HIGH PRESSURE DISSOCIATION OF LACTIC DEHYDROGENASE

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1. Introduction

As shown recently [1–4], polymerization-depolymerization reactions of proteins are accompanied by volume changes which may be explained by changes of solvation or changes in the structure of the solvent. The partial specific volume being increased in the course of association, high hydrostatic pressure is expected to promote dissociation according to

$$\frac{\partial \ln K}{\partial p} = -\frac{\Delta V}{RT}$$

(cf. Johnson and Eyring [5]). On the other hand, there is a volume decrease when denaturation occurs [6]. Therefore, deactivation of oligomeric enzymes by high hydrostatic pressure [7] cannot be interpreted unequivocally in terms of dissociation.

The following experiments give direct evidence for subunit dissociation mediated by high hydrostatic pressure using hybrid formation of LDH* as a tool. Chosing the experimental conditions so that both LDH H_4 and M_4 remain enzymatically active [pH 5.5–7.5, 0.5–4 M KCl, 0°], high pressure in the range of 1000 atm clearly favours dissociation, comparing hybrid formation after restoring the starting conditions to the respective distributions obtained at atmospheric pressure.

* Lactic dehydrogenase; H_4 and M_4, isoenzymes from heart muscle and skeletal muscle.

2. Materials and methods

LDH from pig heart (H_4) and pig skeletal muscle (M_4), and the coenzyme, NADH (NAD), were purchased from Boehringer, Mannheim. Densitometric patterns after electrophoresis on cellulose acetate prove homogeneity of the isoenzymes to be better than 95%.

Activity was measured with pyruvate and NADH (phosphate buffer pH 6.5, $I = 0.1$) and with the tetrazolium technique on agarose gel [8], the specific activity being of the order of 350 I.U./mg. High pressure experiments were performed in an 0.1°-thermostated autoclave according to Suzuki [9]. 0.2 ml samples of H_4 and M_4 (1–3 mg/ml) were enclosed in polyethylene tubes and incubated at $p < 2000$ atmospheres during various periods of time. Probes at 1 atm under equal conditions of the solvent were used as references. In order to check intermediary dissociation, hybrid formation was analyzed electrophoretically after hydrostatic pressure was released. Phosphate buffer ($I = 0.1$) was used as solvent throughout. High voltage electrophoresis (Camag, Berlin) on cellulose acetate (0.067 M veronal buffer pH 8.6) was evaluated using nigrosine or tetrazolium staining and densitometry.

3. Results

Hybridization experiments with LDH have been performed under various solvent conditions proving high ionic strength and low temperatures to be effective parameters. A combination of both parameters,

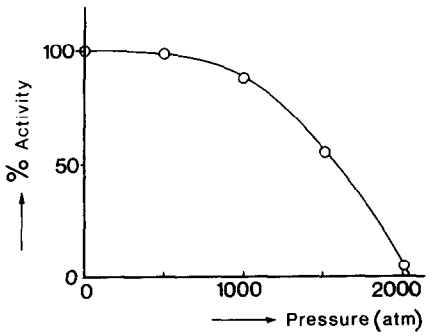

Fig. 1. Influence of hydrostatic pressure on enzymatic activity of LDH. $H_4 + M_4$ (1:1); total concentration c_p = 10 mg/ml; pH 7, I = 0.15, incubation time 1 hr.

Table 1
Influence of high hydrostatic pressure on LDH activity.

[KCl]	% relative activity	
M	0°	20°
0.5	60	90
1.0	61	90
2.0	70	100
4.0	72	92

$H_4 + M_4$ (1:1); pH 6.5, residual relative activity after 4 hr at 1000 atm (identical probes, incubated at 1 atm ≡ 100).

represented by the freeze-thaw technique at high salt concentration was found to produce complete dissociation leading to a binomial distribution of the isoenzymes [10]. The present data refer to isothermal experiments at different hydrostatic pressure (1–1000 atm) under conditions of high electrolyte concentrations. Preliminary results have been published in an earlier review [11].

As shown in fig. 1, the enzymatic activity of LDH turns out to be independent of hydrostatic pressure at pressures up to 500 atm, comparing the final activity under standard conditions (pH 7, I = 0.1, p = 1) after high pressure incubation with the state of the enzyme under identical solvent conditions but normal atmospheric pressure. Increasing salt concentration and raised temperature (20°) provides a stabilizing effect, leading to an increase of the residual activity after a constant period of incubation at high hydrostatic pressure (table 1).

A comparison of the relative amounts of hybrids formed under the two sets of conditions clearly proves high pressure to promote dissociation (table 2). At neutral pH and atmospheric pressure no hybrids can be detected*, while at 1000 atm hybrid formation is observed which increases with increasing time of incubation and decreasing pH. The data confirm earlier results [11] obtained on the basis of the nigrosine staining technique, proving enzymic activity to be preserved upon hybrid formation.

Under all conditions partial denaturation accompanies hybridization, causing an immobile nigrosine band of irreversibly aggregated protein at the start of the electrophoresis. The amount of denaturation depends on the ions applied, the effect of different anions following the sequence of the series of lyotropic ions, while the series of the alkali ions do not show appreciable differences. Only with ions in the middle of the Hofmeister series, high-pressure-induced hybrid formation is observed, pronounced structure breaking ions leading to irreversible denaturation. At pH values below pH 6 (e.g. pH 5.5) and increased temperature (37°) high pressure causes aggregation as well as hybridization, the equilibrium after 1 hr of incubation being characterized by the complete disappearance of H_4 and M_4; at atmospheric pressure the equilibrium is shifted toward the tetramer so that hybridization under this condition proceeds in a slow reaction. At pH 6.5, p = 1 atm, no hybrids are formed. Increasing hydrostatic pressure (1000 atm) again gives rise to dissociation and hybridization; the symmetric hybrid H_2M_2 again predominates in the final distribution, which is of the order of 1:2:10:2:1 (fig. 2).

In general, both deactivation and dissociation (hybrid formation) are inhibited by 1 M glycine or by 10^{-2} M NADH (fig. 3). While the coenzyme is known to stabilize the native quaternary structure, the protecting role of glycine most probably depends on its zwitterion character (cf. Yielding [12], Bern-

* Using polyacrylamide gel disc electrophoresis, instead of high voltage electrophoresis on cellulose acetate, unveils hybrid formation even at low electrolyte concentration, the sensitivity of the method being higher by a factor of > 10.

Table 2
Hybridization of LDH H_4 and M_4 (pig) at high hydrostatic pressure.

$H_4 + M_4$ (1:1) at 0°, c_p = 3 mg/ml									
p (atm)	KCl (I)	pH	H_4	H_3M	H_2M_2	HM_3	M_4	t_i	%A
1	2	5.5	30	5	20	12	33	1	76
1000	2	5.5	< 2	8	70	20	< 2	1	74
1	0.5–2.0	6.5	~50				~50	4	~85
1000	0.5	6.5	40		4		56	4	60
	1.0	6.5	30	~ 1	10	9	50	4	61
	2.0	6.5	24	9	28	13	26	4	70
	4.0	6.5	7	10	49	19	15	4	72

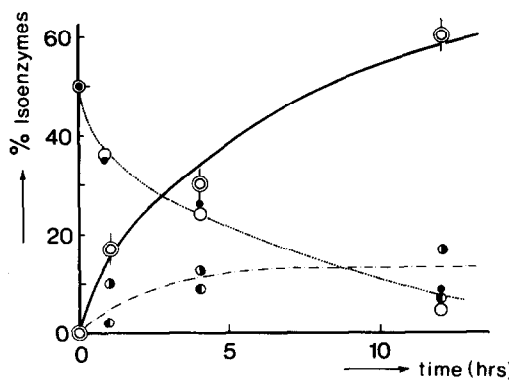

Fig. 2. Time dependence of hybrid formation at 1000 atm. c_p = 3 mg/ml; pH 6.5, 2 M KCl, T = 0°; Tetrazolium staining. ○ H_4; ◐ H_3M; ◍ H_2M_2; ◑ HM_3; ● M_4.

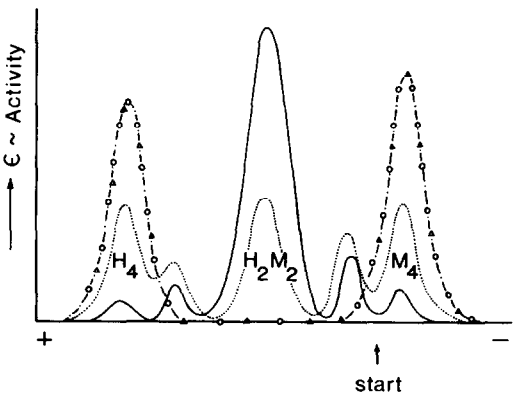

Fig. 3. High pressure hybridization of LDH. c_p = 3 mg/ml; pH 6.5, 2 M CKl, T = 0°, incubation time t_i. –·– Initial components H_4 and M_4 at p = 1 atm, t_i = 4 hr; ····· hybridization at p = 1000 atm (apoenzyme) 4 hr; —— hybridization at p = 1000 atm (apoenzyme) 14 hr; ○—○ holoenzyme: 0.02 mM $H_4 + M_4$ plus 10 mM NADH, p = 1000 atm, t_i = 4 hr; △—△ apoenzyme: 0.02 mM $H_4 + M_4$ plus 1 M glycine, p = 1000 atm, t_i = 4 hr.

feld et al. [13]). Contrary to Bernfeld's observation, bovine serum albumin has no protective effect in the present case.

4. Discussion

It is a generally accepted fact that full biological function of multimeric enzymes is correlated with their unperturbed quaternary structure. Corresponding to this axiom, deactivation has been used as a criterion for subunit dissociation [7, 13].

This criterion, however, is neither necessary nor sufficient, since subunits might be enzymically active, and denaturation (deactivation) may proceed without changing the state of association.

The present experiments give direct evidence that high hydrostatic pressure, which has been reported to deactivate a number of multimeric enzymes, in fact leads to an enhancement of the amount or rate of hybrid formation. The effect of intermediary dissociation suggested by this finding may be easily understood as a consequence of electrostriction and "iceberg" formation around ionic and apolar sidechains exposed to the aqueous medium upon subunit dissociation [14]. Both ion pairs and dehydrophobic interactions require increased volumes as

compared to the separate groups. High hydrostatic pressure will shift the equilibrium of association toward the monomeric state. Considering the range of electrolyte concentration and buffer capacity in the given experiments (table 1), perturbations due to pressure induced pH changes or ionic-strength-dependent hybridization [15] may be excluded. On the other hand, partial aggregation in the final state, which is pronounced at pH 5.5 or elevated temperature (37°), suggests hybrid formation to proceed via associated intermediates. However, direct turbidity measurements at high pressure [16] prove (reversible) aggregation to occur only at $p > 5000$ atm, even under isoelectric conditions in the presence of salt. Therefore, the effect of aggregation rather reflects the fact that loosening of interprotomer contacts necessarily affects intramolecular interactions as well, leading to partial denaturation.

Regarding the specific influence of various ions, electrostatic effects as well as hydration properties of the ions play a role. Typical "structure making" ions counteract the effect of high hydrostatic pressure, which may be interpreted as the conversion of clusters of water molecules to unordered bulk water, increasing entropy of hydration corresponding to increasing denaturation and dissociating power of the solvent.

The effect of the coenzyme and glycine has been demonstrated earlier by independent measurements (ultracentrifugation, proteolysis, heat inactivation etc.); As in the case of other NAD-dependent dehydrogenases, the holoenzyme turns out to be stabilized with respect to its quaternary structure and conformation.

Acknowledgements

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