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THERMODYNAMICS AND MECHANISM OF HIGH-PRESSURE DEACTIVATION AND DISSOCIATION OF PORCINE LACTIC DEHYDROGENASE

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Lactic dehydrogenase (LDH) from pig heart and pig skeletal muscle can be reversibly dissociated into monomers at high hydrostatic pressure. The reaction can be quantitatively fitted by a reversible consecutive dissociation-unfolding mechanism according to $N \rightleftharpoons 4M \rightleftharpoons 4M^*$ (where N is the native tetramer, and M and M* two different conformations of the monomer) (K. Müller, et al., Biophys. Chem. 14 (1981) 101.). At $p \leqslant 1$ kbar, the pressure deactivation of both isoenzymes (H_4 and M_4) is described by the two-state equilibrium $N \rightleftharpoons 4M$. From the respective equilibrium constant and the temperature and pressure dependence of the change in free energy, the thermodynamic parameters of the dissociation/deactivation may be determined, e.g., for LDH-M_4: $\Delta G_{Diss} = 110$ kJ/mol, $\Delta S_{Diss} = -860$ J/K per mol, $\Delta H_{Diss} = -124$ kJ/mol (enzyme concentration 10 μg/ml, in Tris-HCl buffer, pH 7.6, $I = 0.16$ M, 293 K, 0.8 kbar); the dissociation volume is found to be $\Delta V_{Diss} = -420$ ml/mol ($0.7 < p < 0.9$ kbar). Measurements using 8-anilino-1-naphthalenesulfonic acid (ANS) as extrinsic fluorophore demonstrate that the occurrence of hydrophobic surface area upon dissociation parallels the decrease in reactivation yield after pressurization beyond 1 kbar. Within the range of reversible deactivation ($p < 1$ kbar) no increase in ANS fluorescence is detectable, thus indicating compensatory effects in the process of subunit dissociation. ^2H_2O is found to stabilize the enzyme towards pressure dissociation, in accordance with the involvement of hydrophobic interactions in the subunit contact of both isoenzymes of LDH.

1. Introduction

In previous communications from this laboratory, high-pressure studies on dimeric and tetrameric LDH were reported [1–5]. It was shown that in the case of tetrameric pig heart LDH, there exists a two-step mechanism of high-pressure denaturation. At pressures below 1.0 kbar, completely reversible dissociation to catalytically inactive monomers is observed, whereas beyond 1.0 kbar, conformation changes in the dissociated state lead to reduced reactivation yields after decompression to normal atmospheric pressure [4]. The present investigation is concerned with the tetrameric LDH from pig skeletal muscle which has been shown previously to obey a simple unimolecular (bimolecular) dissociation (reconstitution) scheme [6,7].

Upon deactivation at pressures up to 2 kbar the two above-mentioned modes of high-pressure denaturation occur. Making use of the pressure and temperature dependence of the equilibrium of deactivation at $p < 1$ kbar, the thermodynamic parameters ΔG_{Diss}, ΔV_{Diss} and ΔS_{Diss} may be determined. They provide some insight into the energetics of high-pressure dissociation, denaturation and deactivation. Measurements using ANS as extrinsic fluorophore demonstrate that the occurrence of hydrophobic surface areas in the dissoci-

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Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; DTE, dithioerythritol; LDH, lactic dehydrogenase (EC 1.1.1.27, L-lactate:NAD^+ oxidoreductase); H_4 and M_4 refer to isoenzymes from heart and skeletal muscle, respectively.

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ated monomers parallels the decrease in reactivation yield after pressure deactivation above 1.0 kbar. These results together with the solvent isotope effect in the presence of ^2H_2O give insight into the mechanism of high-pressure dissociation of oligomeric enzymes.

2. Materials and methods

LDH-H_4 from pig heart and LDH-M_4 from pig skeletal muscle, NADH and NAD^+ were purchased from Boehringer (Mannheim); DTE was obtained from Roth (Karlsruhe), ANS (magnesium salt), from Serva (Heidelberg). All other reagents were of A-grade purity (Merck, Darmstadt), ^2H_2O (99.7%, Merck, Darmstadt) and water used for buffer solutions were quartz bidistilled.

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 [8]. 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 and 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.

2.1. High-pressure techniques

Quench experiments were performed according to Schade et al. [2]. Fluorescence emission under high pressure was measured as described previously [4]. Details regarding the time and pressure of incubation at 20°C are given in the text. The solvent used was carefully deaerated Tris-HCl buffer ($I = 0.16$ M) containing 1 mM EDTA and 10 mM DTE. The pH was kept constant at pH 7.6. The enzyme concentration during high-pressure incubation was 0.72 μM in all experiments. Acid denaturation was performed in 0.1 M potassium phosphate, pH 2.0, in the presence of 0.1 mM EDTA and 1 mM DTE.

3. Results

3.1. Thermodynamics of high-pressure dissociation of LDH-M_4

Fig. 1 depicts the pressure-dependent deactivation and reactivation of LDH-M_4 at various temperatures between 12.5 and 35.0°C.

Cross-linking as well as hybridization experiments with LDH-M_4 and LDH-H_4 have shown that high-pressure deactivation of the two isoenzymes below 1.0 kbar can be described by the two-state equilibrium

$$N \rightleftharpoons 4M \tag{1}$$

with N and M representing the native tetramer and the dissociated subunit, respectively [2,4]. Since only N is catalytically active, absolute concentrations of N and M can be determined by activity measurements at atmospheric pressure *. The equilibrium constant of deactivation/reactivation according to eq. 1 is defined by

$$K_{eq}(p, T) = \frac{(c_{M,eq})^4}{(c_{N,eq})} \tag{2}$$

The corresponding free energies of dissociation may be calculated according to eq. 3

$$\Delta G^\circ_{Diss} = -RT \ln K_{eq} \tag{3}$$

Volumes and entropies of dissociation are obtained from the pressure and temperature dependence of the free energy:

$$\Delta V^\circ_{Diss} = \left(\frac{\partial \Delta G^\circ_{Diss}}{\partial p}\right)_T, \quad \text{and} \quad \Delta S^\circ_{Diss} = -\left(\frac{\partial \Delta G^\circ_{Diss}}{\partial T}\right)_p \tag{4}$$

The dissociation enthalpy ΔH_{Diss} follows from the Gibbs-Helmholtz equation

$$\Delta H^\circ_{Diss} = \Delta G^\circ_{Diss} + T\Delta S^\circ_{Diss} \tag{5}$$

* As shown previously [2–5], the reassociation in the concentration range applied is slow in comparison with the duration of the enzyme assay.

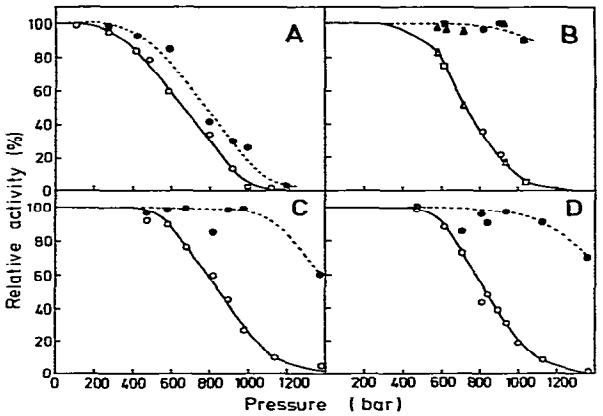

Fig. 1. Pressure-dependent deactivation and reactivation of LDH-M_4. Tris-HCl buffer, $I = 0.16$ M, in the presence of 1 mM EDTA and 10 mM DTE. Enzyme concentration: 10 μg/ml (0.29 μM). Open symbols: residual enzymatic activity after incubation (=24 h) at given pressures; closed symbols: final value of reactivation at ambient pressure after deactivation by incubation (≤24 h) at given pressures. Blank experiments at 20°C (B) prove the pressure dependence of the pH of Tris-HCl buffer to be insignificant. (A) 12.5°C, pH 7.8; $p_{1/2}$ = 670 bar. (B) 20.0°C, pH 7.8 (○, ●), pH 7.6 (□, ■), pH 7.2 (△, ▲); $p_{1/2}$ = 730 bar. (C) 27.5°C, pH 7.4; $p_{1/2}$ = 830 bar. (D) 35.0°C, pH 7.2; $p_{1/2}$ = 860 bar.

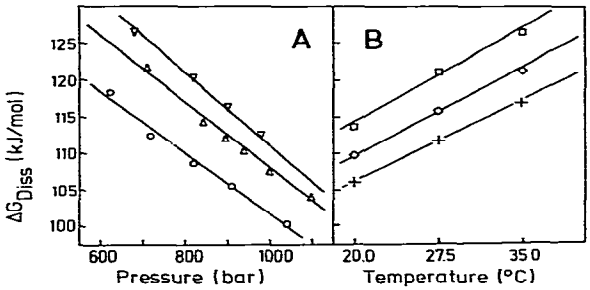

Fig. 2. Determination of the volume and entropy of dissociation of LDH-M_4. Calculated on the basis of a two-state equilibrium M_4 (active)⇌4M (inactive). (A) Dissociation volume at varying temperature. (○) 20.0°C, $\Delta V_{Diss} = -420 \pm 40$ ml/mol; (△) 27.5°C, $\Delta V_{Diss} = -460 \pm 40$ ml/mol; (▽) 35.0°C, $\Delta V_{Diss} = -500 \pm 50$ ml/mol. (B) Dissociation entropy at varying pressure. (□) 700 bar, $\Delta S_{Diss} = -0.87 \pm 0.08$ kJ/K per mol; (◇) 800 bar, $\Delta S_{Diss} = -0.80 \pm 0.08$ kJ/K per mol; (+) 900 bar, $\Delta S_{Diss} = -0.73 \pm 0.08$ kJ/K per mol.

Fig. 2 illustrates the determination of ΔV_{Diss} and ΔS_{Diss}. The complete set of thermodynamic data obtained from the physicochemical analysis of the deactivation/reactivation profiles (fig. 1) is summarized in table 1. Four basic characteristics deserve consideration:

(i) Catalytic activities observed after high-pressure incubation at 12.5°C cannot be used for thermodynamic calculations, since the deactivation is not completely reversible at this temperature.

(ii) Both the volume and entropy of dissociation are negative.

(iii) The dissociation/association equilibrium exhibits entropy-enthalpy compensation [9]. Association is an entropy-driven process; on the other hand, dissociation is enthalpy driven.

(iv) Since the free energy is a function of state, its mixed second derivatives with respect to pressure and temperature must be identical. Within the range of error this holds true; both quantities are close to zero:

$$\left(\frac{\partial}{\partial T}\left(\frac{\partial \Delta G_{Diss}}{\partial p}\right)_T\right)_p = \left(\frac{\partial \Delta V_{Diss}}{\partial T}\right)_p = -5 \pm 6 \text{ ml/K per mol}$$

$$\left(\frac{\partial}{\partial p}\left(\frac{\partial \Delta G_{Diss}}{\partial T}\right)_p\right)_T = -\left(\frac{\partial \Delta S_{Diss}}{\partial p}\right)_T = -7 \pm 8 \text{ ml/K per mol} \quad (6)$$

3.2. ANS binding to LDH-H_4 and LDH-M_4 after denaturation at high pressure and acidic pH

As mentioned above, reactivation of LDH is incomplete after pressure incubation at $p > 1.0$ kbar. Insights into the mechanism of both the reversible and irreversible part of the reaction underlying this observation may be expected from ANS-binding studies. ANS is known to bind specifically to hydrophobic areas in the surface of a protein which are exposed to the aqueous solvent. Upon binding, the fluorophore shows a drastic enhancement of its fluorescence intensity [10–12]. This property was used previously to follow the denaturation of LDH at acidic pH [13].

Fig. 3 shows the kinetics of ANS binding to LDH-H_4 and LDH-M_4 at $p = 900$ bar, followed by a second incubation at $p = 1500$ bar. Intensities are given relative to the denaturation of LDH at

Table 1

Thermodynamic parameters of the pressure-dependent dissociation of LDH-M_4

$c \approx 10$ μg/ml in Tris HCl buffer, pH 7.6, $I = 0.16$ M, in the presence of 1 mM EDTA and 10 mM DTE. Dissociation volume: 293.1 K, $\Delta V_{Diss} = -420 \pm 40$ ml/mol; 300.6 K, $\Delta V_{Diss} = -460 \pm 40$ ml/mol; 308.1 K, $\Delta V_{Diss} = -500 \pm 50$ ml/mol.

Pressure (bar)	Temperature (K)	ΔG_{Diss} (kJ/mol)	ΔS_{Diss} (J/K per mol)	ΔH_{Diss} (kJ/mol)
700	293.1	114	−870	−141
	300.6	121	−868	−140
	308.1	127	−870	−141
800	293.1	110	−800	−124
	300.6	116	−800	−124
	308.1	121	−800	−125
900	293.1	106	−730	−108
	300.6	112	−725	−107
	308.1	117	−730	−108

acidic pH under otherwise identical conditions of high-pressure incubation (fig. 4) (cf. ref. 13). In general, the duration of pressure application was chosen such that the incubation time exceeded the time required to reach the equilibrium of dissociation/association [4]. The presence of ANS is found to have no effect on the yield of reactivation after pressure deactivation. For example, in the case of LDH-H_4, the reconstitution yield after sequential pressurization at 900 bar (1 h), and 1500 bar (1 h) in the absence of ANS and in the presence of 100 μM ANS amounts to 20 and 16%, respectively. Artefacts of ANS binding to the enzyme can therefore be excluded. As taken from the unchanged fluorescence at $p < 1$ kbar (fig. 3), there is no significant increase in hydrophobic surface area upon subunit dissociation of both LDH isoenzymes as long as irreversible denaturation is avoided. At $p > 1.0$ kbar, the increase in ANS fluorescence reflects the exposure of hydrophobic residues, indicating conformational changes within the pressure-deactivated monomers.

This result supports the equilibrium scheme

$$N \underset{}{\overset{p<1.0\ \text{kbar}}{\rightleftharpoons}} 4M \overset{p>1.0\ \text{kbar}}{\rightleftharpoons} 4M^* \qquad (7)$$

which has been previously suggested from high-pressure equilibrium studies on the same enzyme [4].

The occurrence of two conformers of the enzyme with differing ANS fluorescence allows us to interpret the two pressure ranges with different reversibility of pressure deactivation. Below $p = 1.0$ kbar, there is no net increase in water-accessible hydrophobic surface regions; groups which may become exposed upon subunit dissociation are buried again, thus causing the equilibrium

$N \rightleftharpoons 4M$

to be fully reversible. At $p > 1.0$ kbar, the increase in ANS fluorescence corresponding to the increase in ANS binding to the dissociated monomers indicates a significant exposure of apolar areas. As a

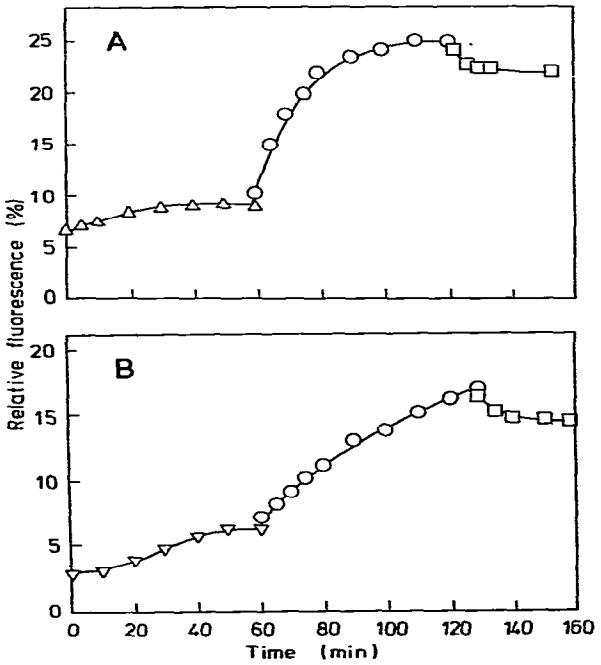

Fig. 3. ANS binding to pressure-dissociated LDH-M_4 (A) and LDH-M_4 (B) (20°C). Enzyme concentration, 25 μg/ml (0.72 μM); ANS concentration, 100 μM. Time dependence of the ANS fluorescence emission at 490 nm ($\lambda_{exc} = 390$ nm); relative fluorescence intensity normalized to the fluorescence of ANS bound to acid-denatured LDH at the given pressure (cf. fig. 4); corrected for the ANS fluorescence in pure buffer. (△) 900 bar, (▽) 1200 bar, (○) 1500 bar, (□) 1 bar (after decompression).

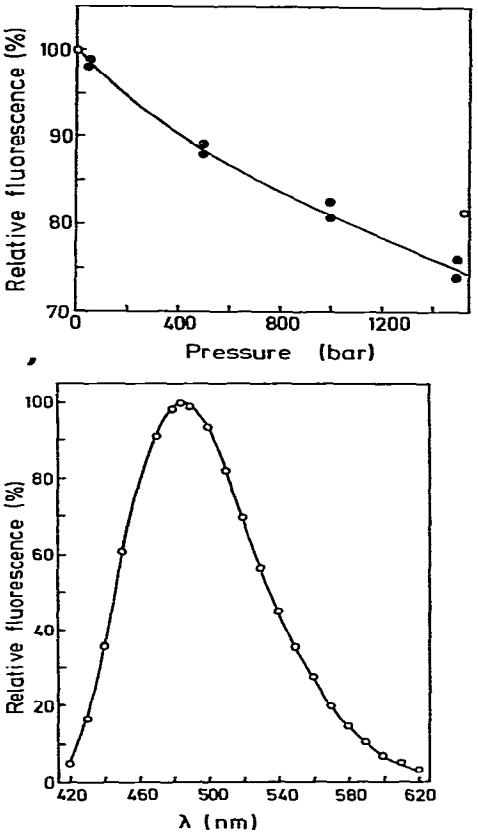

Fig. 4. Fluorescence of ANS bound to acid-denatured LDH-H_4 and LDH-M_4. 0.1 M potassium phosphate buffer, pH 2.0, plus 0.1 mM EDTA and 1 mM DTE; 20°C. Enzyme concentration, 25 μg/ml (0.72 μM); ANS concentration, 100 μM. (A) Apparent pressure dependence of the relative fluorescence emission at 490 nm ($\lambda_{exc}=390$ nm). (○) LDH-H_4, (●) LDH-M_4. Full line represents the reference (100%) used in fig. 3. (B) Relative fluorescence of LDH-H_4 and LDH-M_4 at 1 bar.

consequence, unspecific aggregation of denatured subunits occurs causing the yield of reactivation to be reduced. The situation resembles the hysteresis phenomenon after acid dissociation [14] where metastability in the transition range of dissociation/association has been found to affect the yield of reactivation under quasi-physiological conditions in a similar way.

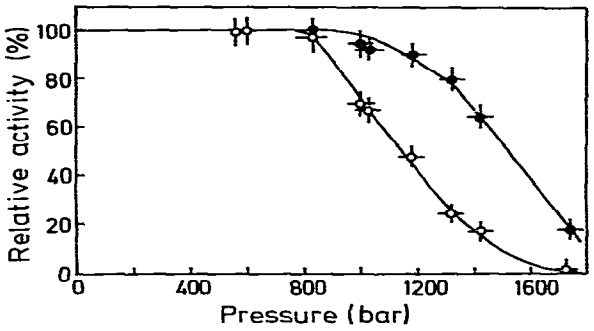

Fig. 5. Pressure-dependent deactivation and reactivation of LDH-M_4 in deuterated buffer. ^{2}H-Tris-HCl buffer, in ^2H_2O, pD 7.6 (corresponding to pH 7.2), $I=0.16$ M; in the presence of 1 mM EDTA and 10 mM DTE; enzyme concentration, 10 μg/ml (0.29 μM); 20°C. (○) Deactivation by 24 h incubation at given pressure. (●) Reactivation yield of (○) after 24 h reactivation at ambient pressure.

As taken from the foregoing results, hydrophobic hydration cannot be the major driving force of dissociation. Solvation of apolar amino acid side chains may only account for the structural changes of the enzyme at $p > 1.0$ kbar.

3.3. Pressure-dependent deactivation and reactivation of LDH-M_4 in deuterated buffer solution

Fig. 5 illustrates pressure-dependent deactivation and reactivation of LDH-M_4 in ^2H_2O/Tris buffer at 20°C. Comparing the data with the profiles obtained in H_2O/buffer solutions, it is obvious that the change $H_2O \rightarrow {}^2H_2O$ (cf. fig. 1B) yields significant stabilization against high-pressure dissociation. This finding, which has been shown to hold also for other proteins [15,16], suggests the stability of the native quaternary structure in ^2H_2O to exceed the stability in H_2O.

4. Discussion

High hydrostatic pressure in the biologically relevant pressure range ($p \leq 1$ kbar) affects the state of association of oligomeric proteins without perturbing the covalent backbone structure [17].

Therefore, the discussion of the present thermodynamic data will deal only with the pressure dependence of the noncovalent interactions stabilizing the native structure of proteins. In connection with the fluorescence labelling experiments using ANS, hydrophobic subunit interactions are of special interest [10–12]. Previous results have shown that the volume and entropy changes characterizing the unfolding of single-chain proteins [18–20] or the dissociation of oligomeric proteins are small considering the number of pair interactions involved. This observation has been generally explained by the assumption that contributions of different sign partially compensate each other yielding small net effects. The negative reaction volume characterizing the unfolding of proteins originates from the hydration of amino acid side chains exposed to the aqueous solvent upon denaturation. As shown for ribonuclease [18], contractions of the proteins in the process of (partial) unfolding do not contribute significantly to the negative denaturation volume. On the other hand, exposure of polar and/or ionic groups is expected to cause a drastic (negative) volume change due to the increased dipole-dipole and ion-dipole interactions accompanying the increase in polar surface area. The corresponding decrease in the degrees of freedom of the solvent molecules gives rise to a strong negative entropy. As taken from measurements of small inorganic ions (e.g., mercury halides), this has been estimated to be of the order of -40 J/K per mol per ion pair [21–23].

Exposure of hydrophobic residues is known to promote 'clathrate' formation in the aqueous solvent giving rise to a decrease in entropy [24,25]. The dissociation volume of aliphatic hydrophobic pairs in water is still controversial regarding its sign and absolute value. The respective value for the unstacking of aromatic residues is $\Delta V_{Diss} = +5$ ml/mol [26–29]. Unfortunately, there are no model systems available which would allow the unambiguous interpretation of the excess volumes of aliphatic hydrophobic substances in water, because the standard state for the hydrophobic pair association in water is experimentally inaccessible. Similarly, the nature of the elementary processes causing the decrease in the excess volume with increasing concentration of the hydrophobic component is still unresolved [30,31]. Ascribing a positive volume effect to the dissociation of hydrophobic 'bonds', the small negative volume of denaturation may be explained by compensatory volume effects of the hydration of charged (polar) groups on the one hand, and hydrophobic residues on the other. This explanation is corroborated by the fact that no ANS binding to LDH is detected upon pressure dissociation of the enzyme at $p < 1.0$ kbar. The significant amount of ANS binding beyond this pressure range does not allow the conclusion that hydrophobic solvation is the driving force of the observed effect. This is clearly shown by the close similarity of the high-pressure ANS binding to the effects observed after acid dissociation and denaturation of the enzyme.

^2H_2O is found to stabilize LDH towards pressure dissociation. The effect cannot be explained as long as the solvent-induced changes of both the pattern of weak intermolecular interactions and the pK values of dissociable groups involved in intersubunit contacts are not known.

As shown by Khalil and Lauffer [32], the endothermic polymerization of tobacco mosaic virus protein at pH 6.5 and 0.1 M ionic strength (which has been interpreted in terms of 'hydrophobic bonding' [33]) is enhanced in the presence of ^2H_2O. Similarly, conductometric measurements on micelles and calorimetric investigations on amino acids with nonpolar side chains [34,35] support the view that the stability of hydrophobic interactions between aliphatic chains is enhanced in ^2H_2O compared to H_2O. Correlating these findings with the increased pressure stability of LDH in the presence of ^2H_2O clearly suggests that hydrophobic interactions contribute to the stabilization of the native quaternary structure. The fact that ANS binding does not corroborate this conclusion may be explained by a consecutive dissociation-transconformation mechanism involving masking of hydrophobic surfaces in the interior of the molecules after subunit separation.

Available evidence from X-ray crystal data confirms the present conclusions, indicating a significant contribution of hydrophobic interactions to the total stabilization energy of the enzyme [36]. For the pressure-induced dissociation, weakening of polar interactions seems to be most important.

Acknowledgments

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