Reversible High-Pressure Dissociation of Lactic Dehydrogenase from Pig Muscle†

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ABSTRACT: Indirect evidence from previous high-pressure experiments on mammalian lactic dehydrogenase led to the conclusion that dissociation, chemical modification by oxidation, and aggregation participate in the mechanism of pressure deactivation [Schmid, G., Lüdemann, H.-D., & Jaenicke, R. (1979) *Eur. J. Biochem. 97*, 407–413]. In the present study, the unperturbed dissociation of porcine muscle lactic dehydrogenase was proved by cross-linking with glutaraldehyde and subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As taken from pressure-dependent measurements at pressures up to 2 kbar, dissociation and deactivation are completely reversible over the whole transition range. The final product of pressure deactivation is the monomer; i.e., high-pressure deactivation corresponds to high-pressure dissociation. The breakdown of the native quaternary structure occurs at pressures considerably lower than those observed upon denaturation of monomeric enzymes. The reaction volumes for the dissociation of LDH M_4 in the absence and presence of NADH were determined as $\Delta V_{apo} = -500 \pm 50$ mL mol^{-1} and $\Delta V_{holo} = -390 \pm 40$ mL mol^{-1}. Specific solvent conditions are of crucial importance with respect to the dissociation equilibrium. Coenzyme binding (NADH) shifts the dissociation transition to higher pressures; the phosphate ion stabilizes the native tetramer even further, inhibiting dissociation completely at $p \leq 2$ kbar. The dissociation rate at elevated pressure is found to be decreased for the holoenzyme compared to the apoenzyme. For the dissociation of both apo- and holoenzyme large negative activation volumes were determined, reflecting the rate increase with increasing pressure: $\Delta V_{apo}^{\ddagger} = -190 \pm 19$ mL mol^{-1} and $\Delta V_{holo}^{\ddagger} = -231 \pm 2$ mL mol^{-1}.

There are two major reasons for analyzing enzymes under high pressure. Firstly, the depth of a considerable part (~86%) of the marine biosphere is beyond 2000 m, corresponding to $p = 0.2$ kbar,[1] so that pressure adaptation is expected to be an important ecological phenomenon. From this the biological significance of high-pressure studies on enzyme structure and function is obvious. Secondly, valuable information about the determinants of the native backbone and quaternary structure of proteins can be obtained by exploring the pressure-dependent limits of stability. In this regard, high-pressure studies are comparable to denaturation studies by temperature variation, extremes of pH, or high concentrations of guanidine hydrochloride or urea. Compared to the chemical denaturants, pressure is a unique tool to obtain unperturbed thermodynamic and kinetic information characterizing denaturation and/or dissociation equilibria (Hawley, 1978; Heremans, 1978).

High hydrostatic pressure affects the structure and function of enzymes in a complex way. The physicochemical basis of the different effects may be described in terms of structural changes involving exposure of groups, changed affinity for the solvent, or changes in electrostriction accompanying the ionization of amino acid side chains and buffer components (Johnson et al., 1974). In general it is not possible to correlate the given overall effects to specific and well-defined molecular interactions. For a number of monomeric enzymes the reversible high-pressure denaturation has been analyzed under equilibrium conditions on the basis of pressure-induced spectroscopic changes (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973; Li et al., 1976). These reversible denaturation transitions occur at relatively high hydrostatic pressures ($4 > p_{1/2} > 8$ kbar).

In the case of oligomeric enzymes high-pressure experiments were in almost all cases concerned with the effect of high pressure on the rate of the enzymatic reaction at nonlimiting substrate concentrations. This implies that only relatively short incubations in the time range of linear enzyme assays were applied so that slow changes (>1 h) necessarily escaped detection. As mentioned, the pressure effects on enzymes can only in the most simple cases be ascribed to the activation volume of the actual catalytic reaction since changes in backbone or quaternary structure may also be involved. Additional information from independent experiments is required in order to provide a correlation of the apparent activation volume to the different molecular processes.

It has been demonstrated previously (Jaenicke & Koberstein, 1971; Schulz et al., 1976; Schade et al., 1978) that assemblies of proteins may be dissociated under the action of high hydrostatic pressure. In the case of oligomeric enzymes indirect evidence for this dissociation came from the hybridization of isoenzymes of lactic dehydrogenase (Jaenicke & Koberstein, 1971) and from deactivation experiments (Penniston, 1971; Schmid et al., 1975, 1979). The direct verification and the characterization of the final product of dissociation were still lacking.

In the present investigation the extent and the kinetics of the pressure-induced dissociation of porcine muscle lactic dehydrogenase were analyzed in a quantitative way. Dissociation into monomers at high pressure, as verified by chemical cross-linking, was found to be reversible in the range of the pressure-dependent transition. The coenzyme (NADH) and

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[1] Abbreviations used: DTE, 1,4-dithioerythritol; EDTA, (ethylenedinitrilo)tetraacetic acid; k_i, rate constants; LDH, lactic dehydrogenase (EC 1.1.1.27); H_4 and M_4 refer to isoenzymes from heart and skeletal muscle, respectively; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; p and $p_{1/2}$, hydrostatic pressure and pressure of half-dissociation, respectively; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; T, D, and M, tetrameric, dimeric, and monomeric states of LDH.

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phosphate ions show a significant influence on both the equilibrium and the kinetics of dissociation. The dissociation at equilibrium was compared to activity measurements performed directly under high pressure prior to equilibration. In a subsequent paper the kinetics of reactivation and reassociation at atmospheric pressure after high-pressure dissociation will be considered.

Materials and Methods

Substances. Lactic dehydrogenase (EC 1.1.1.27) from pig muscle, NAD^+, and NADH were purchased from Boehringer, Mannheim; DTE and hydrazine were from Roth, Karlsruhe; glutaraldehyde (25% aqueous solution) and $NaDodSO_4$ were from Serva, Heidelberg; human serum albumin was from Behring-Werke, Marburg. All other reagents are of A-grade purity (Merck, Darmstadt). Quartz doubly distilled water was used throughout. The pH of all solutions was set to pH 7.6.

Stock solutions of the enzyme (~2 mg/mL) were prepared by dialysis at 4 °C against 0.2 M Tris-HCl buffer or 0.2 M phosphate buffer containing 1 mM EDTA and 10 mM DTE. Enzyme concentrations were determined spectrophotometrically, based on $A_{280nm}^{0.1\%} = 1.40\ cm^2\ mg^{-1}$ (Jaenicke & Knof, 1968); molar concentrations refer to the subunit molecular weight of 36 000.

Enzyme Assays. Enzyme activity was determined at 366 nm in 0.2 M phosphate buffer containing 1 mM EDTA or in 0.2 M Tris-HCl buffer containing 1 mM EDTA and 10 mM DTE in the presence of 0.74 mM pyruvate and 0.2 mM NADH by using a recording Eppendorf spectrophotometer with thermostated cuvettes (20 °C).

Enzyme assays under high hydrostatic pressure were performed in Tris-HCl buffer in the presence of 1 mM EDTA, 10 mM DTE, 0.3 nM LDH, 0.6 mM NADH, and 0.43 mM pyruvate (20 °C). Under these conditions linear kinetics were obtained for at least 30 min. Since the filling operation of the high-pressure cell and the thermal equilibration take ~3 min, this initial period was recorded at atmospheric pressure. After this time the desired pressure was adjusted and the kinetics were measured during a period of 10 min.

High-Pressure Techniques. (a) High-Pressure Optical Transmission Cell. The high-pressure cuvette illustrated in Figure 1a represents a modification of the cell developed previously (Schmid et al., 1975). Changes refer to the improved reproducibility of the spectrophotometric detection and the facilitated handling of the cell in the spectrophotometer. The cone seal inlet was replaced by an O-ring seal, thus permitting the opening of the autoclave and the refilling of the probe container with the cell mounted on the optical bench of a Gilford 2 400 S single-beam spectrophotometer. The side tube of the Teflon container was connected with a silicon rubber hose, the upper end of the hose being sealed by a glass stopper, to facilitate the filling procedure. This way a rapid and bubble-free filling of the cell is rendered possible; in addition, irreversible deformation of the Teflon cylinder is prevented since the volume changes upon pressure application are balanced solely by the flexible silicon rubber.

(b) Quench Experiments. For determination of the pressure-dependent dissociation, enzyme samples were incubated in an autoclave for defined periods of time. After pressure release the probe was quickly removed and the enzymatic activity was measured at ambient pressure under the conditions of the standard assay. An autoclave was designed which allows the enzymatic assay to be started within 15 s after pressure release (Figure 1b). The separating system was checked for leaks by adding a fluorescent dye to the pressurizing liquid (Schmid et al., 1978). 1,2-Ethanediol was chosen as the

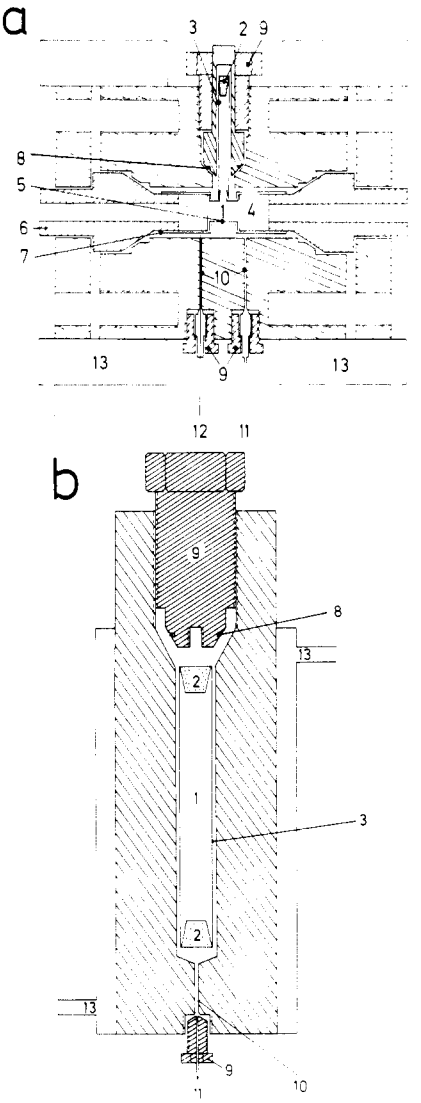

FIGURE 1: (a) High-pressure optical transmission cell for enzymatic tests at elevated pressure. Path length = 12 mm. (b) High-pressure autoclave for quench experiments allowing fast removal of the sample after pressure release. (1) Sample; (2) glass stopper; (3) silicon hose; (4) sapphire window; (5) Teflon cylinder; (6) nipple; (7) window cap; (8) O-ring; (9) sealing screw; (10) holes for thermocouple and oil inlet; (11) connection to press and manometer; (12) thermocouple; (13) brass jacket.

pressure transmitting liquid because of its low compressibility, which keeps temperature changes to a minimum upon variation of the pressure in the autoclave (Schmid, 1977).

Pressure Generation. The pressure generating equipment was the conventional $^1/_8$-in. valve and capillary system with a hydraulic pump supplied by High Pressure Equipment, Erie, PA. Bourdon gauges of quality class 0.6 (A. Wiegand, Klingenberg, West Germany) were used.

Cross-Linking. The degree of pressure-induced dissociation was determined by cross-linking with glutaraldehyde. Cross-linking and subsequent $NaDodSO_4$–polyacrylamide electrophoresis were used to analyze the distribution of monomers and oligomers in the process of dissociation–association, as described by Hermann et al. (1979). The Tris concentration was lowered to 1 mM (pH 7.6) in order to minimize side reactions with the buffer.

Results

Reactivation of porcine muscle LDH after deactivation and dissociation by hydrogen ions is determined by a rate-limiting

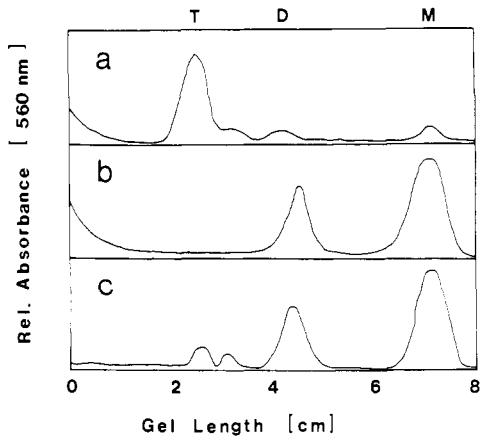

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of porcine muscle lactic dehydrogenase, cross-linked with glutaraldehyde before and after pressure dissociation. Correlation of the bands to tetrameric (T), dimeric (D), or monomeric (M) fractions by calibration with monomeric and dimeric human serum albumin and with monomeric LDH was as described by Hermann et al. (1979). (a) Cross-linked native tetrameric LDH. (b) LDH after 20-min incubation at 2 kbar in 1 mM Tris buffer, pH 7.6, at c = 0.3 μM and t = 20 °C. Cross-linking was performed in less than 15 s after decompression. (c) LDH after 24-h incubation at 1 kbar; conditions were as in (b). The amount of tetramers (~10%) corresponds to the residual activity under equilibrium conditions (cf. Figure 3).

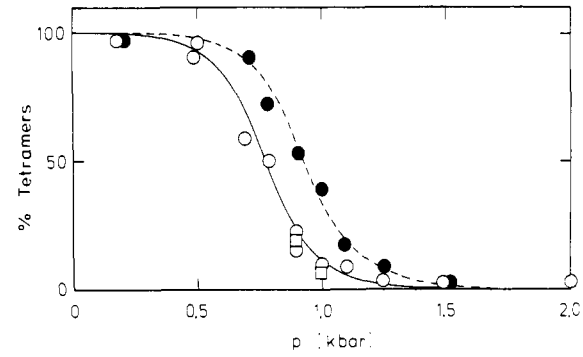

FIGURE 3: Pressure-dependent dissociation equilibrium of apo and holo porcine muscle LDH as determined from the residual activity in quench experiments. Equilibrium values were determined after 24-h incubation at given pressures in 0.2 M Tris buffer, pH 7.6, plus 1 mM EDTA and 10 mM DTE at c = 290 nM and t = 20 °C for the dissociation of native tetramers (○ and ●) and the reassociation of monomers (□), produced by 20-min incubation at 2 kbar. Open symbols refer to apoenzyme; closed symbols refer to holoenzyme formed in the presence of 0.6 mM NADH, under which condition the native enzyme is 99% saturated at atmospheric pressure.

reassociation reaction (Rudolph & Jaenicke, 1976), which implies that the dissociated monomers are enzymatically inactive. This property of the dissociated monomers allows us to determine the pressure-dependent dissociation of LDH simply by determination of the residual activity after pressure release, which should be proportional to the amount of undissociated tetramers. This approach implies that reactivation is slow compared to the time lapse between pressure release and determination of the residual activity and that deactivation is fully reversible. Both requirements are fulfilled for the pressure-dependent dissociation of porcine muscle lactic dehydrogenase: at low enzyme concentration reactivation is slow compared to the time lapse between pressure release and enzymatic test; on the other hand, the pressure-deactivated enzyme can be reactivated to >95% (Schade et al., 1980). Because of the high dilution under test conditions, we can exclude reactivation during the test.

It has to be verified that the product of the pressure-dependent deactivation is actually monomeric. As shown by recent reconstitution studies on LDH (Hermann et al., 1979), cross-linking with glutaraldehyde and subsequent gel electrophoresis can be used to characterize the amount and size of independent species in a heterogeneous population of different states of association. Under the given experimental conditions the specificity of the cross-linking reaction is greater than 90%, as determined with native, tetrameric enzyme (Figure 2a). The enzyme was incubated at 2 kbar for 20 min to analyze the final state of dissociation by this method; immediately after pressure release (<15 s) cross-linking was performed (Figure 2b). It is evident that at the time of cross-linking 65% of the enzyme molecules are still monomeric, while 35% represent dimers, which might be the product of reassociation during the time lapse between decompression and cross-linking. Aside from the equilibrium amount of tetramers (as determined from the residual activity), roughly the same proportion of monomers and dimers can be observed upon incubation for 24 h at 1 kbar (Figure 2c). It can be concluded that in the given pressure range deactivation of lactic dehydrogenase is paralleled by the dissociation of the enzyme into monomers. This dissociation is not caused by pressure-induced chemical modification of some functional groups (Schmid et al., 1978), since dissociation and deactivation are perfectly reversible upon decompression (Schade et al., 1980). Although complete reactivation can be observed after dissociation by 20-min incubation at 2 kbar, some irreversibility occurs upon prolonged incubation at p > 1.5 kbar (cf. Table I). This irreversible deactivation may be caused by the same aggregation process observed earlier during prolonged pressurization of LDH from rabbit muscle (Schmid et al., 1979). Reactivation after pressure-dependent dissociation is characterized by a second-order rate-limiting reaction similar to that observed for the reactivation of acid-denatured monomers (Schade et al., 1980). This finding corroborates the conclusion from the cross-linking experiments that the pressure-induced deactivation (like acid deactivation) is caused by dissociation into inactive monomers (inactive within the error range of reconstitution experiments). We may assume that this dissociation can be measured by determining the residual activity immediately after decompression, provided that the enzyme concentrations are sufficiently low so that reassociation (and reactivation) is slow compared to the time lapse between pressure release and the enzymatic test. In contrast to the dissociation at low pH or at high concentrations of guanidine hydrochloride or urea, where preferential aggregation interferes with the dissociation equilibrium (Zettlmeissl et al., 1979), the pressure-dependent dissociation is reversible in the range of transition (Figure 3). Experimentally this has been shown in the followng way: enzyme was completely dissociated by applying 2.0 kbar for 20 min; then the pressure was lowered to the transition range. After 24 h of incubation at these pressures, enzymatic tests were performed immediately after pressure release. Within an error limit of 5%, identical results are obtained regardless of whether the enzyme was previously pressurized to 2 kbar or brought to the final pressure immediately from ambient pressure.

Since the pressure-induced dissociation equilibrium of LDH from porcine muscle is not perturbed by side reactions such as aggregation, reactivation yields of >95% are observed (Schade et al., 1980). Formation of the binary complex with reduced coenzyme, NADH, results in a stabilization of the native, tetrameric structure of the enzyme against pressure-dependent dissociation (Figure 3). The pressure of half-dissociation, $p_{1/2}$, is shifted from 0.76 kbar for the apoenzyme to 0.91 kbar for the holoenzyme: $\Delta p_{1/2}$ = 0.15 kbar.

Table I: Stabilization of the Native Tetrameric Structure of Porcine Muscle LDH by Phosphate Ions[a]

incubn pressure (kbar)	% tetramers[b] after 20-min incubn in	
	Tris-HCl	phosphate
10^{-3}	100	100
0.5	98	100
1.0	77	100
1.25	30	100
1.5	0	100
2.0	0	100

[a] Comparison of the relative amount of residual tetramers as determined from the enzymatic activity after a 20-min incubation at the given pressures. The buffer solutions (0.2 M, pH 7.6) contain 1 mM EDTA and 10 mM DTE; incubation temperature was 20 °C. [b] Inactive material consists of reversibly dissociated monomers which reassemble completely upon decompression to 1 bar. With increasing pressure or prolonged incubation at $p > 1.5$ kbar, irreversible deactivation occurs, the rate of which is decreased in phosphate compared to Tris-HCl. 24-h incubation at 2 kbar leads to a complete and irreversible loss of activity; this occurs slightly faster in Tris-HCl than in phosphate.

Previous systematic studies regarding the effect of ions on the activity and long-term stability of lactic dehydrogenase (Rudolph & Jaenicke, 1976) have shown that phosphate exhibits a stabilizing effect on the enzyme. This effect holds especially for the deactivation under high pressure. Replacing the standard Tris-HCl buffer used in the foregoing experiments by phosphate buffer of equal pH and ionic strength causes a drastic increase in enzyme stability such that in the given pressure range (≤2 kbar) no deactivation is detectable (Table I). Even after 18-h incubation at 1.5 kbar the residual activity in the presence of phosphate still amounts to 75% of the initial activity.[2]

Reconstitution of the tetrameric structure of LDH must include at least two association steps, i.e., the two association reactions of monomers to dimers and of dimers to tetramers. Including the respective first-order dissociation reactions of the tetramers and the dimers, we may write the following scheme for the dissociation equilibrium of the enzyme:

$$T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} 4M \qquad (1)$$

At complete equilibrium we can write for the individual equilibria

$$[D]^2/[T] = k_1/k_{-1} \qquad (2a)$$

$$[M]^2/[D] = k_2/k_{-2} \qquad (2b)$$

$$[M]^4/[T] = k_1k_2^2/(k_{-1}k_{-2}^2) \qquad (3)$$

i.e., the equilibrium constant K is defined as

$$K = [M]^4/[T] \qquad (4)$$

The cross-linking of LDH M_4 after incubation at 1 or 2 kbar, as well as the transition curves from the tetrameric state present at 1 bar to monomers observed at 1 to 2 kbar (Figures 2 and 3), gives no evidence for the accumulation of a dimeric intermediate at any stage. Therefore, we can neglect dimers and calculate the monomer concentrations at equilibrium directly from the percentage of tetramers given in Figure 3.

[2] A similar stabilizing effect has been observed in the case of the pressure-dependent dissociation of glutamic dehydrogenase (K. Müller, B. Schade, and R. Jaenicke, unpublished experiments).

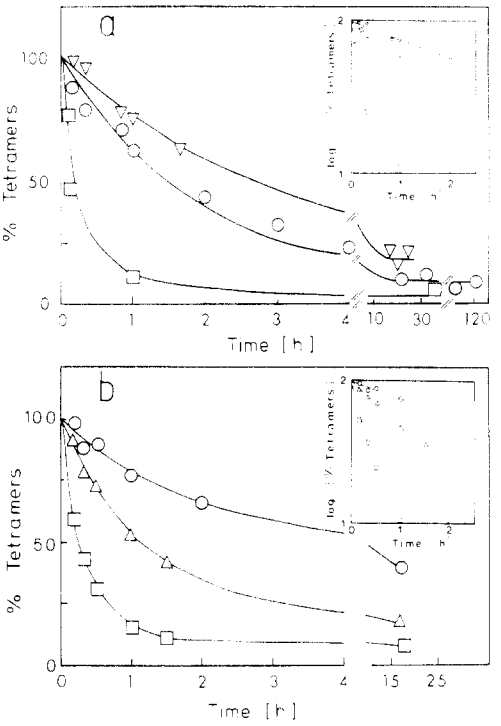

FIGURE 4: Dissociation kinetics of apo and holo porcine muscle LDH upon incubation at selected pressures in the range of dissociation as determined from the residual activity at defined times in quench experiments. Equilibration was at the following pressures (kbar): 0.9 (▽), 1 (○), 1.1 (△), and 1.25 (□) in 0.2 M Tris buffer, pH 7.6, plus 1 mM EDTA and 10 mM DTE, t = 20 °C, at c = 290 nM. Inserts: determination of the rate constants of dissociation, k_1, by first-order linearization; for numerical values, cf. Table II. (a) Apoenzyme. (b) Holoenzyme, formed in the presence of 0.6 mM NADH.

The volume changes for the dissociation of tetrameric LDH into monomers (ΔV) can be estimated from the pressure dependence of the equilibrium constant K according to

$$\Delta V = -RT\frac{d(\ln K)}{dp} \qquad (5)$$

The respective reaction volumes for the dissociation of apo- and holo-LDH M_4, as taken from the slopes of an ln K vs. p plot, are $\Delta V_{apo} = -500 \pm 50$ mL mol^{-1} and $\Delta V_{holo} = -390 \pm 40$ mL mol^{-1}.

The time dependence of the dissociation was determined by incubating native LDH at a given pressure for defined times and determination of the residual activity immediately after decompression. The dissociation kinetics of both apo- and holoenzyme at pressures in the range of the dissociation transition are illustrated in Figure 4.

Reactivation of LDH is mainly governed by a second-order reaction, independent of the extent of the preceding high-pressure deactivation (Schade et al., 1980). Accordingly, within the transition range it is not possible to linearize the time dependence of the pressure-induced deactivation in a first-order semilogarithmic plot. Since at the beginning of deactivation the concentration of dissociated species is small, the reverse reaction (as a second-order reaction) may be neglected. Therefore, linearization of the initial phase according to first-order kinetics will give a reasonable approximation of k_1 (Figure 4, insert). The respective rate constants derived from this linearization are given in Table II.

At a given pressure the rate constants of dissociation are decreased in the presence of NADH. The dissociation rate of both apo- and holoenzyme is greatly enhanced with increasing pressure. The activation volume (ΔV^*) of this dis-

Table II: Rate Constants (k_1) of the Pressure-Induced Dissociation of Porcine Muscle LDH in the Range of Reversible Dissociation[a]

	$k_1 \times 10^4$ (s^{-1})	
p (kbar)	apoenzyme	holoenzyme (+0.6 mM NADH)
0.9	0.78 ± 0.03	
1.0	1.4 ± 0.5	0.67 ± 0.20
1.1		1.7 ± 0.3
1.25	11.0 ± 2.0	6.9 ± 1.0

[a] k_1 was determined by first-order linearization of the initial rates of the dissociation kinetics (Figure 4, inserts).

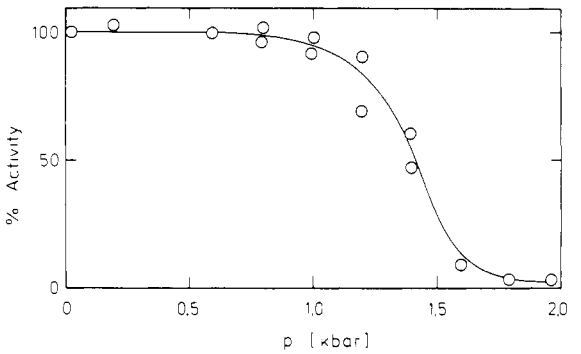

FIGURE 5: Enzymatic properties of porcine muscle LDH determined under high hydrostatic pressure. Enzymatic activity at the given pressure was determined in the high-pressure optical transmission cell (cf. Figure 1a) as described under Materials and Methods.

sociation reaction can be estimated from the pressure dependence of k_1 according to

$$\Delta V^{\ddagger} = -RT\frac{d(\ln k_1)}{dp} \quad (6)$$

The respective $\Delta V^{\ddagger}$ values, taken from the slopes of an ln k_1 vs. p plot, are $\Delta V_{apo}^{\ddagger} = -190 \pm 19$ mL mol^{-1} and $\Delta V_{holo}^{\ddagger} = -231 \pm 2$ mL mol^{-1}.

Using the transmission cell shown in Figure 1a, we can determine the enzymatic activity directly under pressure. However, these values are not the activities at equilibrium but apparent activities of native LDH at a given pressure, 3–10 min after pressurization. During this period, the enzymatic activity is not affected by pressures below 0.8 kbar (Figure 5). A further increase in pressure causes the activity to be decreased; complete deactivation is reached at 1.8 kbar. Varying the substrate concentration shows that significant changes in K_m do not occur unless the pressure exceeds 0.8 kbar; V_{max} remains unchanged under the given conditions of pressure and incubation time (Schade, 1979).

It cannot be decided whether the deactivation observed at $p > 0.8$ kbar reflects the onset of dissociation or pressure effects on the actual catalytic reaction.

Discussion

Available high-pressure data on lactic dehydrogenase from earlier investigations are based on three types of experiments: activity measurements in crude extracts (Moon et al., 1971; Gillen, 1971), high-pressure enzyme assays with the purified enzyme (Schmid et al., 1975, 1978), and hybridization of isoenzymes (Jaenicke & Koberstein, 1971). In view of the present results, all three approaches may be subject to artefacts caused by undefined experimental conditions, as well as systematic errors in the evaluation of the results. In connection with the slow kinetics of deactivation and dissociation reported here, this holds especially for the short-term incubation method underlying the enzymatic assay under conditions of high pressure, since there is no way of deciding whether or not the observed effects refer to an equilibrium. High-pressure deactivation of LDH had been attributed to dissociation into subunits, and the occurrence of all three hybrids aside from the homogeneous isoenzymes during high-pressure hybridization was considered to corroborate this hypothesis (Jaenicke & Koberstein, 1971). Since hybridization gives only indirect evidence for dissociation, an independent test for the equilibrium distribution between native tetramers and dissociated enzyme was developed. If deactivation is actually caused by dissociation into inactive monomers, then the amount of tetramers at equilibrium should be equivalent to the activity determined immediately after decompression. Reassociation to native tetramers during the time lapse between pressure release and the enzymatic test can be neglected since this reaction is slow, as determined by reactivation (Schade et al., 1980).

The dissociation of LDH at high pressures, suggested by the hybridization experiments, was finally established by covalent cross-linking and gel electrophoresis immediately after pressure release (Figure 2). The kinetics of reactivation after pressure deactivation, which are virtually identical with those observed after acid dissociation into monomers, corroborate these findings (Schade et al., 1980). The dissociation reaction of LDH M_4 is reversible within the transition range (Figure 3), and the yield of reconstitution after pressure dissociation is very high (>95%). Previous attempts to realize equilibrium conditions in the process of deactivation–reactivation of LDH and various other oligomeric enzymes failed because of side reactions involving irreversible denaturation (Rudolph, 1977; Jaenicke, 1978). In these studies the denaturation–renaturation conditions had to be far off the transition range to achieve maximum yields. The reason for this is that the ratio of renaturation and "irreversible" aggregation is kinetically determined by the relative rates of these processes and depends on the extent of denaturation and on the concentration of the enzyme in the process of reconstitution (Jaenicke, 1979; Rudolph et al., 1979).

Considering the reversibility of the pressure dissociation over the whole transition range, we may conclude that in this case the monomers (which are at equilibrium with the tetramers inside this transition) are more structured than those monomers produced by acid or guanidine hydrochloride. Further studies are required to characterize the monomers at high pressure in greater detail. The same holds for the irreversible loss of their capacity to reactivate upon prolonged incubation at high pressure. This irreversibility might be caused by slow pressure-induced aggregation, as observed in the case of LDH from rabbit muscle (Schmid et al., 1979).

Comparison of the pressure denaturation and dissociation in Tris-HCl and in phosphate buffer of equal ionic strength proves that the phosphate ion exercises a strong stabilizing effect on the structure and function of native LDH. While in 0.2 M Tris-HCl or 0.2 M KCl 20-min incubations at 2 kbar are sufficient to completely deactivate the enzyme, no deactivation is observed in phosphate buffer (Table I). The stabilization of the native structure of globular proteins by phosphate or sulfate ions is a well established fact (von Hippel & Wong, 1965). For LDH from dog fish, X-ray crystallographic analysis (Adams et al., 1973) revealed specific binding sites for bivalent ions in the vicinity of the active center and between the subunits. It may be assumed that these sites can also be occupied by the phosphate ion which in the given pH range is bivalent too and of comparable size. The shift of the

characteristic pressure of half-dissociation by 0.15 kbar toward higher pressure upon formation of the binary complex with NADH corroborates the stabilization of the backbone and quaternary structure of LDH upon coenzyme binding, which has been frequently reported in earlier investigations (Holbrook et al., 1975).

The dissociation rates at respective pressures are found to be decreased for the holoenzyme as compared to the apoenzyme (Figure 4). The large negative activation volumes determined for the dissociation for both apo- and holoenzyme reflect the rate increase with increasing pressure.

Assuming that the volumes of the dissociated states do not depend on the presence of the coenzyme, the smaller reaction volume of the holoenzyme as compared to the apoenzyme ($\Delta\Delta V$ = 110 mL mol^{-1}) suggests a volume contraction accompanying NADH binding to the enzyme. This finding is supported by sedimentation data and measurements of the partial specific volume (Jaenicke et al., 1979).

Determination of enzymatic activity at $p \approx 1$ kbar shows that under this condition, i.e., during the relatively short time span of the enzymatic assay, the enzyme is still perfectly active, though after establishment of equilibrium it turns out to be inactive and dissociated (Figure 5). It cannot be decided whether the deactivation observed in these experiments is caused by the onset of dissociation or by volume effects on the catalytic reaction. A discussion of this deactivation in terms of its biological relevance is obviously futile since these values do not represent the final equilibrium state. For porcine muscle LDH the dissociation equilibrium is characterized by a pressure of half-dissociation $p_{1/2}$ = 0.76 and 0.91 kbar for the apo- and holoenzyme, respectively. Obviously, dissociation of this enzyme occurs at considerably lower pressure than the denaturation reactions observed for a number of monomeric proteins which are characterized by $p_{1/2}$ = 4–8 kbar [cf. Li et al. (1976) and Hawley (1978)].

The hypothesis that successful colonization of deeper marine regions necessitates pressure adaptation of the enzymatic properties of LDH (Siebenaller & Somero, 1978) seems unlikely if we consider that the porcine enzyme (i.e., an enzyme from an organism never subjected to pressure adaptation) is perfectly stable and active up to ~0.5 kbar. Since monomeric enzymes denature at even higher pressures, we may propose that pressure adaptation is not primarily regulated on an enzyme level.

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