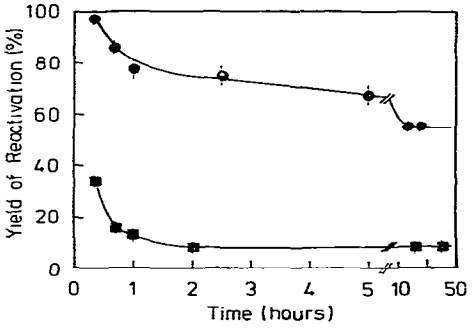

Fig. 7. Reactivation yield of apo LDH-H_4 as a function of deactivation time at $p = 1200$ bar (●), and $p = 2000$ bar (■). Conditions of incubation as given in fig. 2. Reconstitution at 1 bar (20°C) for ≥24 h.

3.3. Characterization of the pressure-deactivated enzyme

Deactivation of oligomeric enzymes may be caused either by first-order conformational transitions, or by changes in the state of association characterized by higher-order processes. In order to obtain information with respect to conformational changes, high-pressure fluorescence spectroscopy was applied. Insight into the state of association of pressure-deactivated LDH is provided by hybridization experiments [21,22].

Fluorescence spectra of native and pressure-deactivated LDH-H_4 at $700 \leq p \leq 1200$ bar are given in fig. 8. Denaturation (deactivation) causes a decrease in fluorescence intensity, but does not shift the emission maximum. The spectrum of the pressure-denatured enzyme is identical to that of LDH-H_4 after acid denaturation, suggesting that similar structures prevail under both conditions of denaturation. As indicated by the lack of any red shift in the high-pressure emission spectrum, no significant unfolding seems to occur at high pres-

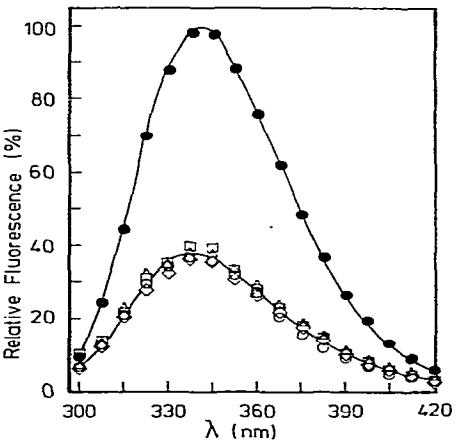

Fig. 8. Fluorescence emission spectra ($\lambda_{exc} = 285$ nm) of native (closed symbols), and pressure-denatured (open symbols) apo LDH-H_4. Conditions of incubation as given in fig. 2. Spectra of the (partially) denatured enzyme are corrected for residual native enzyme present at equilibrium (cf. fig. 4). Incubation at high pressure at 700 and 820 (□), 900 (○), 1000 (△), 1100 and 1200 bar (◇). The duration of high-pressure incubation was < 1 h throughout.

sure. In the case of the acid denaturation of the enzyme this observation has been corroborated by circular dichroism measurements [23] *.

Previous high-pressure hybridization experiments with LDH [5,24] provided clear evidence for subunit dissociation under pressure. However, they cannot be used for the quantitative determination of the extent to which dissociation occurs, since (a) the technique necessarily requires both isoenzymes to be present, and (b) no details are known with respect to the relative rates of reassociation of the two isoenzymes and their hybrids under the given experimental conditions.

In order to obtain quantitative information regarding the amount of dissociated material present at high-pressure equilibrium, the original hybridization procedure was modified by denaturing the two enzymes separately by high pressure; hybridization and joint reconstitution were then achieved by mixing the two solutions immediately after decompression.

As shown in fig. 9, all five possible hybrids of the two isoenzymes are formed during the joint reconstitution after separate denaturation (deactivation) at $p = 1200$ bar (20°C, Tris-HCl buffer, 2 h incubation). The relative amount of the hybrids formed is compatible with the assumption that the inactive species at $p \geq 1200$ bar is the homogeneous monomeric subunit. Dissociation to dimers only is ruled out by the occurrence of hybrids of the A_3B type. Deviations from the binomial distribution of the hybrids should become negligible if the accumulation of H_4 and M_4 is correlated with the residual activity at $p = 1200$ bar. The observed dissociation to the monomer level is in agreement with the previously mentioned fluorescence measurements. As pointed out, monomers after acid dissociation and denaturation exhibit the same fluorescence spectrum as the pressure-denatured species. If pressure dissociation were to yield dimers, one would expect differences in the change of fluorescence intensity comparing the monomeric product of acid dissociation on the one hand, and high-pressure dissociation on the other.

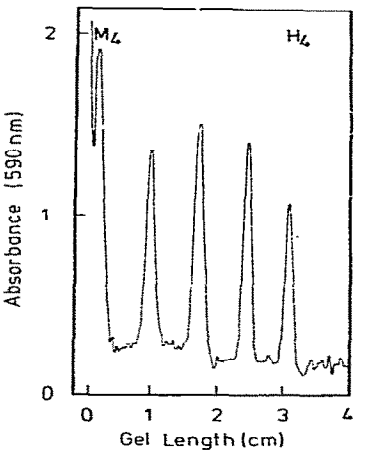

Fig. 9. Gel electrophoresis of LDH hybrids, formed upon joint reconstitution, after separate deactivation at 1200 bar (2 h). Conditions of incubation as given in fig. 2. Solutions of the deactivated enzyme (residual activity ≈ 10%) were combined 30 s after decompression. After 24 h of reconstitution at 1 bar (reactivation yield 90%), solutions were concentrated (20-fold) by ultrafiltration (Amicon Diaflo with PM 10 filters). For details of staining and destaining see section 2.

4. Discussion

4.1. Thermodynamic and kinetic considerations

The action of high pressure on LDH from pig heart causes reversible deactivation, denaturation, and dissociation. As shown by the effect of pressure on the reversibility of the three processes various elementary steps must be involved in the deactivation of the enzyme. The results of hybridization experiments indicate that at $p \geq 1200$ bar the inactive species is the homogeneous monomer. This conclusion is supported by the similarity of the fluorescence spectra of the acid-denatured and pressure-denatured enzyme. Furthermore, the emission spectrum of deactivated LDH-H_4 does

* Circular dichroism measurements at high pressure would be desirable to quantitatively characterize the enzyme in its deactivated state. At present, there is no way of measuring high pressure circular dichroism with sufficient accuracy. Measurements at atmospheric pressure immediately after decompression suffer from the ill-defined structure of the enzyme during the process of reconstitution.

not change in the pressure range between 700 and 1200 bar. Taking into account that the deactivation transition is found to be monophasic, we may conclude that only tetramers (which represent the active species at normal atmospheric pressure [6,9]), and (inactive) monomers are present at equilibrium.

The reaction scheme

$$N \rightleftharpoons 4\,M' \rightleftharpoons 4\,M^*$$

permits description of the high-pressure denaturation in a quantitative fashion. The detailed analysis of the reversibility of the pressure-induced changes shows that the measurements cannot be quantitatively described by a model containing one monomeric species only. Instead, two different monomers have to be considered: M', undergoing fully reversible deactivation, denaturation, and dissociation (reconstituting quantitatively to the native enzyme, N), and M*, representing the fraction which undergoes irreversible denaturation. The given kinetic results provide information with regard to the volume effects involved in both reactions.

As pointed out, the deactivation $N \rightarrow 4\,M'$ exhibits an activation volume of $\Delta V^{\neq}_{De} = -140 \pm 10$ cm^3/mol. Since the loss of reversibility after long incubation at a constant pressure is accelerated with increasing pressure, the activation volume of the process $M' \rightarrow M^*$ must also be negative. There are two possible reasons for the dependence of the yield of reconstitution on the deactivation pressure: irreversible aggregation of pressure-denatured monomers under high pressure [2], or unspecific aggregation competing with the reconstitution after decompression; the latter was shown to govern the concentration-dependent decrease in the yield of reconstitution after acid dissociation of LDH-M_4 [25].

Comparing these earlier results with the present study, there are basic differences with respect to the experimental conditions: LDH-H_4 instead of LDH-M_4, low enzyme concentration, and chloride instead of phosphate as buffer anion (cf. ref. [3]). Under these conditions aggregation is considered to be a consecutive reaction of dissociation and unfolding.

The fact that for each pressure a plateau value of the reconstitution yield is reached after a certain time of deactivation suggests that in the case of LDH-H_4 unspecific aggregation after the decompression is responsible for the effects observed. In contrast to the present experimental results, pressure-induced unspecific aggregation should decrease the reversibility of reactivation continuously down to zero with increasing time of deactivation. As in the case of acid deactivation [25,26] this is not observed. Instead a tetramer $\rightleftharpoons$ monomer equilibrium is postulated which involves monomeric species differing in their ability to return to the native state after reestablishing ambient pressure.

The reaction volume of deactivation characterized by the pressure dependence of the equilibrium $N \rightleftharpoons 4\,M$ is found to be $\Delta V_{Diss} = -360 \pm 30$ cm^3/mol. As mentioned the reversibility of the tetramer $\rightleftharpoons$ monomer transition is restricted at elevated pressure; therefore, the reaction volume of the equilibrium $M' \rightleftharpoons M^*$ must also be negative.

The fact that there is no stabilizing effect of bivalent anions against pressure deactivation represents a remarkable difference between the two isoenzymes LDH-H_4 and LDH-M_4 [6]. It is well known that the chemical nature of buffer ions has a marked influence on the thermal stability of proteins [27]. Apparently, in the case of LDH-M_4 a similar influence is also observed for the pressure-induced denaturation. On the other hand, LDH-H_4 does not show significant differences in its pressure-dependent structural changes on replacing Cl^- by SO_4^{2-} or HPO_4^{2-}. From this we conclude that the change in solvent structure, connected with the exchange of a monovalent anion for a bivalent one, cannot be decisive in the stabilization of LDH-M_4 by phosphate. The finding that in the presence of 1 mM HPO_4^{2-} no deactivation of LDH-M_4 is observed at pressures as high as 2000 bar supports this hypothesis. The collected evidence suggests that it is the specific binding of HPO_4^{2-} to the muscle isoenzyme which causes the significant stabilization. The unspecific variation of the solvent structure is too small to cause measurable shifts of the high-pressure equilibria of LDH-H_4.

4.2. Comparison with other proteins

Three types of proteins can be distinguished with respect to their sensitivity towards high hydrostatic pressure: The least sensitive class are small monomeric proteins like ribonuclease, myoglobin, chymotrypsinogen, and lysozyme; under quasi-physiological buffer conditions these proteins exhibit unfolding at pressures beyond the biologically relevant pressure range ($p > 3000$ bar) [28–30]. The most pronounced effects are observed in highly organized superstructures like tobacco mosaic virus protein, tubulin, ribosomes, or polymerized glutamic dehydrogenase; in these systems dissociation is caused by pressures of only a few hundred bar [31–36]. Therefore, assuming the in vitro experiments to reflect the in vivo situation in the deep sea, we must conclude that barotolerant (or barophilic) organisms require pressure adaptation in order to perform metabolism, biosynthesis, and protein assembly. Oligomeric enzymes show a behaviour which may be considered intermediate between those of the monomeric proteins and protein superstructures.

The present results exhibit properties of both classes mentioned above: Restricted reversibility and decreased rates in the reactivation kinetics after denaturation at $p \geq 1000$ bar monitor the slow unfolding of dissociated monomers, resembling the pressure-induced exposure of hydrophobic residues from the interior of monomeric proteins to the aqueous solvent. The fully reversible dissociation and deactivation at lower pressure parallels the behaviour of protein superstructures.

The hypothetical pressure-induced dissociation of oligomeric enzymes [37,38] has gained increasing support from a number of independent experimental approaches. In the present work dissociation to monomers was monitored by joint reassociation and hybridization after separate pressure incubation of the two isoenzymes LDH-H_4 and LDH-M_4. This method provides direct and unambiguous evidence for the dissociation of the native tetramers to monomers [39].

High hydrostatic pressure affects the structure of oligomeric enzymes in a way different from that of other destabilizing agents. This is shown by the following features: (a) no hysteresis region in the transition of deactivation-reactivation; (b) significant stabilization of the holoenzyme compared to the apoenzyme; (c) full reversibility in the transition range in terms of a tetramer $\rightleftharpoons$ monomer equilibrium.

Conclusions from in vitro high-pressure studies with respect to barophilic adaptation of proteins should in general be taken with care. The present experiments show that the chemical nature and the ionic strength of buffers, as well as the presence of cofactors, may exercise a strong influence on high-pressure deactivation and dissociation. In this context, it should be kept in mind that high-pressure biotopes normally represent low-temperature biotopes. Since variation of temperature strongly affects hydrophobic interactions, adaptation has necessarily to cope with both extremes (apart from darkness and low oxygen concentrations). If barophilic organisms exist, our understanding of their adaptation mechanisms in molecular terms is at best rudimentary [40].

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