

Effects of High Pressure upon the Kinetic Constants of the Enzyme Fumarase

BJØRN ANDERSEN and POUL ERIK BROE

Chemical Laboratory IV, H. C. Ørsted Institute, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark

This paper describes an apparatus enabling continuous spectrophotometric measurements to be made at variable high pressures up to 10 000 atm. Any predetermined pressure can be established within 15 sec.

The apparatus has been used to examine the high pressure kinetics of the enzyme fumarase, which catalyzes the interconversion of fumarate to L-malate.

A series of determinations of the initial velocity as a function of concentration and pressure has allowed the determination of the pressure dependence of the kinetic parameters V and K_m . When either fumarate or L-malate is the starting material increasing pressure leads to a decrease in the initial velocity. The decrease is due to a decrease in V , since K_m does not alter with pressure. From the theoretical expressions for V_m and K_m it is seen that this is the case when the activation volumes ΔV^\ddagger for the different enzyme forms are of same order of magnitude.

A review of investigations of the effects of high pressure upon catalytically active proteins shows that these may be classified into two groups. In the first the degree of inactivation of the protein is described as a function of pressure and time.¹⁻⁶ Exposure of the enzyme to a certain pressure for a given time is followed by a determination of the activity of the enzyme after it has returned to normal pressure. A technique for assays of this type has been described by Brandts *et al.*⁷ investigating the denaturation of ribonuclease up to 3000 atm.

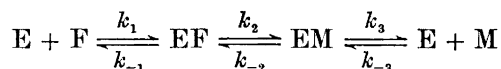
In the other group of studies the high pressure is applied to an incubation mixture of the enzyme and an appropriate substrate. Examples of investigations of this type include the systems chymotrypsin-casein⁸ and invertase-sucrose.⁹ All the experiments described have employed the discontinuous technique mentioned above.

Information concerning the mechanism of a given enzyme-substrate system could be derived from the pressure dependence of the kinetic parameters K_m (Michaelis constant) and V (maximum velocity). And these constants may be

obtained from a series of determinations of the initial velocity, v , as a function of the substrate concentration, s , and pressure. Such determinations require the rapid application of high pressure after the start of the reaction and continuous measurement of the degree of reaction.

This paper describes an apparatus for performing such experiments, and its application to an enzyme-substrate system. For several reasons, the system chosen was the reversible, fumarase-catalyzed transformation of fumarate to L-malate. Firstly, the kinetics of this system in phosphate buffer at normal pressure has been investigated carefully.¹⁰ At substrate concentrations too low to give rise to substrate activation¹¹ the system behaves in a purely hyperbolic manner. Secondly, continuous determination of the fumarate concentration may easily be made from spectrophotometric measurements in the UV region. Thirdly, equilibrium in the reaction occurs when the concentration of L-malate is about four times that of fumarate, making it possible to measure the velocity of both the forward and reverse reactions. Finally, the choice of fumarase at a given pressure has the advantage that measurements of the equilibrium constant for the overall reaction can be carried out so that the kinetic parameters determined can be verified, as described below, using the Haldane relation.

The reaction between enzyme and its substrates can be described by



where F and M denote fumarate and L-malate, respectively, and E is the free enzyme. $(E_0) = (E) + (EF) + (EM)$ represents the total amount of enzyme. With this model the initial velocities for the two substrates are

$$v_F = \frac{k_1 k_2 k_3 (F)(E_0)}{(k_1 k_2 + k_1 k_{-2} + k_1 k_3)(F) + k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3}$$

and

$$v_M = \frac{k_{-1} k_{-2} k_{-3} (M)(E_0)}{(k_{-1} k_{-3} + k_2 k_{-3} + k_{-2} k_{-3})(M) + k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3}$$

respectively. From these two equations can be derived the expressions for the parameters V and K_m , where V is the maximum attainable velocity in the given direction and K_m the corresponding Michaelis constant. They are

$$V^F = \frac{k_2 k_3 (E_0)}{k_2 + k_{-2} + k_3}; \quad K_m^F = \frac{k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3}{k_1 (k_2 + k_{-2} + k_3)}$$

and

$$V^M = \frac{k_{-1} k_{-2} (E_0)}{k_{-1} + k_2 + k_{-2}}; \quad K_m^M = \frac{k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3}{k_{-3} (k_{-1} + k_2 + k_{-2})}$$

From these equations the so-called Haldane relation can be written down. Thus, at equilibrium ($v=0$),

$$\frac{V^F K_m^M}{V^M K_m^F} = \frac{(M)}{(F)} = K_{eq}$$

where K_{eq} is the (pressure dependent) equilibrium constant.

EXPERIMENTAL

Pressure generator. In a two-stage process a pre-set pressure up to 10 000 atm can be delivered by the generator in 15 sec. The connection to the measuring cell is stainless steel tubing of 8 mm O.D., 2 mm I.D. The pressure transmitting medium is a mixture of 50 % Esso white spirit and 50 % Shell Dialac. A detailed description of the apparatus has been given by Grønlund and Andersen.¹²

Measuring cell. The measuring cell (Fig. 1) is constructed from Arne Steel SIS 2140. In the construction process the steel cell is hardened to 350° Brinell and afterwards hard chromium plated, while the end parts are hardened to 450° Brinell before plating. The surfaces against the sapphire windows are polished to optical smoothness. The windows are cylindrical sapphires, diameter 6 mm, length 6 mm. As can be seen in Fig. 1 they are

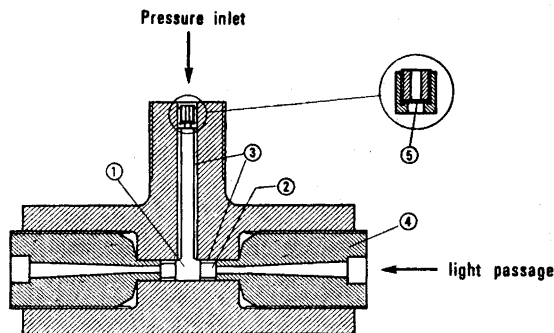


Fig. 1. Measuring cell. 1. Reaction mixture. 2. Sapphire window. 3. Teflon lining. 4. Window supports, threaded end parts, 5. Rubber membrane.

placed unsupported in the teflon lining and are surrounded by the liquid. This was found necessary to avoid crushing the sapphires. To hold them in the correct position before pressure is applied, a steel spring is placed between the sapphires to push them against the window support area on the end parts. In the vertical section of the tube a membrane separates the pressure liquid from the reaction mixture. The light path between the two sapphires is approximately 10 mm long, but is determined more precisely after each polishing by measuring the apparent absorption extinction coefficient of a standard calibration solution.

Optical path and detection equipment. The light source is a deuterium lamp. Light passes through a lens system to the measuring cell and, after passage through the reaction mixture, is focussed by another lens system on the slit of a Heath monochromator E. U. 800. An EMI 9558 Q. C. photomultiplier tube detects the selected light. The signal from this tube passes to a Keithley 4145 amplifier, the output of which is registered on a Philips chart recorder PM 8100.

Procedure. L-Malic acid and Na₂-fumarate were each dissolved in 0.05 M, pH 6.5, phosphate buffer. The concentration ranges were 0–10 mM for L-malate and 0–2 mM for fumarate. A stock solution of enzyme was made from crystalline fumarase suspended in (NH₄)₂SO₄, 2 mg/ml (Boehringer) by dissolving 10 μl of this in 2 ml 0.05 M, pH 6.5, phosphate buffer. Before each day's assay the activity of the enzyme was standardized.

A typical experiment for the determination of initial velocity was performed as follows: 5 ml of substrate solution was incubated with enzyme at time zero. The incubation mixture was transferred to the measuring cell. For 5–10 sec the recorder followed the reaction at 1 atm. Then during the next 10–15 sec the pressure was raised to the pre-set value and, at this constant pressure, the transmission of the reaction mixture was followed on the recorder as a function of time.

Equilibrium determinations were made from the L-malate side using a somewhat higher enzyme concentration. When equilibrium had been attained at 1 atm, the pressure

was raised to the desired value and maintained until the system was again in equilibrium. After some time the pressure was lowered to 1 atm again to ensure that the system had returned to its initial condition.

Calculation. From the series of measurements of the initial velocity as a function of concentration and pressure the kinetic constants were evaluated using a computer program in which normal hyperbolic kinetics are assumed as the basis for a weighted, iterative least squares fit of the experimental data.

RESULTS AND DISCUSSION

Pressure influence on the protein during the reaction. In the first series of experiments the influence of pressure upon the catalytic action of the protein was investigated by making identical assays of the system at different pressures.

The speed of reaction, $-dc/dt$, decreases with increasing pressure up to 3000 atm, at which it is nearly zero. This decrease is not due to time-dependent irreversible denaturation of the protein since, even after a considerable time at pressures higher than 3000 atm, where the reaction has totally stopped, there is no sign of destruction of the catalytic activity when the system is at normal pressure again. Fig. 2 shows the results of two identical assays

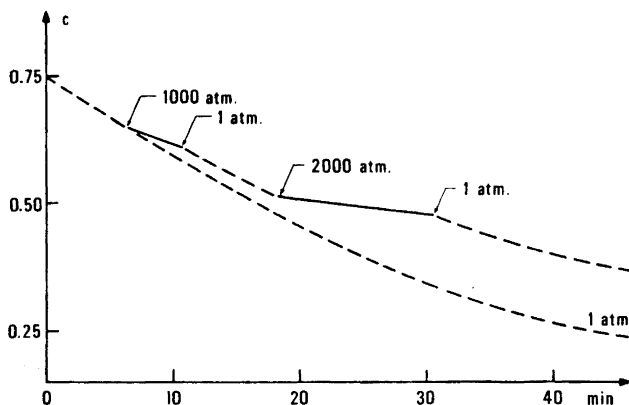


Fig. 2. Fumarase-catalyzed conversion of fumarate to L-malate. The lower curve shows the concentration of fumarate as a function of time at a constant pressure of 1 atm. The upper curve shows the influence of increased pressure during two periods. The speed of reaction $-dc/dt$ is diminished in the pressure periods.

starting with fumarate and measuring the time-dependent change of concentration of fumarate. For one of the experiments the pressure was constant at 1 atm while in the other it was 1 atm for about 6 min after which it was raised to 1000 atm for the next 6 min. After returning to 1 atm for a short period, the pressure was again raised, this time to 2000 atm for about 13 min. The concentration-dependent velocity $-dc/dt$ decreases in the high pressure periods by an amount depending upon the magnitude of the pressure.

Since the time required to make a determination of initial velocity is considerably shorter than that of any of the pressure intervals used here, it was concluded that, at least between 1 and 3000 atm, initial velocity could be measured without any risk of irreversible denaturation of the protein.

Whether the expression "reversible denaturation" might be used to describe the action of pressures from 1–3000 atm upon the enzyme is, at this stage, entirely a matter of terminology and definition.

Influence of pressure upon initial velocity and kinetic parameters. Since the concentration-dependent speed of reaction clearly decreased with increasing pressure, it was expected that the initial velocity was also reduced. Assuming hyperbolic kinetics, decrease of initial velocity will arise from (a) maximal velocity V decreasing, (b) Michaelis constant K_m increasing, or (c) both kinetic parameters simultaneously changing as in a and b.

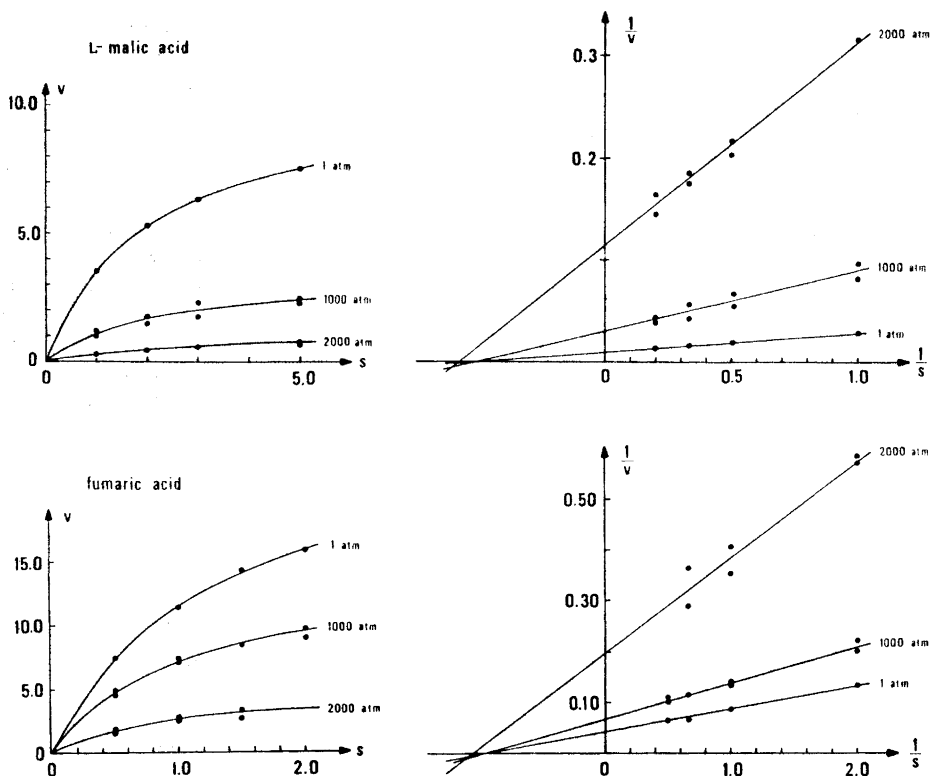
Note that a, b, c correspond to non-competitive, competitive, and uncompetitive inhibition, respectively.

Table 1. Initial velocity v as a function of concentration for L-malate and fumarate.

v in $\mu\text{M}/\text{min}$							
L-Malate				Fumarate			
mM	1 atm	1000 atm	2000 atm	mM	1 atm	1000 atm	2000 atm
1.0	3.56	1.05 1.23	0.31	0.5	7.40	4.46 4.88	1.76 1.67
2.0	5.32	1.48 1.75	0.45 0.49	1.0	11.49	7.22 7.40	2.80 2.48
3.0	6.38	2.37 1.78	0.57 0.54	1.5	14.29	8.46	3.47 2.74
5.0	7.56	2.33 2.49	0.60 0.69	2.0	15.87	9.71 9.05	—

Table 2. Pressure dependence of the kinetic constants K_m and V as calculated from the values in Table 1. Graphs corresponding to these results are shown in Figs. 3 and 4.

P atm	L-Malate		P atm	Fumarate	
	K_m^M mM	V^M $\mu\text{M}/\text{min}$		K_m^F mM	V^F $\mu\text{M}/\text{min}$
1	1.95 ± 0.02	10.52 ± 0.02	1	1.26 ± 0.08	26.00 ± 0.74
1000	2.08 ± 0.69	3.43 ± 0.48	1000	0.95 ± 0.13	13.91 ± 0.80
2000	1.75 ± 0.40	0.87 ± 0.07	2000	1.01 ± 0.50	5.23 ± 1.24



Figs. 3 and 4. Initial velocity v as a function of substrate concentration, s , plotted at 3 different pressures. To the left a direct (hyperbolic) plot, to the right the same values in a double reciprocal plot. v in $\mu\text{M}/\text{min}$; s in mM .

Measurements of the initial velocity for various substrate concentrations were performed at 1 atm, 1000 atm, and 2000 atm. They were made with both fumarate and L-malate as substrate. The initial velocities (Table 1) were calculated from the recorder traces, and values of V^F , K_m^F , V^M , and K_m^M were calculated from these using the computer program briefly described above (Table 2). Graphs corresponding to these results are shown in Figs. 3 and 4.

As an independent verification of the accuracy of the values which were found of V^F , V^M , K_m^F and K_m^M , measurements were made of the equilibrium constant at different pressures. As expected the equilibrium was displaced against malate formation with increasing pressure. The results are listed in Table 3, together with corresponding values calculated from the kinetic measurements using the Haldane relation. The agreement between the two sets of experiments is good.

Table 2 shows that the decrease of initial velocity with increasing pressure is due to a corresponding decrease in the maximal velocity, the K_m values

Table 3. The pressure dependence of the equilibrium constant K_{eq} as measured directly in equilibrium studies and as calculated from the kinetic measurements using the Haldane relation.

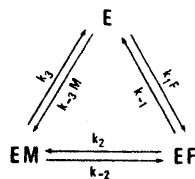
	1 atm	1000 atm	2000 atm
$K_{\text{eq}} = \frac{(M)}{(F)}$	4.0	5.9	9.0
$K_{\text{eq}} = \frac{V^F K_m^M}{V^M K_m^F}$	3.8	7.4	10.1

being unchanged. This observation is similar to those found for non-competitive inhibition, in which the decomposition of the enzyme-substrate complex is affected.

The molecular change of the reactions may be written



where the molecular volume was 88.99 ml/mol for $F + H_2O$ and 84.09 ml/mol for M . From these values might have been predicted the increase in the final malate concentration with pressure that was observed experimentally (Table 3). The reaction is not spontaneous, but implies transient complexes with the enzyme. In agreement with this the velocity decreased, with both fumarate and malate as starting material. So it was reasonable to assume that the pressure dependence was connected with an effect on the volume of the transient activated complexes. The enzyme can exist in three forms, E , EF , and EM . These are related in the following way:



The velocity constants here are those appropriate at 1 atm. The pressure dependence for the velocity constants, according to the transition state theory, are

$$\frac{\delta \ln k}{\delta P} = \frac{-\Delta V^\ddagger}{RT}$$

where ΔV^\ddagger is the difference in molecular volume between one enzyme form and the activated transient intermediate, calculated in the direction of the appropriate velocity constant. Integration from P_0 to P gives

$$\ln \frac{k_P}{k_0} = \frac{-\Delta V^\ddagger}{RT} (P - P_0)$$

Since P_0 is 1 and is negligible in the present experiments,

$$k_p = k_0 \exp(-\Delta V^\ddagger P/RT)$$

Thus to introduce the pressure dependence of the velocity constants they must be multiplied by an exponential factor containing the relevant ΔV^\ddagger . The corresponding kinetic parameters V_p^F , V_p^M , K_{mP}^F and K_{mP}^M may be derived in the same way. For example,

$$V_p^F = \frac{k_2 \exp(-\Delta V_2^\ddagger P/RT) \times k_3 \exp(-\Delta V_3^\ddagger P/RT) \times E_0}{k_2 \exp(-\Delta V_2^\ddagger P/RT) + k_{-2} \exp(-\Delta V_{-2}^\ddagger P/RT) + k_3 \exp(-\Delta V_3^\ddagger P/RT)}$$

If the various ΔV^\ddagger values in these four expressions are of approximately the same size, then the exponential terms in the expressions for K_m cancel out, but one remains in each of the expressions for V . That is,

$$V_p^F = V^F \exp(-\Delta V^\ddagger P/RT)$$

$$K_{mP}^F = K_m^F$$

$$V_p^M = |V^M| \exp(-\Delta V^\ddagger P/RT)$$

$$K_{mP}^M = K_m^M$$

This is in agreement with the experimental results.

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