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Review

Pressure Effects on Enzyme Functions

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Hydrostatic pressure is a well-known method for studying protein dynamics and hydration. Recent developments in molecular biology allow us to obtain and investigate recombinant proteins from deep-sea organisms living in high-pressure environments. The aims of this review are to give a brief introduction of the thermodynamic principles of pressure effects on proteins and to highlight the effects of hydrostatic pressure on various enzymes, especially enzymes from deep-sea organisms.

Keywords: activation volume, hydrostatic pressure, volume change

Introduction

High hydrostatic pressure is the only technique for disrupting protein dynamics and protein-ligand or protein-protein interactions without altering the temperature and composition of the experimental system. Therefore, pressure effects on proteins have been studied for a long time (1−11) and have been applied in the food industry (12−15). Combination of hydrostatic pressure with various techniques such as NMR (16), Raman (17), absorbance (18, 19) or fluorescence (20, 21) spectroscopy, X-ray (22) or light (23) scattering, crystallography (24), and computer simulations (25) have provided valuable information on the mechanisms of protein stability and function, especially hydration and dynamics. From the viewpoint of protein stability, moderate pressure, usually below 200 MPa, shifts the equilibrium between oligomers and monomers towards subunit dissociation in oligomeric proteins. Much higher pressure, up to 500 MPa, induces unfolding of proteins (26). Thus, pressure effects on protein functions are generally studied under moderate pressures, and pressure effects on protein stability are studied up to higher pressure.

Protein functions can be classified into two categories. One is binding with ligands, ions, lipids, or macromolecules such as subunits, other proteins, carbohydrates, and nucleic acids. The other is an acceleration of chemical reaction rates, so-called enzymatic catalysis. Since enzyme functions are constructed from multiple steps – binding with substrates, chemical reactions, and dissociation of products –pressure effects on enzymatic catalysis can also be divided into two categories: the effects on equilibrium constants and the effects on rate constants. The thermodynamic principles for pressure effects on these constants are summarized briefly in the next two sections.

Pressure Effects on Equilibrium Constants

From thermodynamics, pressure effects on an equilibrium constant, K, can be represented by the following equation.

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = -\frac{\Delta V}{RT} \qquad (1)$$

Here P is the pressure, ΔV is the volume change from the initial-state to the final-state, R is the gas constant, and T is the absolute temperature. If ΔV is positive, the equilibrium shifts towards the initial-state when the pressure increases, and vice

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However, for enzyme systems, there exist multiple simultaneous equilibria: the association or dissociation of subunits, substrate binding, product releasing, conformational changes such as folding-unfolding or open-closed, ionization, and hydration. Thus it needs to be taken into account that experimentally measured ΔV values include contributions from other equilibria in the system. Since the pressure is applied to the entire system, all the equilibria are affected and each is shifted in the direction such that the total volume of the system decreases. Therefore, it is possible that some equilibria shift in the opposite direction to that predicted from the corresponding volume change.

The total volume of a system, V_t, can be represented by

$$V_t = \sum_i n_i \cdot V_i \tag{2}$$

where n_i and V_i are the molar quantity and partial molar volume of component i, respectively. If the system is a 'closed system' and no chemical reaction is occurring (for example, the pressure-induced unfolding of a protein), molar quantities of all components do not change. Therefore, when we measure the pressure dependence of an equilibrium constant, the corresponding total volume change of the system can be calculated as follows.

$$\begin{aligned}\Delta V_t &= \sum_i n_i^{\mathrm{F}} \cdot V_i^{\mathrm{F}} - \sum_i n_i^{\mathrm{I}} \cdot V_i^{\mathrm{I}} = \sum_i n_i \left(V_i^{\mathrm{F}} - V_i^{\mathrm{I}}\right) \\ &= \sum_i n_i \Delta V_i\end{aligned} \tag{3}$$

The superscripts F and I indicate the final state and the initial state, respectively, and ΔV_i is the partial molar volume change for component i. Since the volume changes are usually normalized by a molar quantity of protein, n_p, the experimentally reported volume change, ΔV_{exp}, is given by

$$\begin{aligned}\Delta V_{exp} &= \sum_i \left(n_i / n_p\right) \Delta V_i = \sum_i \left(c_i / c_p\right) \Delta V_i \\ &= \Delta V_p + \sum_{i \neq p} \left(c_i / c_p\right) \Delta V_i\end{aligned} \tag{4}$$

where c_i and c_p are concentrations of the component i and protein, respectively, and ΔV_p is the partial molar volume change of protein.

It is noteworthy that experimental protein concentrations usually range from the order of mM (NMR methods) to nM (fluorescence or activity methods) while the concentration of water is 55.6 M. Therefore, the weight term of Equation 4, c_i/c_p, for water ranges from the order of 10^5 to 10^{10}. Of course, the volume of bulk water, which does not interact with the other components, is not changed. However, one protein molecule has hundreds or thousands of hydrated water molecules, and the contribution of the volume change of water, ΔV_w, to ΔV_{exp} value cannot be neglected. Put simply, a 10 ml/mol change in ΔV_{exp} corresponds to only a 10 μl/mol change in the average volume of the hydrated water.

Other components, such as buffers, salts, and reductants, can also contribute to the volume change. However, if the ΔV_i values of these components are negligible, *i.e.*, interactions between these components and other components are almost same in the initial and final states of the equilibrium, the contribution of these components to the ΔV_{exp} can be neglected.

In 1959, Kauzmann modeled the partial molar volume of a protein in water, V_p, by the following equation (27).

$$V_p = V_{atm} + V_{cav} + V_{hyd} \tag{5}$$

The first term, V_{atm}, is the van der Waals volume of atoms. The second term, V_{cav}, is the volume of cavities, which are interatomic spaces in a protein molecule. The last term, V_{hyd}, is the contribution of hydration. The sign of this term is usually negative since the volume of a hydrated water molecule is smaller than that of a bulk water molecule. This equation is appropriate when the system contains only protein and water, or when the interactions of the other components in the system can be neglected. Similarly, ΔV_{exp} can be represented by

$$\Delta V_{exp} = \Delta V_p + \left(c_w / c_p\right) \Delta V_w = \Delta V_{atm} + \Delta V_{cav} + \Delta V_{hyd} \tag{6}$$

where ΔV_{atm} is the change in the van der Waals volume of atoms and is negligible in the pressure

range explored in this article. ΔV_{cav} is the volume change of cavities and is strongly compressed by pressure. ΔV_{hyd} is the contribution of hydration and increase in hydration causes a decrease in the total volume of the system.

Pressure Effects on Kinetic Constants

Pressure effects on a kinetic constant, k, can be represented by the following equation from transition-state theory.

$$\left(\frac{\partial \ln k}{\partial P}\right)_T = -\frac{\Delta V^*}{RT} \quad (7)$$

Here ΔV^* is the activation volume, the volume difference between the reactants and the transition-state. Although the activation energy of any reaction is positive, the activation volume could be positive or negative. When the sign of the activation volume is negative, the reaction is accelerated when the pressure is increased. When the sign is positive a pressure increase suppresses the reaction.

As in the equilibrium case, the total reaction of an enzyme is constructed from multiple steps such as substrate binding, product releasing, chemical reactions, and conformational changes. The turnover rate of enzyme catalysis is limited by the slowest reaction step and the different pressure dependences of each reaction step can lead to a change of the rate-limiting reaction. This produces a kink in the plot of ln k against pressure (26). Non-linear pressure dependence is also observed when the enzyme conformation is changed by pressure. If a pressure unfolding occurs in the pressure range of the experiment, the activity of the enzyme depends on its concentration.

Pressure Effects on Enzymes from Organisms Living under Atmospheric Pressure

Many researchers have investigated pressure effects on the functions of enzymes obtained from organisms living under atmospheric pressure. Groβ *et al*. reported ΔV^* values for four monomeric proteins (hen egg-white lysozyme, thermolysin, trypsin, and octopine dehydrogenase from scallops) over a pressure range of 0.1−100 MPa (26). They found that thermolysin and lysozyme showed a simple linear dependence of ln k on pressure with ΔV^* values of −30.2 and 10.9 ml/mol, respectively. On the other hand, trypsin showed a biphasic pressure dependence of activity: it was linearly activated to 40 MPa with a ΔV^* value of −8.8 ml/mol, and retained constant efficiency at higher pressure. The other enzyme, octopine dehydrogenase showed a complex pressure dependence for both octopine synthesis and degradation. The maximum activities of both reactions were obtained at 60 MPa, and the ΔV^* values for the activation of synthetic and degraded reactions were −12.9±3.5 ml/mol and −11.6±0.6 ml/mol, respectively.

Pressure effects on thermolysin were also carefully investigated by Kunugi and coworkers (28−31). They showed that the maximum activity of thermolysin was obtained at 200−250 MPa for a dipeptide substrate, 3-(2-furyl) acryloyl-Gly-Leu-NH_2, and at 100−120 MPa for a heptapeptide substrate, MeOcAc-Pro-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 [where MeOcAc and A_2pr(Dnp) indicate (7-methoxycoumarin-4-yl)acetyl and N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl, respectively] (29). Surprisingly, the magnitude of activation for the former peptide increased 45-fold at about 220 MPa, and the activation volumes changed from −53±3 ml/mol at 10 ºC to −95±5 ml/mol at 45 ºC. Spectroscopic change in the fourth-derivative absorption and fluorescence spectra suggested that the enzyme lost its activity due to conformational change at pressures higher than 200 MPa. On the other hand, complex formation by thermolysin and a proteinaceous inhibitor from *Streptomyces nigrescens* was suppressed by pressure with a volume change of 8.1±0.3 ml/mol (30). These results suggest that high-pressure and high-temperature conditions are favorable for thermolysin.

Other enzymes have been investigated in less detail. Dallet and Legoy reported that the alcohol dehydrogenase from a thermophilic bacterium, *Thermoanaerobium brockii,* was activated by a pressure of 100 MPa, but yeast alcohol dehydrogenase was inactivated by pressure (32). Fujiwara *et al.* reported that the hydrolysis activity of pepsin and proteinase A on a synthetic oligopeptide substrate were reduced, but that for acid-denatured myoglobin was induced by a pressure of 100 MPa (33). Masson *et al*. showed

that the rate-determining step for the hydrolysis of benzoylcholine by human butyrylcholinesterase was changed at a pressure of 100 MPa (34). Kornblatt *et al*. reported that yeast enolase was dissociated from dimer to monomer by pressurization, but that the activity was maintained with 1mM manganese or 5mM magnesium ions (35).

Recently, pressure effects have been used to understand the reaction mechanisms of enzymes in more detail. It is noteworthy that the chemical reaction step is much faster than the rate-limiting step for many enzymes. Hence classical steady-state studies on enzyme kinetics do not directly reveal the chemical step of the reaction. To investigate the enzymatic hydrogen-tunneling reaction, Northrop and coworkers have studied the effect of pressure on kinetic isotope effects in hydride-transfer reactions involving NAD or NADP. They found large negative activation volumes of −38±1 ml/mol for the hydride-transfer reaction of yeast alcohol dehydrogenase and −9.7 ±1.0 ml/mol for folmate dehydrogenase (36, 37). Hay *et al*. also showed that the hydride-transfer from NADH to flavin mononucleotide catalyzed by molphinon reductase from *Pseudomonus ptida* had an activation volume of −15.6±0.8 ml/mol (38). These results suggest that pressure accelerates the enzymatic tunneling reaction and is an important factor for the activation of enzyme catalyzed reactions.

Davydov *et al*. investigated the role of protein-bound water molecules in a catalytic cycle of cytochrome P450s using high-pressure spectroscopy. They reported that pressure induced a high- to low-spin shift of a compound I, which was an oxyferryl heme, and the subsequent P450 to P420 transition in three cytochrome P450s – P450cam, P450BMP, and P450 2B4 (39−41). They also found that the volume changes from low- to high-spin conformations of these P450s without substrate were 20−23 ml/mol, but those of substrate-bound complexes were 91, 49, and 16 ml/mol for P450cam, P450 2B4, and P450BMP, respectively.

Pressure Effects on Deep-sea Enzymes

Recent developments in molecular biology allow us to study the enzymes from deep-sea organisms. The deep sea is an extreme environment with little or no light, low temperature, and high hydrostatic pressure. It was believed that there were no living organisms in the deep sea, but recent exploration with submersibles has shown that there are many organisms which have adapted to that extreme environment. Many bacteria have even been isolated from the Mariana Trench, the deepest sea in the world (42−44). The inside of the cell of such deep-sea organisms must have the same hydrostatic pressure as the outside of the cell. Thus the biological components in the cell, especially proteins, must work under high-pressure conditions. So it is conceivable that proteins from deep-sea organisms have adapted to high-pressure conditions themselves (45−47).

There are only few reports of pressure effects on the deep-sea enzymes. Ferrer *et al*. reported one esterase obtained from the metagenome expression library of the Urania deep-sea hypersaline anoxic basin (3,552m) had about two times higher activity at 20 MPa than at atmospheric pressure, although another esterase was almost unchanged and three others had slightly decreased activity (48). Clark and co-workers reported that a protease from the deep-sea hyperthermophile, *Methanococcus jannaschii*, was activated by a hydrostatic pressure of 50 MPa, although the 20S proteasome from the same bacterium was inactivated by the same pressure (49, 50). Kawano *et al* reported that the transcriptional activity of a recombinant RNA polymerase from a piezophilic bacterium, *Shewanella violacea*, which was isolated from the Ryukyu Trench at a depth of 5,112 m, was enhanced at pressures from 50 to 100 MPa (51). We also found that dihydrofolate reductase from this bacterium was activated by a pressure of 100 MPa (52). Abe *et al*. showed that the activity of two polygalacturonases from the yeast *Cryptococcus liquefaciens* strain N6, which was isolated from the Japan Trench at a depth of 6,500 m, remained almost constant or slightly decreased when the pressure increased from 0.1 to 100 MPa. Interestingly, they also found that the same enzyme from *Aspergillus japonicus*, which is common fungus living under atmospheric pressure, was increased by about 50% at the same pressures (53). These results indicate that a simple proportional relation between enzyme activity under high

pressure and the depth at which the organisms were obtained is not observed.

More detailed experiments were performed by Saito and Nakayama. They reported that the k_{cat} value of a recombinant malate dehydrogenase (MDH) from a deep-sea bacterium, *Moritella* sp. strain 2D2, was increased about 2.5-fold at 62.1 MPa compared to the value under atmospheric pressure (54). However the k_{cat} value of a recombinant MDH from a psychrophilic bacterium, *Moritella* sp. strain 5710, was increased only 1.5-fold by compression. This is despite the sequence similarity of these two MDHs being 94.9%. From site-directed mutagenesis, they found that the histidine-229 of 2D2 MDH was important for thermal stability and activity under high-pressure conditions. Site-directed mutagenesis is a powerful technique for clarifying the adaptation mechanisms of enzymes to high hydrostatic pressure, and investigations with this method are awaited for other deep-sea enzymes.

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