High-pressure enzyme kinetics

Lactate dehydrogenase in an optical cell that allows a reaction to be started under high pressure

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A newly designed optical cell allows an enzyme reaction to be started under high pressure and makes it possible to begin measurement of the reaction rate after a 'dead time' no longer than 1–2 s. This device was used to study the kinetics of lactate dehydrogenase reaction at 1 kbar. At this pressure lactate dehydrogenase from rabbit muscle exhibited a rapid deactivation in the presence of NADH if pyruvate was absent. After addition of pyruvate the reaction was initiated and proceeded at a constant rate, i.e., without loss of enzyme activity. It is suggested that pyruvate markedly increases the association constant of this tetrameric enzyme.

High pressure *Kinetics* *Optical cell* *Lactate dehydrogenase* *Deactivation* *Stabilization*

1. INTRODUCTION

High-pressure experiments have proved useful in studying the change in molecular volume that may accompany enzyme functioning [1]. Furthermore hydrostatic pressure is a convenient means of investigating reversible dissociation reactions between subunits of complex enzymes [2].

The effect of pressure on the reaction rate is usually studied either by sampling or spectrophotometrically. The major problem in these measurements is the 'dead time' between the onset of a reaction and the very beginning of recording the kinetics. To decrease this period, a high-pressure device should allow the reaction to be started after the pressure has already been raised and the accompanying increase of temperature compensated. The first attempt to design such a device, to our knowledge, was described in [3]. However, in that device the onset of reaction was carried out outside the spectrophotometer, and therefore, the dead time still remained too long, about 2–4 min. A stopped-flow technique under a pressure up to 1.2 kbar has been developed for fast reactions, allowing measurements to be made after just 20 ms [4,5]. This apparatus allowed the study of reactions with $\tau_{1/2}$ up to 20 ms but had intrinsic shortcomings: firstly, all reagents in the syringes were under high pressure for several minutes before mixing (as will be shown later, this can be essential for enzyme kinetics); secondly, the stopped-flow technique is rather complicated and expensive; thirdly, it cannot be combined with a conventional spectrophotometer.

A simple and handy high-pressure spectrophotometric cell for kinetic measurements of moderately fast reactions with a half-time of a few seconds has been suggested [6]. This paper describes a modified cell [6] and presents results of a study of the effect of high hydrostatic pressure on the LDH reaction carried out with this new

Abbreviation: LDH, lactate dehydrogenase (EC 1.1.1.27)

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technique. Authors in [7–10] have shown that high hydrostatic pressure causes deactivation of this enzyme; in the presence of pyruvate and NADH, however, LDH is stable at a pressure of 1 kbar. The experiments described below confirm that pyruvate (in the presence of NADH) stabilizes the enzyme but NADH, when present separately, does not.

2. MATERIALS AND METHODS

2.1. *Materials*

LDH from rabbit muscle and NADH was purchased from Serva (Heidelberg), dithioerythritol, pyruvate (sodium salt) and Tris from Sigma (USA), and EDTA from Reanal (Hungary). Quartz-bidistilled water was used throughout. Stock solutions of LDH were prepared by desalting a small volume of 1:10 diluted $(NH_4)_2SO_4$ suspension of the enzyme by dialysis against 0.2 M Tris–HCl buffer (pH 7.6) containing 10 mM dithioerythritol and 2 mM EDTA. For assaying LDH activity, the buffer was supplemented by 0.2 mM NADH, 1 mM pyruvate and 76 ng/ml LDH (initial spec. act. 500 IU/mg).

2.2. *Instrumentation*

2.2.1. Spectrophotometer

A Specord UV VIS spectrophotometer (Zeiss, Jena) with a tungsten lamp was employed to monitor the absorption change of NADH at 340 nm and 25°C.

2.2.2. High-pressure apparatus and operation

The enzymatic reaction was conducted in the transparent cylindrical cell sketched in fig.1. It was made of plexiglass polished on both the inside and outside. The volume was about 2.5 ml. On the outside of the bottom, the cell had a recess in which a steel disk (2) was fastened and sealed. A small hard-plastic (Kel-F) cup (3) with a flat polished flange was filled with enzyme or substrate solution and pressed onto the cell bottom as shown in fig.1. A little disk-shaped permanent magnet (4) glued to the cup held it firmly on the bottom opposite to the steel disk (2). (The flanges of the cup may be finely smeared with vaseline to make the contact reliably impermeable.) The filled cup being in this position, the cell was filled with excess assay solution and closed with a cover (5). The latter had a conical inner surface with a hole in it to facilitate the bubble-free filling of the cell and serving for pressure transmission; the hole was sealed with a strip of thin elastic polyethylene greased with vaseline.

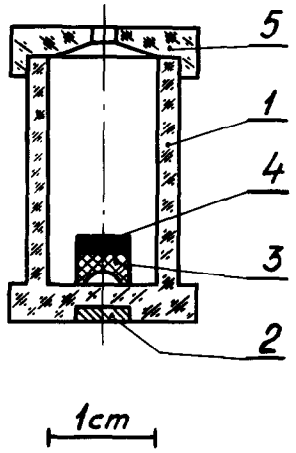

Fig.1. A cell for initiating and studying an enzyme reaction under high hydrostatic pressure. (1) Cylindrical transparent reaction cell, (2) steel disk secured in a recess, (3) plastic (Kel-F) cup, (4) permanent magnet, (5) conical cover with a hole for pressure transmission and for bubble-free filling of the cell.

The cell thus assembled could be installed in the high-pressure optical bomb shown in fig.2. The cell had to be immersed in a cylindrical well (1) previously filled with water. The bomb was sealed by screwing down the cap (2), and by means of the port (3) was connected with a hydraulic high-

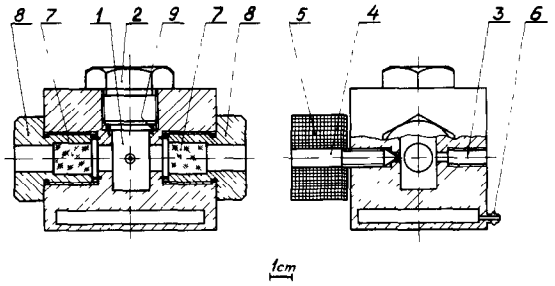

Fig.2. High-pressure optical bomb (made from stainless steel). (1) Cylindrical well for installing the reaction cell, (2) screw cap for sealing the bomb, (3) channel for connecting the bomb to a high-pressure hydraulic pump, (4) magnetic core, (5) removable solenoid coil, (6) couplings for connecting to a water ultrathermostat, (7) quartz windows, (8) window fastening nuts, (9) Teflon sealing gasket.

pressure pump and mounted in the spectrophotometer on a specially designed stand. On the protruding core (4) the coil of a solenoid (5) was mounted. After the couplings (6) were connected with an ultrathermostat, the cover of the spectrophotometer cell compartment was closed and the pressure could be raised.

The assay solution in the cell was under the same pressure as the surrounding water, owing to the elasticity of the polyethylene seal. At the same time, the surrounding water did not penetrate the cell, provided it was filled without bubbles. It is essential to note that the solution in the cup before the onset of the reaction was not subjected to pressure noticeably exceeding that of the atmosphere. All other components of the assay, being isolated from the contents of the cup, could be incubated at high pressure for as long as desired.

To set off the reaction, the solenoid was switched on for ~0.5 s in an a.c. power source. The voltage, being dependent on the parameters of the solenoid, had to be adjusted for the alternating magnetic field to detach the cup with the pasted-on magnet from the bottom of the cell, and to force the reagents to be vigorously mixed. The absorption recording was started a short time before the solenoid was energized (see fig.3). The moment the reaction started could thus be visualized and determined quite accurately, and a check could be made whether the reaction had begun before the energization, due to the reactant's leakage from the cup. Abrupt deflections of the pen seen in fig.3 marked the onset of the reaction, and one can see that the monitoring of the reaction began after a dead time of no longer than 2 s.

3. RESULTS AND DISCUSSION

The apparatus described was used in studying the effect of pyruvate and NADH on high-pressure deactivation of LDH. For this purpose, the pyruvate-containing solution (9 μl, 0.28 M) was placed into the cup (3) (fig.1). After mixing, the reaction began at an initial concentration of 1 mM pyruvate. The enzyme (76 ng/ml) and NADH (initial concentration 0.2 mM) were in the cell medium under high pressure from the outset. This made it possible to incubate LHD at a high pressure (1 kbar) in the presence of NADH for various periods before starting the reaction with

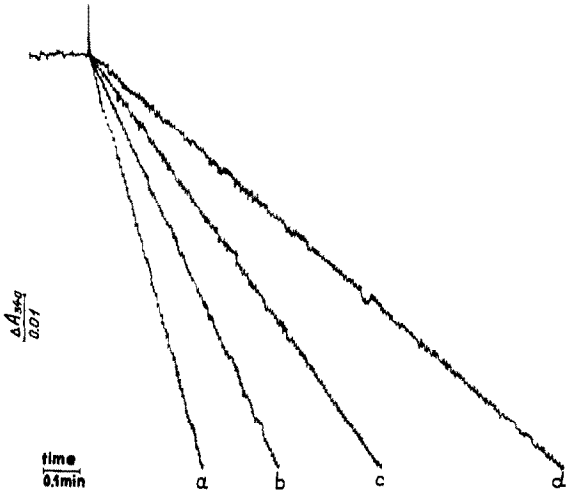

Fig.3. Superimposed kinetic absorption change traces after different times of incubation of LDH under high pressure in the absence of pyruvate. (a) At atmospheric pressure (test of native enzyme activity), (b) 1 min incubation at 1 kbar, (c) 3 min incubation at 1 kbar, (d) 5 min at 1 kbar; 340 nm, 25°C, 76 ng/ml LDH, Assay solution: 0.2 M Tris–HCl (pH 7.6), 10 mM dithioerythritol, 2 mM EDTA, 0.2 mM NADH, 1 mM pyruvate.

pyruvate. The results are shown in fig.4. The curve is drawn through the points where the reaction was started, and indicates a rapid deactivation of LDH under a pressure of 1 kbar. It is essential to note

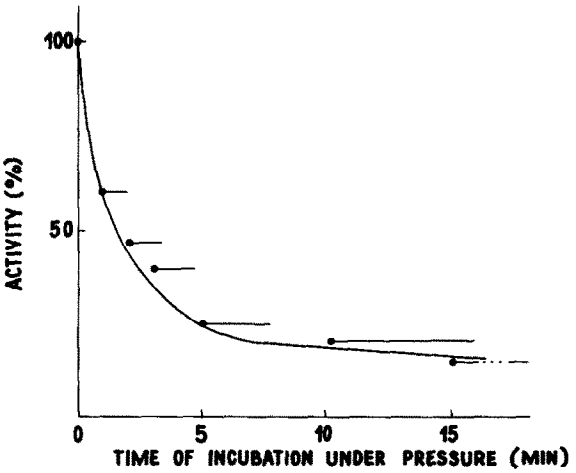

Fig.4. The time course of LDH deactivation under a pressure of 1 kbar. The point at $t = 0$ corresponds to atmospheric pressure (curve a in fig.3) and was taken as 100% of activity (for details see text).

that NADH, when present separately, did not prevent deactivation. Pyruvate, on the other hand, being introduced at any time after application of high pressure stopped deactivation, as can be seen from the constant reaction rate throughout the measurement time (see fig.3). The horizontal line segments on fig.4 that rise from the points corresponding to the onset of the reaction indicate that the activity of the reacting enzyme remained constant for a sufficient period of time, while decreasing noticeably in the absence of pyruvate.

As shown in [10], deactivation of LDH by high pressure is the result of dissociation of this tetrameric enzyme into inactive dimers. The stabilizing effect of pyruvate may, therefore, signify that this substrate causes a substantial increase in the association constant of LDH dimers.

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