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HIGH PRESSURE EFFECTS ON THE ACTIVITY OF GLYCOLYTIC ENZYMES

Dedicated to Professor Hans Netter on occasion of his seventy-fifth birthday

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High hydrostatic pressure inhibits growth in most organisms; this may be explained by a deactivation of enzymes involved in essential metabolic pathways. In order to check this hypothesis the enzymic activity of rabbit muscle lactic dehydrogenase and yeast glyceraldehyde-3-phosphate dehydrogenase was investigated in the presence of the coenzyme and excess of substrate at pressures up to 2 kbar.

Kinetic analysis of an initial phase of pressure induced activation and of a second phase of reversible deactivation shows that the two enzymes respond to high pressures in different ways leading to a volume of activation of $\Delta V^{\neq}$(LDH) $= 0 \pm 1$ cm^3 mol^{-1} and $\Delta V^{\neq}$(GAPDH) $= 60 \pm 4$ cm^3 mol^{-1}, respectively. Comparing the lower limits of pressure deactivation, LDH is found to be more stable towards pressure than GAPDH. At $p \approx 2$ kbar total deactivation of both enzymes is observed. A concentration dependent lag of GAPDH reactivation proves dissociation to participate in the process of deactivation, while the effects for LDH are explicable on the basis of reversible denaturation alone.

1. Introduction*

The structure of water is of considerable importance in determining the structural and biological properties of cell components. High hydrostatic pressure affects the interactions in aqueous media in a significant way [1,2] because ionization and solvation of solute molecules are usually accompanied by large volume changes of activation and reaction. For macromolecules such as enzymes or nucleic acids these volume changes may be quite large so that most life processes, from the molecular level to the organismic level depend strongly on the external pressure.

Since 70% of the earth's surface is covered by the oceans (86% of which exceeds a depth of 2000 meters [3]), high pressure is a common parameter in the biosphere. Searching for bacteria in sediment samples from extreme depths, e.g., in the Philippine trench, proved that there is life under pressures higher than 1 kbar [4]. There exist barophilic bacteria which grow preferentially with increasing pressures [5]; as a rule, however, bacterial growth is inhibited at pressures significantly above the pressure of the normal habitat [6]. The nature of this inhibition is unknown. Two potential mechanisms are (a) the suppression of nucleic acid or protein synthesis [7–9] and (b) denaturation or dissociation of enzymes involved in essential metabolic pathways [10,11].

Indirect evidence for (b) comes from the protecting effect of increased ATP concentration in the pressure induced blocking of the furrowing strength of fertilized sea urchin eggs [12]. Hill and Morita [13] proved that pressure induced inactivation of enzymes participating in the tricarboxylic acid cycle deprives the cells of an important source of energy.

Most high pressure studies on bacteria were performed under anaerobic conditions. In this case the generation of ATP requires the unperturbed state of the enzymes involved in glycolysis. Little is known

* Abbreviations:
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
LDH, lactic dehydrogenase
PGK, 3-phosphoglycerate kinase.

about the influence of high pressure on the activity of these enzymes. Available data on lactic dehydrogenase [14,15] and pyruvate kinase [16] from fishes are based on measurements in crude extracts rather than purified systems, thus obscuring the true mechanism of deactivation. In order to throw some light on the elementary processes of growth inhibition, it seemed desirable to investigate the effect of elevated pressure on purified enzymes of the glycolytic chain. Since bacterial GAPDH and LDH are very sensitive to autoxidation [17,18] the purified enzymes from yeast and rabbit were investigated in the present experiments. The results show that pressures of 0.5–2 kbar lead to reversible deactivation caused by dissociation and/or conformational changes (denaturation) of the enzymes.

2. Materials and methods

Lactic dehydrogenase from rabbit muscle (LDH, E.C. 1.1.1.27), phosphoglycerate kinase (PGK, E.C. 2.7.2.3) and NADH were purchased from Boehringer, Mannheim; glyceraldehyde-3-phosphate dehydrogenase from baker's yeast (GAPDH, E.C. 1.2.1.12) was prepared according to Kirschner and Voigt [19]. 2-Mercaptoethanol and dithiothreitol were products of Fluka, Buchs, and Calbiochem, Lucerne, respectively. All other reagents were of A-grade purity (Merck, Darmstadt); quartz bidistilled water was used throughout.

Stock solutions of LDH in potassium phosphate buffer, pH 7.0, $I = 0.1$, were prepared by desalting 0.1 ml of the ammonium sulfate suspension (10 mg/ml) on a short sephadex G-25 column (5 × 1 cm) at 25°C. Portions of the ammonium sulfate suspension of GAPDH were dialyzed for 24 hours at 5°C against 0.1 M potassium phosphate buffer, pH 7.5, in the presence of 1 mM K_3-EDTA and 0.2 mM dithiothreitol. Stock solutions of GAPDH were diluted with cold water.

The activity of the enzymes was tested spectrophotometrically using the decrease of the NADH absorption at 366 nm. LDH activity was measured in potassium phosphate buffer, pH 7.0, $I = 0.1$ in the presence of 0.71 mM pyruvate + 0.18 mM NADH (saturating conditions). In the case of GAPDH the enzymic test was performed in 0.05 M triethanolamine buffer with 6.5 mM MgK_2EDTA, 5 mM 2-mercaptoethanol and 0.26 mM NADH. 2-Mercaptoethanol was used instead of cysteine·HCl since the standard test with cysteine in the presence of traces of heavy metal ions and oxygen led to changes in NADH absorption with time also in the absence of the enzyme. The substrate 1,3-diphosphoglycerate for the reverse reaction of GAPDH was generated from 1.1 mM ATP plus 3.2 mM phosphoglycerate using phosphoglycerate kinase as auxiliary enzyme (equilibrium concentration 0.08 mM). Under the conditions of the standard test given above GAPDH is > 50% saturated with NADH; saturating conditions ([NADH] = 2 mM) would cause an absorbance at 366 nm too high to allow the optical test in the pressure cell. Therefore the following experiments with GAPDH refer to the activity at half saturation regarding the coenzyme. For PGK saturation conditions are maintained. The fact that $\Delta A_{366}/\Delta t$ is constant over a relatively wide range under any conditions used in the present study seems to be due to a compensatory effect of the decrease of [NADH] and the activation of GAPDH by NAD^+ [19a].

In order to compare the rates of the enzymic reaction at different hydrostatic pressures, and to correlate the rates to the concentration of active enzyme, the saturation of the enzyme does not play a decisive role as long as linearity of the assay is provided. This statement is based on the assumption that the K_M(NADH) does not show significant pressure dependence.

The specific activities of LDH and GAPDH were 540 IU/mg and 133 IU/mg, respectively; enzyme concentrations were calculated from $A_{280}^{0.1\%} = 1.40$ [20] and 0.89 $cm^2\ mg^{-1}$ [19].

Measurements at elevated pressure used a thermostated transmission cell (optical pathlength 12 mm) mounted in the optical path of a Gilford 2400 S single beam spectrophotometer. The pressure generating equipment is the conventional 1/8" valve and capillary system with an oil filled hydraulic pump from High Pressure Equipment Co., Erie, Pa. Bourdon gauges, quality class 0.6 (A. Wiegand, Klingenberg, FRG) were used. A mixture of oil and kerosene served as pressure transmitting medium. The temperature was monitored within the autoclave by a miniature chromel–alumel thermocouple (A) (Philips, Industrie Elektronik, Hamburg) silver soldered into a metal

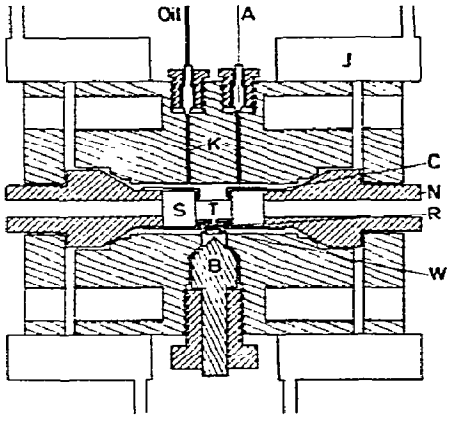

Fig. 1. Optical transmission cell for enzymic tests at elevated pressures, pathlength 12 mm. (A) Thermocouple, (B) cone seal, (C) window cap, (J) brass jacket, (K) holes for oil inlet and thermocouple, (N) nipple, (R) rubber seal, (S) sapphire window, (T) teflon container, (W) sealing cap and screw, (for details see text).

cone seal. The basic features of the cell are given in fig. 1 (cf. [21,22]). All metallic parts were made from a copper–beryllium alloy (Berylco 25, Deutsche Beryllium GmbH, Oberursel FGR). The cylindrical windows (S) of synthetic colourless sapphire are pressed by caps (C) against the flat polished ends of the metal nipples (N). To obtain a good seal, a 0.01 mm thick teflon foil is used between the sapphires and the surface of the nipples. In addition the caps seal between the sapphires and the thin walled teflon cylinder (T), the metal free container for the enzyme solutions. This container is cleaned and refilled with a syringe, without removing the windows, by opening the bigger side cone seal (B) and removing screw (W) and silicone rubber plate (R). One of the small holes (K) on the opposite side serves as oil-inlet and the thermocouple (A) is led into the cell through the second hole. The autoclave was kept at constant temperature by pumping water from a thermostat through the brass jacket (J). To insure that the pressure transmitting fluid had not penetrated into the solution during the enzymic test, the tightness of the cell was checked after each experiment.

Pressures $\leqslant 2$ kbar were generated within < 2 min. The temperature change caused by adiabatic compression of the oil was found to be of the order of 1°C; the temperature change in the test solution cannot exceed $(\partial T/\partial p)_{water}$ which is 1.8°C/kbar [23]. All measurements were corrected for the compressibility of water at 25°C [24,25].

3. Results

It is well-known from experiments of Suzuki and coworkers [26] that the effects of elevated hydrostatic pressure on enzymes are complex. Depending on pressure, activation or deactivation may occur, the rate of the catalytic reaction being influenced by alterations of the rate constant and/or the equilibrium constants of substrate binding. Apparently different conformational states of the protein are favoured under varying conditions of pressure, temperature, duration and number of compressions, etc. For different isoenzymes of LDH quench experiments proved deactivation at $p \geqslant 0.5$ kbar [27]. Since this result was obtained under conditions quite different from those in the enzymic test applied in the present study the following experiments start from the basic characteristics of rabbit muscle LDH and yeast GAPDH at elevated pressure.

3.1. General features of LDH and GAPDH at $p \leqslant 2$ kbar

Measurements of the enzymic activity of LDH and GAPDH from 1 bar to 2 kbar proves LDH to be much more stable than GAPDH against pressure deactivation. Consistent with the quench experiments [27] the optical test of LDH remains unchanged below ~ 1 kbar; on the other hand GAPDH shows deactivation at very much lower pressures (< 0.1 kbar). For a fixed pressure in the specific range of deactivation, the profiles in fig. 2 are observed. As shown by blank experiments with enzymes absent the initial increase of absorption upon application of pressure is caused by two effects: (a) the compressibility of the solution, which leads to an increase of the NADH concentration in the enzymic test, and (b) the change of the molar extinction coefficient of the coenzyme with pressure. The subsequent changes of activity ($\sim \Delta A_{366}/\Delta t$) suggest the mechanisms for LDH and GAPDH to be rather different. While, for LDH the initial slope differs only within the range of the pressure induced shift of enzyme concentration from the slope at

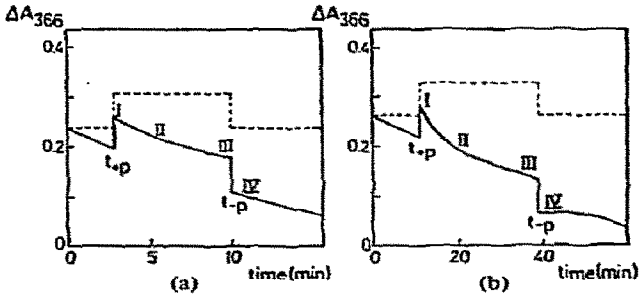

Fig. 2. Schematic representation of typical enzymic tests under high pressure. t_{+p} and t_{-p}, moment of pressure application and pressure release; I, initial phase of pressure induced activation; II, phase of pressure deactivation; III, equilibrium value under pressure; IV, residual activity after pressure release.

a. Lactic dehydrogenase ($c = 1.1 \times 10^{-10}$ M) in phosphate buffer pH 7.0, $I = 0.1$, $T = 25°C$, 0.71 mM pyruvate, 0.18 mM NADH, $p = 1500$ bar.

b. Glyceraldehyde-3-phosphate dehydrogenase ($c = 1.3 \times 10^{-11}$ M) in 0.05 M triethanolamin pH 7.5, $T = 25°C$, 0.26 mM NADH, 1 mM ATP, 6.5 mM MgK_2-EDTA, 3.2 mM phosphoglycerate, 5 μl phosphoglycerate kinase (10 mg/ml), $p =$ 500 bar. Dotted line: blank experiment with enzyme absent.

atmospheric pressure, the activity of GAPDH is enhanced considerably by high pressures. In both cases the reaction rate then decreases to reach a constant final value of activity, as shown by a linear A_{366} versus t profile. Upon relasing the pressure the change of absorption due to compressibility is reversed. The final activity of LDH remains more or less unchanged. For GAPDH only a low residual activity is observed after release of pressure; after a lag of several minutes, a slow regain of activity occurs.

3.2. Initial velocity after application of pressure

Measuring the rate of the enzymic reaction at different pressures immediately after application of pressure shows that there is no change in the rate of the reduction of pyruvate catalyzed by LDH (fig. 3); on the other hand with GAPDH a strong enhancement of $\Delta A_{366}/\Delta t$ is observed which is reflected by the increased slope at the point t_{+p} in fig. 2. Accurate determination of this slope is increasingly more difficult with increasing pressure since the initial curvature as well as experimental errors due to thermal equilibration etc. render the precision of the data more

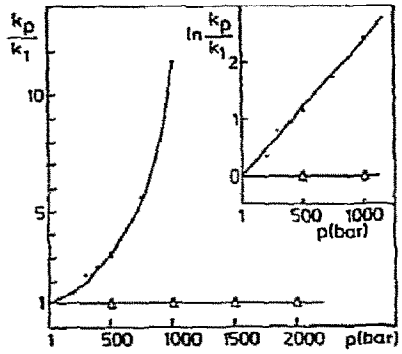

Fig. 3. Reaction rate immediately after pressure application (cf. phase I in fig. 2). k_1 and k_p, rate constants of the enzymatic reaction at 1 and p bar respectively. (△) Lactic dehydrogenase. (●) Glyceraldehyde-3-phosphate dehydrogenase, values taken from a plot of ln activity versus time extrapolating activity to t_{+p}.

and more insufficient for a quantitative evaluation. Since a single exponential describes the initial decrease of the slope (see below, fig. 4b), the activity of GAPDH immediately after applying pressure may be taken from a plot of the logarithm of enzymic activity versus time extrapolating the activity to t_{+p}. Contrary to results gained from the uncorrected initial slopes which lead to an apparent maximum of activation at ~ 0.5 kbar, the extrapolation procedure reveals a steady increase of initial rate in the whole pressure range $p \leq 1$ kbar (fig. 3). The enhancement of GAPDH activity could be attributed to a pressure induced decrease of the binding constant for NADH. Since we start with the > 50% saturated enzyme this mechanism should yield a *saturation* profile with a maximum activation of *twice the initial activity*. The effect observed is characterized by an *exponential increase* of about an *order of magnitude*; therefore the activation of GAPDH is determined by a change of the rate constant for the enzymic reaction.

In transition state theory [28] the pressure dependence of the reaction velocity constants is described by

$$k_p = k_1 \exp(-\Delta V^{\ddagger} \Delta p / RT), \qquad (1)$$

with

k_1 and k_p = rate constants at 1 and p bar, respectively,
$\Delta V^{\ddagger}$ = volume of activation ($cm^3\ mol^{-1}$),

T = absolute temperature (K),
R = gas constant (cm^3 bar mol^{-1} K^{-1}),
Δp = pressure difference (bar).

According to this equation a semi-logarithmic plot of the ratio of the activities at high pressure and at 1 bar ($\sim \ln k_p/k_1$) versus p, should yield a straight line, with a slope $-\Delta V^{\ddagger}/RT$. As shown in the insert in fig. 3, linearity is indeed obtained for both enzymes, leading to volumes of activation of

$\Delta V^{\ddagger}$(LDH) = 0 ± 1 (cm^3 mol^{-1})
$\Delta V^{\ddagger}$(GAPDH) = 60 ± 4 (cm^3 mol^{-1}).

3.3. Deactivation under pressure

Above a critical value of pressure both enzymes are slowly deactivated to a final value, the rate of deactivation increasing with pressure. After reaching a final value of deactivation at a given pressure the activity profile becomes linear again proving the deactivation to be not merely a K_M-effect. Two mechanisms may account for the deactivation, (i) conformational changes towards an equilibrium of denaturation under preservation of the native quaternary structure and (ii) dissociation into inactive or partially inactive subunits. The time resolution of the method does not allow to separate equilibria of several species of the enzymes showing various specific activity. Assuming therefore a two-state model with the equilibirum

$$E_{active} \rightleftharpoons E^{*}_{inactive}$$

to be sufficient to analyze the data, the theory of chemical kinetics [29] describes the two processes by the following equations:

Denaturation: $-dc_a/dt = k_d c_a - k_r(c_0 - c_a)$, (2)

Dissociation: $-dc_a/dt = k_d c_a - k_r c_s^n$
$= k_d c_a - k_r n^n (c_0 - c_a)^n$, (3)

with

c_0 = concentration of active enzyme at zero time,
c_a = concentration of active enzyme at time t,
c_s = $n(c_0 - c_a)$ = concentration of inactive subunits,
n = number of subunits,
k_d, k_r = rate constants of deactivation and reactivation.

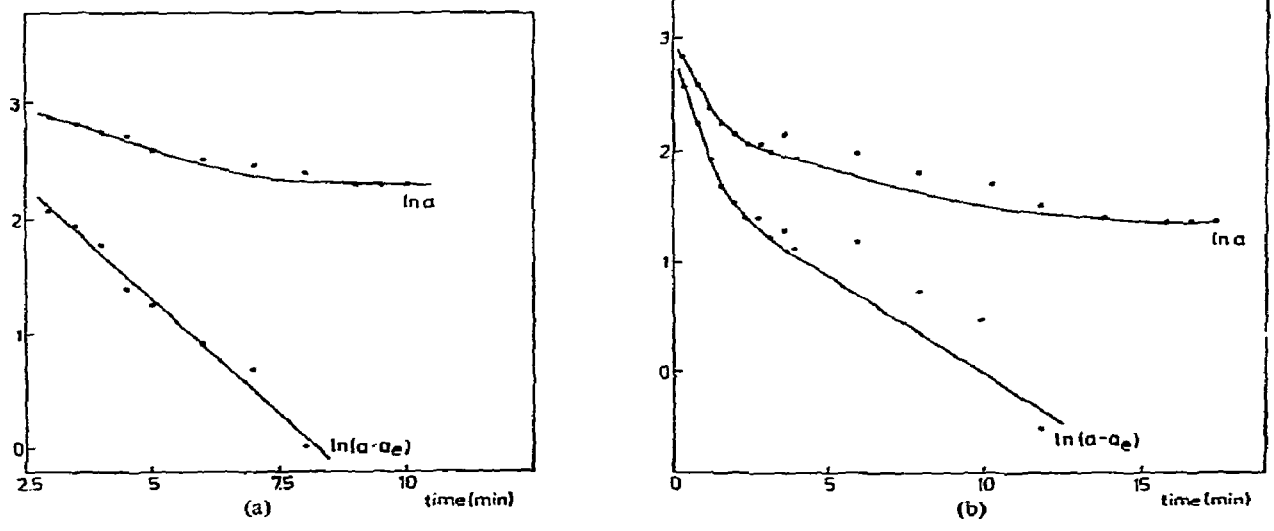

Fig. 4. Semi-logarithmic plot of enzymic activity as function of time (for details see text, conditions as given in fig. 2, activity in arbitrary units).
a. Lactic dehydrogenase, $p = 1600$ bar.
b. Gyceraldehyde-3-phosphate dehydrogenase, $p = 500$ bar.

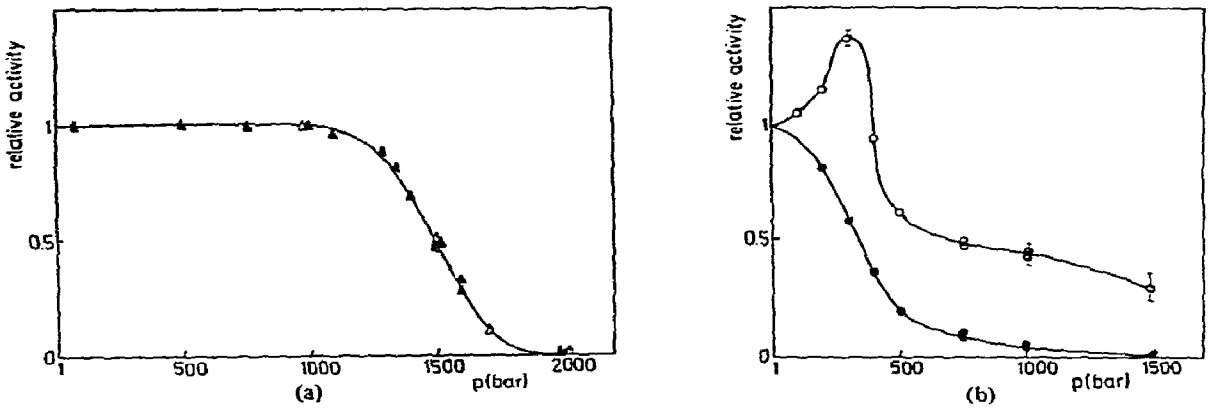

Fig. 5. Equilibrium values of enzymic activity as a function of pressure (cf. phase III, fig. 2, conditions as given in this figure).
a. Lactic dehydrogenase, (▲) without DTT, (△) in the presence of 2 mM DTT.
b. Glyceraldehyde-3-phosphate dehydrogenase. (○) Deactivation relative to the activity at 1 bar, demonstrating the pressure induced alteration of enzymic activity. (●) Deactivation relative to the initial activity immediately after application of pressure, demonstrating the percentage of enzyme deactivated (data from fig. 3).

Since concentration and activity are correlated by a defined value of the specific activity under given experimental conditions, eqs. (2) and (3) can be rewritten:

$$\text{Denaturation: } -\frac{da}{dt} = k_d a - k_r(a_0 - a), \qquad (2a)$$

$$\text{Dissociation: } -\frac{da}{dt} = k_d a - n^n k_r \left(\frac{1}{a_{sp}}\right)^{n-1} (a_0 - a)^n, \qquad (3a)$$

with

a_0 = activity at zero time,
a = activity at time t,
$a_{sp}(p)$ = specific activity (dependent on pressure).

At the beginning of the reaction the second term in both equations can be neglected, leading to a linear relationship of $\ln a$ versus time. For *unfolding* as the process of deactivation, plotting $\ln(a - a_e)$ versus time (with a_e = activity at equilibrium, i.e., $da/dt = 0$) should yield a linear relation until complete deactivation is reached; for *dissociation*, however, deviations from linearity would be expected. The respective plots for LDH and GAPDH are shown in fig. 4.

3.4. Final value of deactivation under pressure

As shown in fig. 5 deactivation at different values of pressure leads to reproducible final values of activity, a_e, proving the existence of pressure-dependent equilibrium states. Apparently enzymic activity of both enzymes vanishes completely at sufficiently high pressures. Fig. 5b presents the final values of deactivation of GAPDH for two reference states: the open circles refer to the ratio of the residual activity ($\Delta A_{366}/\Delta t$) under pressure at equilibrium, compared to the activity at normal atmospheric pressure. These data, which represent the biologically relevant decrease of enzymic activity under pressure are brought about by two counteracting effects: an activation process due to an increased rate constant of the enzymic reaction and a deactivation process due to a pressure induced denaturation and/or dissociation of the enzyme. The full circles refer to the deactivation relative to the initial activity immediately after application of pressure (cf. fig. 3); these values represent the fraction of active enzyme left. In these measurements phosphoglycerate kinase is needed in the coupled optical test. In order to exclude potential artifacts caused by the deactivation of the auxiliary enzyme varying PGK concentrations were applied. Since a tenfold excess of the kinase did not produce significant differences in the GAPDH profile, pressure effects on PGK may be excluded.

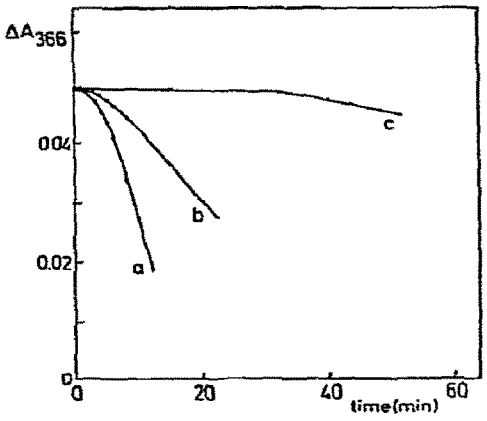

Fig. 6. Concentration dependence of glyceraldehyde-3-phosphate dehydrogenase reactivation after release of pressure from p = 1 kbar to 1 bar. (Conditions as in fig. 2). Enzyme concentrations: 6.2×10^{-10} M (a), 9.0×10^{-11} M (b), 3.5×10^{-11} M (c). Final values of reactivation: 88% (a), 25% (b), 13% (c).

3.5. *Reactivation after release of pressure*

There is no indication of reversible equilibria for denaturation or dissociation in LDH from the time dependence of activity after release of pressure (fig. 1); the specific activity remains unchanged and the variation of enzyme concentration has no effect on the characteristics of A_{366} versus time. On the other hand, GAPDH shows a slow reactivation, the rate and yield of which depend on the concentration of the enzyme (fig. 6).

4. Discussion

As demonstrated in the results section, LDH and GAPDH show divergent pressure effects although both enzymes belong to the closely related group of NAD^+ dependent oxidoreductases which catalyze the transfer of H^- to a carbonyl group.

The observed different effects on the rate constant immediately after applying pressure (I in fig. 2) may be caused by either the different catalytic mechanism of the two enzymes or by specific properties involving their structure. It is obvious that structural effects of elevated pressure cannot be predicted because of the subtle equilibrium of intramolecular and intermolecular interactions responsible for the specific three-dimensional structure of enzymes. On the other hand there is a close similarity in the overall structural properties of LDH and GAPDH. Therefore one may attempt to interprete the data on the basis of the mechanism of the two enzymes.

In the case of LDH, kinetic analysis proves the conversion of pyruvate to lactate to be characterized by a rate determining isomerization reaction E·NADH $\rightleftharpoons$ E′·NADH which is followed by the hydride transfer [30]:

$$E + NADH \rightleftarrows E{\cdot}NADH \rightleftarrows E'{\cdot}NADH \rightleftarrows \underset{\text{pyruvate}}{E'NADH} \rightleftarrows \underset{\text{lactate}}{E'{\cdot}NAD^+}$$

$$\rightleftarrows E'{\cdot}NAD^+ \rightleftarrows E{\cdot}NAD^+ \rightleftarrows E + NAD^+ \qquad (4)$$

E′ = isomerized form of E.

According to X-ray [31], ORD and CD data [32,33] the conformational changes upon coenzyme and substrate binding consist of slight structural changes which are expected to be accompanied only by a small volume change. From this the influence of pressure is expected to be negligible, in accordance with the result given in fig. 3.

The pressure induced activation of GAPDH cannot be explained by a shift of the allosteric T–R transition. This transition in fact is accompanied by a volume contraction [34,35]; however, the presence of catalytic amounts of NAD^+ causes the R state to be predominant under all conditions in the present experiments [36]. In addition a shift of the T $\rightleftharpoons$ R equilibrium would result in a profile of activation different from the one observed (see section 3.2).

To explain the high value of the volume of activation, $\Delta V^{\ddagger}$, the various steps in the enzymic reaction of GAPDH have to be considered regarding their rate determining character. As shown for a number of dehydrogenases hydrogen transfer and substrate binding are rapid reactions [30,37]. Therefore the formation of the acyl–enzyme or the cleavage of the aldehyde from the hemi-thioacetal may be considered rate determining in the reverse reaction of GAPDH. Comparing analogous chemical reactions proves $\Delta V^{\ddagger}$ to be too high to corroborate this explanation [38]. We conclude that conformational changes in the enzyme molecule participate in the transition state of the rate

determining step. Transient kinetic studies on the lobster and sturgeon enzyme [37] allow to localize the respective step in the reaction sequence between the formation of the acyl–enzyme complex (connected with the dissociation of NAD^+) and the redox reaction. The volume of activation for the yeast enzyme may be explained on the basis of this mechanism considering a conformational change of the acyl–enzyme on binding NADH.

The slow deactivation observed for both LDH and GAPDH (II in fig. 2) can be attributed to pressure-generated structural changes. pH shifts with pressure are small [39] and cannot result in drastic changes of the enzymic activity since the experimental pH corresponds to the broad pH optimum of the enzymes. Oxidation of essential SH groups by hyperbaric oxygenation can also be neglected, because this effect starts at very low pressures [6], and addition of DTT or 2-mercaptoethanol does not prevent the deactivation.

The structural changes underlying the pressure induced deactivation may consist of dissociation and/or unfolding equilibria which are shifted by pressure. Considering an all or none *conformational transition* between an active form of the enzyme, E_a, and an inactive form, E_i, leads to

$$c_i/c_a = K\exp(-\Delta V\Delta p/RT) = c_{total}(1-\beta)/c_{total}\beta, \quad (5)$$

with

c_i = concentration of inactive enzyme,
c_{total} = total concentration of the enzyme,

and

$$\beta = \frac{a_e}{a_0} = \frac{\text{activity at equilibrium}}{\text{initial activity}}.$$

Deactivation caused by *dissociation* of an active form of the enzyme into inactive subunits S is described by

$$c_S^n/c_a = K\exp(-\Delta V\Delta p/RT). \quad (6)$$

For $n = 2$ and $n = 4$, eq. (6) yields

$$[4(1-\beta)^2/\beta]\,c_{total} = K\exp(-\Delta V\Delta p/RT),$$

and

$$[256(1-\beta)^4/\beta]\,c_{total}^3 = K\exp(-\Delta V\Delta p/RT), \quad (7)$$

respectively.

Consequently unfolding and dissociation may be distinguished by plotting the experimental data in a log $[(1-\beta)^n/\beta]$ versus p plot. A linear relationship for $n = 1$ would point to an unfolding mechanism, while pressure dependent dissociation to dimers or monomers will show a straight line for $n = 2$ or $n = 4$. The data for LDH in fig. 7a obey eq. (5), this way confirming the unfolding mechanism. This result does not contradict earlier hybridization experiments in the absence of coenzyme [27], since the tetrameric quaternary structure of LDH is strongly stabilized by the presence of NADH [33]. Chosing plausible values of n for GAPDH does not lead to a linear relation. Consequently a more complex mechanism of deactivation has to be considered.

Evidence for the participation of a dissociation process comes from the concentration-dependent reactivation (IV in fig. 2 and fig. 6); the onset of deactivation at $p \approx 0.2$ kbar points to the same because protein denaturation normally is observed in the high pressure range [26]. On the other hand, the extent of reactivation is also dependent on the concentration of the enzyme. This clearly indicates unfolding to be involved since varying concentrations will yield

Fig. 7. Semi-logarithmic plot of $(1-\beta)^n/\beta$ versus pressure according to eqs. (5) and (7). a. For lactic dehydrogenase. b. For glyceraldehyde-3-phosphate dehydrogenase.

different fractions of native tetramers and aggregates of incorrectly folded subunits, depending on the relative rates of the 1st order refolding and the 2nd order reassociation reaction [40].

The preceding results show that LDH and GAPDH respond to elevated pressure in different ways; while the catalytic properties of LDH remain unchanged in the whole range of pressure realized in the hydrosphere, GAPDH is deactivated reversibly at $p \approx 0.5$ kbar. At higher pressure both enzymes show deactivation which is caused by dissociation and/or denaturation. The results hold for the enzymes in the presence of the coenzyme and excess of substrate, i.e., under conditions of maximum stability. In vivo most enzymes do not operate under saturating substrate concentrations [41]. Therefore the deactivation discussed in the present study is expected to be even more pronounced under physiological conditions.

Acknowledgement

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