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The catalytic activities of monomeric enzymes show complex pressure dependence

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High hydrostatic pressures in the biologically relevant range (≤ 1,200 bar) are known to cause dissociation of oligomeric enzymes in vitro, whereas protein denaturation requires pressures far beyond this range. Pressure-induced inactivation phenomena attributable to neither of these effects are shown to occur in monomeric enzymes. Three different types of pressure dependence can be distinguished: (1) a linear dependence of catalytic rate constants on pressure, as predicted by the activated complex theory, observed for lysozyme and thermolysin; (2) a biphasic profile consisting of two linear contributions, found for trypsin; (3) maximum curves, as observed for both directions of the octopine dehydrogenase reaction. The third case may be ascribed to a pressure-induced decrease in the partial specific volume of the protein, resulting in reduced flexibility of the active site. This mechanism may also apply to the pressure-induced inactivation of assembly systems stabilized against dissociation in the cell.

Activation volume; Compressibility; Hen egg-white lysozyme; Octopine dehydrogenase; Thermolysin; Trypsin

1. INTRODUCTION

The pressure-induced inhibition of biochemical processes and hence of bacterial growth has been attributed to the dissociating effect of pressure on assembly systems such as ribosomes, tubulin or oligomeric enzymes [1]. However, recent findings have shown that simulation of in vivo conditions may stabilize these systems against pressures even beyond the biologically important range [2]. Thus, an alternative explanation has been proposed [3], which relies on two well-established facts: (1) biological activity in macromolecules commonly requires structural flexibility [4,5]; (2) many globular proteins have positive intrinsic compressibilities, which correlate with partial specific volumes as well as with indicators of flexibility such as protease digestability [6]. Based on these observations, we propose that the pressure-induced reduction in partial specific volume may reduce flexibility and hence inhibit biological activity.

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Abbreviations: BAPA, α-*N*-benzoyl-L-arginino-*p*-nitroanilid; FA-Gly-Leu-NH_2, *N*-(3-[2-furyl-]acryloyl-)glycyl-leucyl-amide; NAD, nicotinamide adenine dinucleotide; NADH. nicotinamide adenine dinucleotide, reduced; ODH, octopine dehydrogenase; $\Delta V^{\neq}$, activation volume.

Enzymes: lysozyme (E.C. 3.2.1.17.), octopine dehydrogenase (E.C. 1.5.1.11.), thermolysin (neutral protease from *Bacillus thermoproteolytic us*, E.C. 3.4.24.2.) trypsin (E.C. 3.4.21.4.).

Globular monomeric enzymes provide model systems for testing this hypothesis which, however, refers to a broad range of biochemical systems. So far, high pressure effects on monomeric enzymes have mainly been studied in the range of pressure-induced denaturation around 4 kbar [1,7,8]. These studies do not allow conclusions with respect to the biologically relevant pressures.

Commonly, the dependence of the logarithm of the rate of a chemical reaction on pressure ($\ln k$ vs. p) is a linear function, with the activation volume as the proportionality factor. Deviations from linearity may reflect compression phenomena. In the present study, we investigated the effects of pressures ranging from 1 to 1,000 bar on a number of monomeric enzymes including lysozyme (14.4 kDa), trypsin (23.5 kDa), thermolysin (34.6 kDa) and octopine dehydrogenase (38 kDa). The latter allows to measure rate constants in both directions at similar conditions, thus providing the opportunity to distinguish kinetic effects originating from the catalyzed reaction from structural effects reflecting the altered flexibility of the enzyme.

Octopine dehydrogenase (ODH) from scallops, one of the very few monomeric NAD-dependent dehydrogenases, has been characterized with respect to its kinetic mechanism [9] and to its folding behaviour [10]; no sequence or structural data are available. From its physiological role and its physical properties, the enzyme may be presumed to be homologous to lactate dehydrogenase.

2. MATERIALS AND METHODS

Enzymes and substrates were purchased from Boehringer

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Table I

Concentrations used in the enzyme assays

Protein	Conc. μg/ml (mM)	Substrate(s)	Conc. mg/ml (mM)	Cofactor	Conc. μg/ml (mM)	[Tris-HCl] (mM)
Trypsin	4.3	L-BAPA	0.044 (0.10)	–	–	48
Lysozyme	3.7	*Micrococcus lysodeikticus*	83	–	–	41
Thermolysin	5.6	FA-Gly-Leu-NH_2	3.0 (102)	–	–	39
ODH (octop. synthesis)	4.0	Pyruvate	0.14 (2.5)	NADH	0.18 (0.25)	23
		Arginine	0.44 (5.0)			
ODH (octop. degradation)	9.5	Octopine	2.96 (12.0)	NAD^+	1.33 (2.0)	12.5

Mannheim (Germany), except for thermolysin, ODH, FA-Gly-Leu-NH_2 and octopine, which were from Sigma. Conditions for the activity assays were optimized to obtain constant activity for at least 10 minutes after the dead time (3 min) needed for loading, mounting and pressurizing the cell in the photometer (Gilford 2000 S). Concentrations of the enzymes, substrates and coenzymes are displayed in Table I. The standard buffer system was Tris-HCl pH 8.0, except for ODH, which was assayed at pH 8.3. The standard temperature was 20°C.

The high pressure UV-transmission cell was described previously [11]. Enzyme assays were mixed in a separate vessel and then transferred into the cell.

Each point in the experimental graphs (Figs. 2–4) represents the initial slope of an independant kinetic experiment; each graph shows one of at least two series of experiments with identical results.

3. RESULTS

3.1. *General considerations*

The rates of chemical reactions depend on the pressure according to

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT} \qquad (1)$$

Thus, the slope of the $\ln k = f(p)$ plot allows one to calculate the activation volume $\Delta V^{\neq}$. This holds for both catalyzed and uncatalyzed reactions. Negative activation volumes correspond to a pressure-induced enhancement (apparent enzyme 'activation') and vice versa (Fig. 1a). In the case of two consecutive reactions with different activation volumes, a crossing over of the corresponding profiles may lead to a change in the rate-limiting step at a certain pressure and hence to a (downward) kinked profile (Fig. 1b). Any pressure-induced structural changes that affect the enzymatic activity may induce deviation from this linear dependence. In the pressure-range studied here, and in the absence of denaturants or other destabilizing agents, such effects cannot be ascribed to unfolding.

3.2. *Linear dependence of* ln*k on pressure: lysozyme and thermolysin*

Out of the four monomeric enzymes studied, lysozyme and thermolysin show the simple linear ln*k* vs. *p* dependence predicted from equation 1. Obviously,

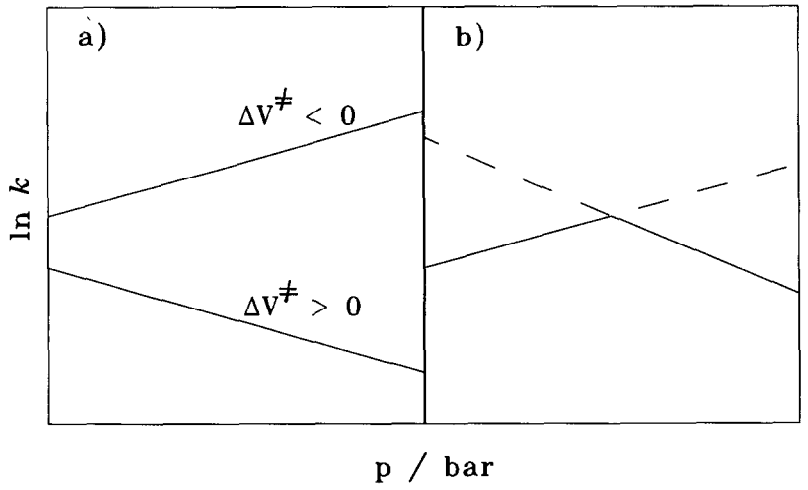

Fig. 1. Pressure dependence of rate constants as expected from equation 1. (a) Simple reactions with $\Delta V^{\neq} < 0$ (top) and $\Delta V^{\neq} > 0$ (bottom); (b) two-step-reaction in which superposition of the profiles leads to a change of the rate limiting step.

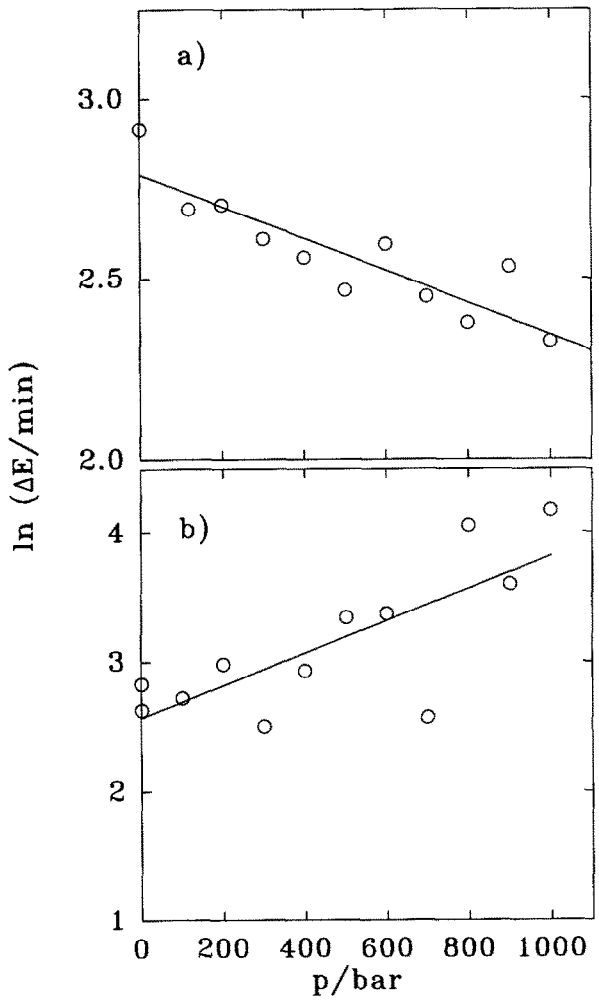

Fig. 2. Pressure dependence of the catalytic activities of hydrolases. (a) Hen egg-white lysozyme; (b) thermolysin (neutral protease from *Bacillus thermoproteolyticus*).

there are neither pressure effects on the enzyme nor an overlap of different kinetic features of consecutive reactions. As shown in Fig. 2a, lysozyme is apparently inactivated by pressure. The corresponding positive activation volume ($\Delta V^{\neq}$ = 10.9 ml/mol) must be attributed to the catalyzed reaction, i.e. the hydrolysis of the peptidoglycan in the cell walls of *Micrococcus lysodeikticus*. On the other hand, thermolysin is apparently activated (Fig. 2b): the catalyzed hydrolysis of the dipeptide substrate FA-Gly-Leu-NH_2 is connected to a negative activation volume amounting to $\Delta V^{\neq}$ = −30.2 ml/mol. This result is consistent with former studies on this enzyme [12]. Pressure-induced deactivation by denaturation of thermolysin only begins beyond 2,000 bar [13].

3.3. *Biphasic pressure dependence: trypsin*

Like thermolysin and other proteolytic enzymes, trypsin is activated by moderate hydrostatic pressures, revealing a negative activation volume in the rate-limiting step. However, at 430 bar, there is a kink in the profile leading to a phase, where the rate is virtually independent of pressure (Fig. 3). According to the general considerations (section 3.1.), a reaction with $\Delta V^{\neq}$ = 0 becomes rate-limiting at this pressure. The corresponding activation volumes are $\Delta V^{\neq}_1$ = −8.8 ml/mol and $\Delta V^{\neq}_2$ = 0.0 ml/mol.

3.4. *Complex behaviour: octopine dehydrogenase*

The pressure dependence of the ODH activity was monitored for both directions, octopine synthesis (Fig. 4a) and oxidative degradation of octopine resulting in arginine and pyruvate (Fig. 4b). For a reaction with more than one reversible step, no simple relationship between the behaviour in both directions can be predicted.

Both reactions are enhanced by moderate pressures; however, beyond 400 bar the slope decreases and becomes negative, thus leading to optimum curves with maxima at 600 bar, which may be approximated according to:

$$y = a + bx - \exp[(cx - d)^2] \quad (2)$$

Based on the assumption that some rapidly growing inhibitory effect causes the deviation from linearity, this approach allows one to calculate activation volumes for the first, linear part of the profile and to determine at which threshold of pressure, p_{dn}, a defined deviation from linearity, Δ_n, is attained. The results of these calculations and the pressures of maximum activity are summarized in Table II. It turns out that the pressure of maximum activity as well as the deviation parameters are identical for both directions of the reaction within the experimental error.

From the findings, first, that the ln$k$ vs. p plots for both reactions turn downward at the same pressure, and second, that the profiles are fitted by equation 2 better

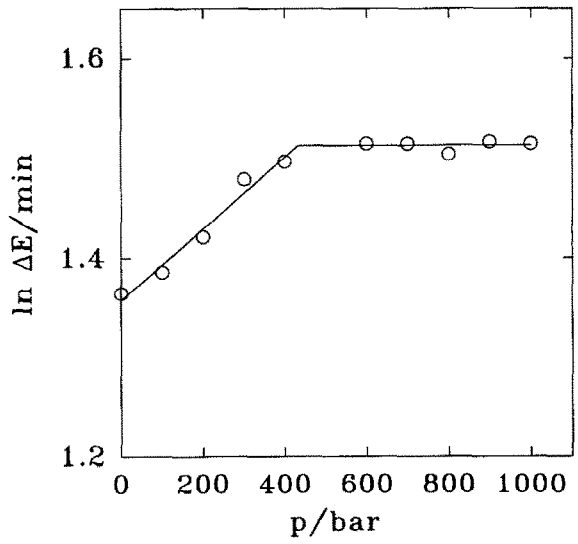

Fig. 3. Pressure dependence of the catalytic activity of trypsin from bovine pancreas.

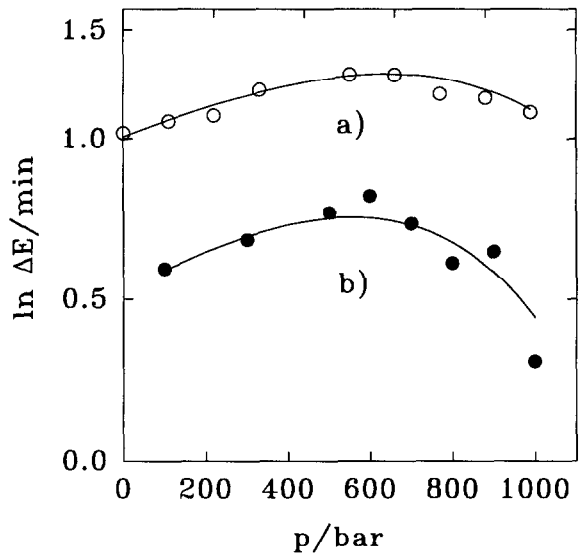

Fig. 4. Pressure dependence or the catalytic activity of octopine dehydrogenase. (a) Octopine synthesis; (b) octopine degradation.

than by two straight lines, we conclude that the decrease in the reaction rate of both octopine synthesis and degradation may be ascribed to a pressure effect on the protein rather than on the catalyzed reactions. The most simple explanation would refer to changes in the conformation or domain structure of the protein and/or its packing density.

4. DISCUSSION

Until now, the pressure-dependence of catalytic rate constants in monomeric enzymes has been assumed to follow equation 1 between 1 and 1,000 bar. Investigations of pressure-induced deactivation usually did not contain more than one point within this range. We have shown that this simple model holds for lysozyme and thermolysin, but cannot be generalized. For trypsin, a biphasic profile is found, and even more complex patterns are observed for both directions of the octopine dehydrogenase reaction.

Lysozyme is the only enzyme whose structure at high hydrostatic pressure (1,000 bar) has been resolved by X-ray crystallography [14]. Volume changes induced by hydrostatic pressure are localized in the a-helical domain and in the active site cleft, but do not provoke any major structural rearrangements. The assumption of pressure-induced changes of conformation or hydration of lysozyme in oversaturated solution has been useful for the understanding of the pressure-induced inhibition of lysozyme crystallization [15]. However, no compression-induced deactivation of lysozyme could be detected in the present study.

The effect of biologically relevant pressures on the activity of thermolysin has been studied using both, dipeptides [12], and β-lactoglobulin [13] as substrates. Both studies revealed a negative activation volume for the catalyzed reaction, as is confirmed by the present data. Although measured under rather different conditions, the activation volumes reported in [12] (FA-Gly- Leu-NH_2, pH 4.8: 35 ml/mol; FA-Gly-Phe-Ala, pH 6.88: 23 ml/mol) are comparable to the present results. In the latter study, no activation volumes were given; quantitative evaluation of the data may prove difficult, because effects on the substrate protein, on the cleavage kinetics and on the enzyme itself cannot be distinguished.

In the case of trypsin, the biphasic pressure dependence may be related to the well-established two-step mechanism of serine proteases, which includes a covalently linked acyl-enzyme complex as an intermediate [16]. Early high pressure studies on this enzyme only referred to irreversible inactivation in the 6–8 kbar range [17].

The mechanism of octopine dehydrogenase is more complex, since it includes the formation of dead-end complexes as well as 'memory' effects [9]. In the direction of octopine synthesis, the uncatalyzed formation of a Schiff-base between pyruvate and arginine is followed by the NADH-dependent enzymatic reduction of the C-N double bond leading to octopine.

In the present study, ODH is shown to exhibit most intriguing deactivation phenomena at pressures between 500 and 1,000 bar, which are similar for both directions. Interpretation of the data as superposition of two kinetics with different $\Delta V^{\neq}$ appears unlikely considering the shape of the profiles and the fact that for both directions the deactivation begins at the same pressure. The similarity of the profiles for octopine synthesis and octopine degradation also rules out possible explanations in terms of substrate binding constants.

In order to elucidate the detailed mechanism, further

Table II

Characteristic data for the pressure dependence of the octopine dehydrogenase reaction in both directions

	s_i ($kbar^{-1}$)	$\Delta V^{\neq}$ (ml/mol)	p_{max} (bar)	p_{d1} (bar)	p_{d2} (bar)	p_{d3} (bar)
Octopine synth. (Fig. 4a)	67.4	−16.4	600	400	500	600
Octopine synth. (not shown)	95.1	−9.4	600	550	575	650
Octopine degrad. (Fig. 4b)	38.6	−11.0	600	400	500	600
Octopine degrad. (not shown)	50.5	−12.3	600	550	600	700

Initial slope (s_i), activation volume ($\Delta V^{\neq}$), pressure of maximal activity (p_{max}) and pressure p_{dn} at which the deviation from linearity surpasses a certain threshold $\Delta_n = s_i \times p_n$ with $p_1 = 50$ bar, $p_2 = 100$ bar and $p_3 = 200$ bar.

studies, including variations in substrate concentrations and determination of the various binding constants as a function of pressure, are required. In addition, sequence and structural data would be of utmost importance in localizing the observed effects.

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REFERENCES

[1] Jaenicke, R. (1981) Annu. Rev. Biophys. Bioeng. 10, 1–67.
[2] Groß, M. (1993) Dissertation, University of Regensburg.
[3] Groß, M., Jaenicke R. & Nierhaus K.H. (1992) in: High pressure and Biotechnology (Balny, C., Hayashi, R., Heremans, K. and Masson P. Eds.) pp. 159–161, John Libbey Inc./INSERM, Paris.
[4] Tsou, C.-L. (1986) Trends Biochem. Sci. 11, 427–429.
[5] Huber, R. (1988) Angew. Chem. Int. Ed. Engl. 27, 79–88.
[6] Gekko, K. and Hasegawa, Y. (1986) Biochemistry 25, 6563–6571.
[7] Suzuki, K. and Taniguchi, Y (1972) in: The effects of pressure on organisms (Sleigh, M.A. and Macdonald, A.G. Eds.) pp. 103–124.
[8] Weber, G. and Drickamer, H.G. (1983) Quart. Rev. Biophys. 16, 89–112
[9] Monneuse-Doublet, M.O., Olomucki, A. and Buc, J. (1978) Eur. J. Biochem 84, 441–448.
[10] Zettlmeißl, G., Teschner, W., Rudolph, R., Jaenicke, R. and Gäde, G. (1984) Eur. J. Biochem. 143, 401–407.
[11] Schade, B.C., Lüdemann, H.-D. and Jaenicke, R. (1980) Biochemistry 19, 1121–1126.
[12] Fukuda, M. and Kunugi, S. (1984) Eur. J. Biochem. 142, 565–570.
[13] Dufour, E., Hervé, G. and Haertlé T. (1992) in: High pressure and Biotechnology (Balny, C., Hayashi, R., Heremans, K. and Masson P. Eds.), pp. 147–150, John Libbey Inc./INSERM, Paris.
[14] Kundrot, C.E. and Richards, F.M. (1987) J. Mol. Biol.
[15] Groß, M. and Jaenicke, R. (1993) Biophys. Chem. 45, 245–252.
[16] Kraut, J. (1977) Annu. Rev. Biochem. 46, 331–358.
[17] Miyagawa, K. and Suzuki, K. (1963) Rev. Phys. Chem. Japan 32, 43–49.