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Review

Pressure effects on intra- and intermolecular interactions within proteins

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Abstract

The effects of pressure on protein structure and function can vary dramatically depending on the magnitude of the pressure, the reaction mechanism (in the case of enzymes), and the overall balance of forces responsible for maintaining the protein's structure. Interactions between the protein and solvent are also critical in determining the response of a protein to pressure. Pressure has long been recognized as a potential denaturant of proteins, often promoting the disruption of multimeric proteins, but recently examples of pressure-induced stabilization have also been reported. These global effects can be explained in terms of pressure effects on individual molecular interactions within proteins, including hydrophobic, electrostatic, and van der Waals interactions, which can now be studied in greater detail than ever before. However, many uncertainties remain, and thorough descriptions of how proteins respond to pressure remain elusive. This review summarizes basic concepts and new findings related to pressure effects on intra- and intermolecular interactions within proteins and protein complexes, and discusses their implications for protein structure–function relationships under pressure.

Keywords: High pressure; Molecular interaction; Protein function; Protein stability

1. Introduction

The Earth is predominantly a high-pressure environment, and 62% of the total biosphere is characterized by pressures greater than 100 bar [1]. Nonetheless, information on the effects of pressure on biomolecules has been relatively scarce until recently. Recent advances in analytical techniques for studying proteins under pressure [2] have facilitated the investigation of molecular interactions within proteins and protein complexes, and have provided new insights into the structure and function of biomolecules at high pressures. Moreover, because pressure effects on proteins often differ from those of temperature, pH, and chemical denaturants, pressure is now regarded as an important variable for examining protein structure–function relationships [3]. High pressure has also proven to be useful for refolding recombinant, oligomeric proteins as an alternative to concentrated chaotropes such as guanidine [4,5].

Pressure has long been recognized as a potential denaturant of proteins; however, the pressure range for protein denaturation differs depending on the structural characteristics of protein. Whereas primary and secondary structures of proteins are not affected by pressures as high as 10 kbar, oligomeric proteins can readily dissociate at pressures below 3 kbar [2,3]. On the other hand, hydrostatic pressure has been used to increase the activity and/or stability of several enzymes, including thermolysin [6], hy-

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drogenase from *Methanococcus jannaschii* [7], and a putative protease from *M. jannaschii* [8] (since determined to be a proteasome).

In general, the effect of pressure on a physicochemical process at equilibrium is governed by the volume change of the process, ΔV. The relationship between ΔV and K, the equilibrium constant for the process, is given by Eq. 1:

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

Thus the application of pressure will cause the equilibrium to shift in favor of the state with the lowest overall volume. The magnitude and sign of ΔV for changes in protein structure will depend on the specific molecular interactions [3,9], but the precise details of ΔV for different interactions in proteins are often difficult to define (a case in point is the controversy that has surrounded the role of hydrophobic interactions in pressure-induced denaturation [10,11]). Whereas the activity and stability of proteins are influenced by myriad interactions, the major factors that contribute to ΔV and hence govern pressure effects include the electrostriction of charged and polar groups, elimination of packing defects, and the solvation of hydrophobic groups [11]. This review summarizes key concepts and recent advances toward the elucidation of pressure effects on molecular interactions within proteins and protein complexes, and discusses important implications of these effects for protein activity and stability. Finally, it should be noted that the molecular forces involved in intramolecular interactions are the same as those that effect intermolecular interactions; thus, the distinction between the two types of interactions is a somewhat artificial construct intended to simplify organization of this review.

2. Intramolecular interactions

2.1. Ionic interactions

Ion pairs in proteins are attractive, short-range interactions that occur between negatively and positively-charged amino-acid side chains over a distance of 4 Å or less. Sometimes ion pairs are also termed salt bridges, but this usually refers only to ion-pair interactions that are also hydrogen bonded [12]. The role of ion pairs in proteins is to stabilize the tertiary [13] and quaternary structure. For example, interprotein salt bridges provide a mechanism for solvent exclusion from the interfacial domain of the cytochrome b_5–cytochrome c complex, in addition to maintaining the stability and specificity of the complex formed [14].

Dissociation of ion pairs leads to electrostriction, which is the contraction of solvents such as water and alcohols [15] due to alignment of dipolar solvent molecules in the electric field of an exposed charge. Because contraction of solvents leads to a volume decrease, pressure is expected to favor ion-pair disruption [16]. However, as discussed by Michels et al. [9], the volume reduction associated with electrostriction should be counteracted by high temperature due to disruption of the highly ordered structure of electrostricted water. Indeed, dissociation of ion pairs upon application of pressure has been shown to produce an overall volume decrease in the range of 10–30 ml/mol [17]. There is a limit to the solvent's compressibility, however, due to repulsive interactions caused by van der Waals repulsion at close distances [18].

Because pressure favors electrostriction [19], pressure can promote disruption of protein salt bridges and induce protein denaturation. In the case of chymotrypsin, disruption of a salt bridge in the proflavin binding site produced a volume change of −30 ml/mol [20]. Furthermore, when a pressure of 500 bar was applied to *Pyrococcus furiosus* rubredoxin, which contains multiple electrostatic interactions [21–23], the protein was destabilized at 110°C [7], possibly as a result of pressure-induced ion-pair dissociation.

On the other hand, pressure may bring about protein stabilization if there are weak intersubunit ion-pair interactions not exposed to solvent, which may be strengthened by pressure. In studies of glutamate dehydrogenase (GDH) from *P. furiosus* by Sun and colleagues, a tetrapeptide extension (Gly–Ser–Gly–Cys) was added to the C-terminal end of the protein [24]. The dangling tail was believed to transmit increased thermal fluctuations throughout the protein, leading to weakened intersubunit ion-pair interactions and destabilization relative to the native form. When 500 bar was applied to the protein at 103°C, the recombinant GDH was stabilized by 24-fold,

whereas the native GDH was stabilized only 3-fold. The enhanced stabilization was attributed to stronger intersubunit ion-pair interactions accompanying decreased thermal fluctuations.

2.2. Hydration

Water molecules exert a profound influence on protein conformation through interactions with amino acids [25]. In proteins, water fills crevices between amino acids, which serves to screen repulsive forces [3] and facilitates motion of the side chains and polypeptide backbone [26]. Upon application of relatively low pressures (<2 kbar), increased conformational fluctuations provide pathways for water to penetrate into the interior of the native protein [27]. These fluctuations are enhanced by pressure due to increased water exchange between the protein interior and bulk solvent. On the other hand, hydration is opposed by pressure-induced reduction of cavity sizes, rendering pressure-enhanced fluctuation inapplicable to some proteins such as apoazurin and basic pancreatic trypsin inhibitor (BPTI) at pressures below 1.5 kbar and 1.2 kbar, respectively [28–30]. This is consistent with the apparent reduction of thermal fluctuations in glutamate dehydrogenases observed by Sun et al. [24] with pressures up to 750 bar. It should be noted that in some cases amino acids can act as two-way valves controlling the exchange of water between the bulk solvent and the protein, whereas in other cases amino acid residues can act as 'one-way check valves' that enable water molecules to be released from the protein interior while impeding penetration into the core [31]. This effect may be responsible for the increased conformational fluctuations with increasing pressure observed for some proteins but not others. The current level of understanding, however, does not allow us to draw general conclusions about pressure effects on protein hydration, or about water penetration in particular.

As a result of penetration of water penetration into the protein interior, pressure likely leads to conformational transitions resulting in unfolding [32]. Water penetration under pressure can induce the protein to adopt the conformation of a molten globule [32], a compact, partially folded conformation without specific tertiary structure [33]. This molten globule form has been observed in the pressure dissociated-denatured Arc repressor, and is thought to be the result of water penetration and non-polar side-chain exposure to solvent [33]. However, water penetration can also lead to increased reactivity as in the case of β-amylase [28], where enhanced activity upon an increase in pressure from 1 to 4 kbar may have been due to decreased rigidity [34] resulting from hydration of the β-amylase binding site.

At very high pressures (>5 kbar), protein aggregation and loss of secondary structure can occur due to hydration. Protein unfolding becomes irreversible at pressures above 5 kbar [35], and aggregation occurs because of increased protein exposure to solvent [36]. Wroblowski et al. [37] proposed that loss of secondary structure above 10 kbar results from the different compressibilities of water and protein, which is thought to disturb the balance of hydrophobic/hydrophilic protein interactions by allowing the solvent to interpenetrate the protein.

Besides protein unfolding and aggregation, increased hydration of the polypeptide can cause changes in protein compressibility and flexibility. Pressure can induce increased hydration of the polypeptide [29,38] leading to decreases in compressibility due to electrostriction of solvent around charged and polar groups and loss of void volume [39]. The decrease in compressibility is expected to decrease mobility of internal regions [29]. On the other hand, pressure-induced hydration can also reduce the number of intramolecular hydrogen bonds, due to the formation of intermolecular hydrogen bonds with water, resulting in increased conformational fluctuations of peripheral protein segments that are generally more mobile [29].

One way to counteract protein unfolding due to hydration under hydrostatic pressure is through application of osmotic pressure, e.g., by addition of glycerol. Addition of glycerol raises the chemical potential of the protein due to preferential exclusion of the glycerol from the protein surface, which is thermodynamically unfavorable [40]. However, the increase in chemical potential is counteracted by a contraction of the protein-solvent interface, decreasing the extent of preferential hydration and rendering the system less unfavorable thermodynamically. Compression of the protein, and reduction of the protein–solvent interface, is accomplished by the release of water from voids, which has the effect of

increasing the core density and decreasing amino acid motion [41].

Addition of 40% (v/v) glycerol to α-chymotrypsin at 1.8 kbar reduced protein denaturation at 50°C [42], probably via decreased hydration as a result of the osmotic pressure produced by glycerol [43]. Supporting the beneficial effects of osmotic pressure, sodium chloride was also found to stabilize the dimeric state of the Arc repressor by decreasing the available water concentration [18]. Providing further evidence for the potentially disruptive role of water in protein–protein interactions, Kornblatt and Hui Bon Hoa showed that glycerol clearly increased the affinity of pulsed cytochrome *c* oxidase for reduced cytochrome *a* [44]. Recently, Sun et al. [24] found that adding 25% (v/v) glycerol increased the half-life of a recombinant GDH mutant from *P. furiosus* 14-fold at 105°C and 5 atm as shown in Table 1. It was proposed that pressure and glycerol affect proteins through similar mechanisms, whereby the enzyme adopts a more compact and rigid structure in some cases [29], diminishing thermal fluctuations [24]. Trehalose was also found to stabilize GDH from *Thermococcus litoralis* against thermoinactivation [45]. Oliveira and co-workers even went so far as to suggest that pressure denaturation would not occur in the absence of water by extrapolating ΔV values for dissociation of Arc repressor at glycerol concentrations of 0–50% up to 100% glycerol [33] (see Fig. 1). However, Sun et al. [24] and Mozhaev et al. [42] showed that glycerol stabilizes proteins maximally at 25% and 40%, respectively, when the addition of 0–80% (v/v) glycerol was tested for protein stabilization. These data illustrate that a single water exclusion model is insufficient to fully explain the stabilizing effects of cryosolvents for all proteins.

Table 1
Glycerol stabilization of a recombinant GDH mutant (containing an extra tetrapeptide at the C-terminus) from *Pyrococcus furiosus* at 105°C and 5 atm. Reproduced from Sun et al. [24]

Glycerol (% v/v)	Half-life[a,b] (min)	$t^{a}_{1/2}$ (glycerol)/$t_{1/2}$
0	13 ± 0.5	1
25	175 ± 7	14
50	120 ± 3	9.2
75	80 ± 5	6.3

[a]Half-lives are averages of two trials.
[b]Errors represent mean deviations.

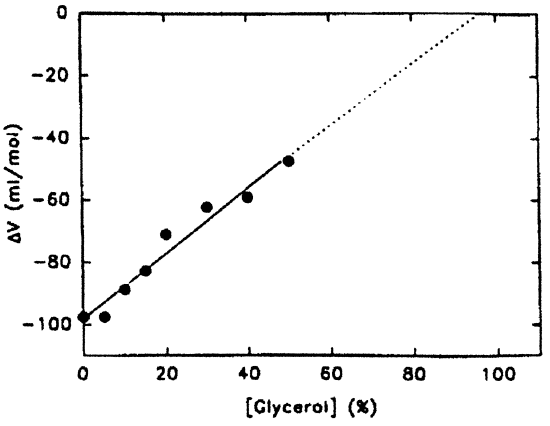

Fig. 1. Effect of glycerol on the volume change for dissociation of the Arc repressor. Extrapolating to 100% (v/v) glycerol suggests that, without the presence of water, the Arc repressor will not dissociate under pressure. Reproduced from Oliveira et al. [33].

2.3. Hydrophobic interactions

Hydrophobic interactions direct non-polar side chains to cluster inside proteins and are a major driving force for proper folding [10]. Hydrophobic interactions are expected to be stabilized by elevated pressure (although this conclusion is not universally accepted in the literature). Stabilization of these interactions by pressure has been attributed to the unfavorable volume increase that results from the solvation of apolar surfaces during protein unfolding [7,9]. This volume increase results from clathrate formation around the apolar groups when they are exposed to water.

Hydrostatic pressure has been utilized to probe the role of hydrophobic interactions in protein stability. Hei and Clark [7] investigated the stability of GAPDHs isolated from several sources in relation to the hydrophobicity of the so-called s-loop region (residues 178–201) of each subunit. A positive correlation between the hydrophobic transfer free energy (a measure of the unfavorable energy difference associated with transfer of a hydrophobic side chain from a hydrophobic to an aqueous phase) and pressure-induced stabilization was observed, as shown in Fig. 2. Thus, the observed correlation indicated that hydrophobic interactions between the s-loops, which are an important determinant of GAPDH thermal stability, were favored by increased pressure (0.5 kbar).

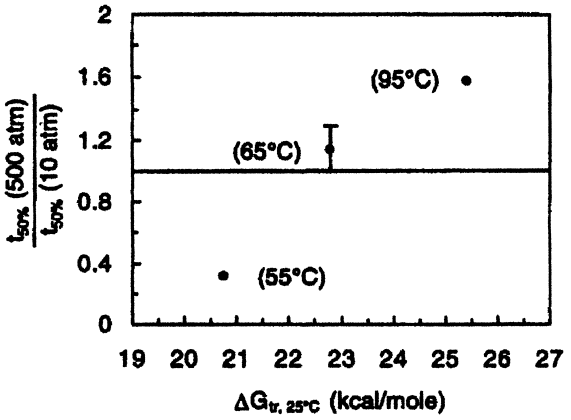

Fig. 2. Inactivation ratios for GAPDHs versus transfer free energies (ΔG_{tr}) of the s-loops. The source of GAPDH and temperatures at which the inactivation experiments were performed are, from left to right, pig muscle (55°C), *B. stearothermophilus* (65°C), and *T. maritime* (95°C). Data shown are averages of duplicate trials (in some cases, the error bars are smaller than the symbols). This graph indicates that hydrophobic interactions between s-loops (an important determinant of GAPDH thermal stability) are favored by increasing pressure. Reproduced from Hei and Clark [7].

Hydrostatic pressure was also used to study the role of hydrophobic residues in the stability of azurin, a single-chain blue-copper protein [46]. Stability of azurin is practically unaffected by hydrostatic pressure up to 3 kbar. Through site-directed mutagenesis, Mei et al. [46] substituted a hydrophobic residue, either Ile7 or Phe110, in both holo- and apo-azurin with serine, and compared the stabilities of the mutants with their wild-type counterparts under hydrostatic pressure ($\sim$3 kbar). As shown in Fig. 3, each mutation caused destabilization at high hydrostatic pressure. The positive correlation between pressure-induced stabilization and hydrophobicity of the azurin core is consistent with the earlier results obtained for GAPDH [7].

Although hydrostatic pressure is an important tool for investigating the role of hydrophobic residues in the stability of proteins, the actual behavior of hydrophobic residues within proteins under pressure remains unclear. An important and relevant question concerns the nature of the hydrophobic core within proteins. The original 'oil-drop' or 'liquid-hydrocarbon' model for the hydrophobic core of proteins, suggested by Kauzman several decades ago [47], was challenged for failing to explain the effect of pressure on protein unfolding (the infamous 'fly in the ointment') [10,11,48]. Kauzman wrote later [10] that the reason for the apparent inapplicability of the model arose from contradictory values of ΔV: ΔV upon protein unfolding was positive at moderate pressures and became negative at pressures higher than 1–2 kbar; however, ΔV for the transfer of hydrocarbons into water (a model process for unfolding of the liquid-like protein) showed the opposite signs of ΔV at both pressure ranges for protein unfolding. However, considering it is still arguable whether hydrocarbons such as low molecular mass alkanes accurately represent hydrophobic side chains in the core of a protein [49], Kauzman's ointment may have been free of flies after all.

In some respects the interiors of proteins more closely resemble a solid than a liquid [50–53]. A semi solid-like protein interior was first proposed on the basis that molecular densities of several proteins were estimated to be much higher than those of

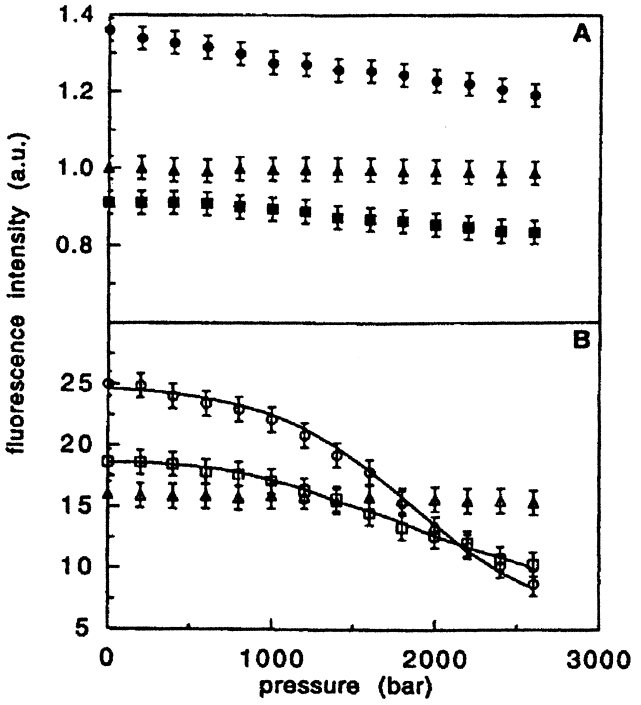

Fig. 3. Effects of pressure on azurin as indicated by relative fluorescence intensity as a function of hydrostatic pressure for the holo- (A) and apo-proteins (B). Symbols represent WT azurin (triangles), Ile7Ser mutated azurin (squares), and Phe110Ser mutated azurin (circles). The fluorescence intensity of holo-wt azurin at 1 bar has been normalized to 1. In each case, the mutations resulted in destabilization at high pressures. Reproduced from Mei et al. [46].

organic liquids, and proteins are relatively less compressible [9,50]. Supporting this model, the first reported measurement of the isothermal compressibility of an enzyme's interior showed that mesoporphyrin-substituted horseradish peroxidase behaved as a fully elastic solid at pressures up to 20 bar [51]. However, binding of a small aromatic substrate molecule in the heme pocket produced a significant change in protein compressibility, effecting a transition from a solid-like to a liquid-like structure [52]. An efficiently packed, solid-like interior was also assumed by Hummer et al. [48,53], who used information theory to predict that water molecules penetrate into the tightly packed protein interior at hydrostatic pressures up to 7 kbar.

2.4. Hydrogen bonds

Based on negative volume changes associated with hydrogen bond formation in organic compounds such as formic acid, ε-caprolactam, phenol, and poly-L-lysine [20,54], hydrostatic pressure has been proposed to promote hydrogen bond formation within proteins [9,55]. As noted in the hydration section, pressure may promote intermolecular hydrogen bonds at the expense of intramolecular hydrogen bonds thus causing increased conformational fluctuations [29].

The lengths of existing hydrogen bonds within proteins have been observed to shorten under hydrostatic pressure. Shortening of hydrogen bonds at high pressure was first detected with ^{1}H nuclear magnetic resonance (NMR) for liquid water and ethanol [56,57]. Furthermore, low-frequency Raman spectra of liquid amides (formamide, *N*-methylformamide, *N*-ethylformamide, and *N*-methylpropionamide) reflected the formation and compression of hydrogen bonds under hydrostatic pressure up to 4 kbar, due to hindered rotational motion as evidenced by reduction in the pressure dependence of low frequency bands [58]. Recently, Li et al. [59] observed compression of hydrogen bonds within BPTI at 2 kbar using two-dimensional ^{1}H NMR. However, compression was not uniform, with estimated reductions in distance varying considerably in the range of 0–0.11 Å. Thus, the shortening of hydrogen bonds, in addition to the collapse of internal cavities, can contribute to the compression of proteins under pressure [60,61].

2.5. Van der Waals forces

Van der Waals (VDW) forces are weak attractions produced by all atoms and molecules as a result of interactions related to induced polarization effects at close distances [12]. These forces can be represented by a Lennard-Jones potential. Although generally considered as a distinct force among the various intramolecular interactions that govern protein folding and stability, as well as protein–protein interactions [14], VDW forces also contribute enthalpic and entropic components to the hydrophobic effect [12,62]. Sites where local VDW interactions are weakened may lead to imperfect packing and cavity formation, which could in turn serve to undermine protein stability [63].

VDW forces are likely to be favored by pressures since they tend to maximize the packing density of proteins, thus reducing the volume. Packing density is maximized by VDW interactions because the dipole moment attractions they produce improve packing of the hydrophobic core [34,64]. This conclusion is supported by the work of Lange and co-workers, who replaced the phenylalanine residue of ribonuclease from *Sulfolobus solfataricus* with alanine, which probably weakened the former aromatic network, destabilizing the protein at 2.5 kbar [65]. This result points to the importance of the aromatic network for ribonuclease stability, although the effect may also have resulted from creation of a non-polar cavity that was filled with water under pressure.

On the other hand, VDW forces can also contribute to protein destabilization under pressure. Pressure below 3 kbar promotes dissociation of oligomers resulting in replacement of the weakest non-covalent interactions between amino acid residues with amino acid–water interactions and changes in peptide chain conformations [18]. These protein–water interactions are promoted by pressure because they produce stronger and shorter bonds that decrease the overall volume [2,18].

2.6. Covalent bonds

Even though covalent bonds are affected by temperature, these bonds are negligibly compressible under pressure [66]. This statement is consistent with the fact that covalent bonds define the primary

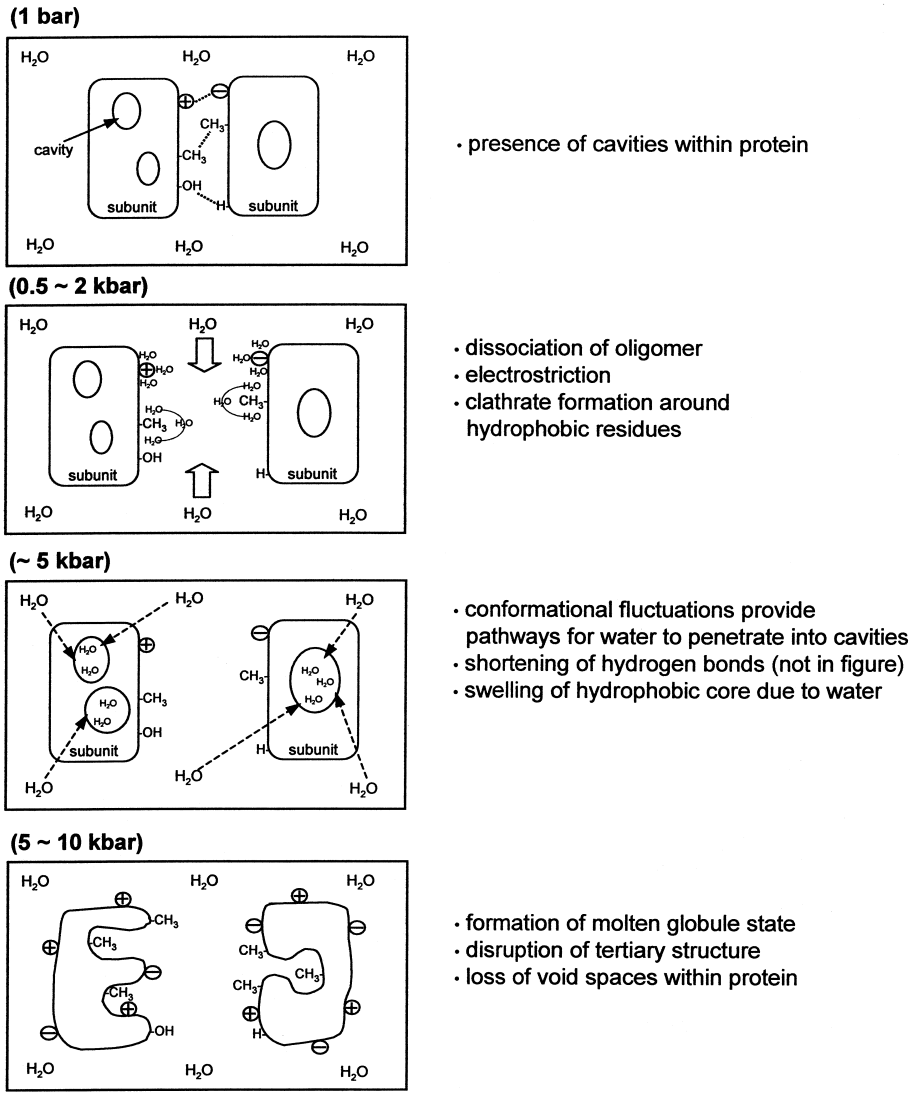

Fig. 4. Major effects of pressure on molecular interactions causing dissociation of oligomeric proteins and subunit denaturation. Note that water molecules in the 5–10 kbar range have been omitted for clarity.

structure of proteins, and the primary structure of hen egg-white lysozyme was unperturbed by pressures greater than 10 kbar [34,67]. It is also worth noting that when the active conformation of chymotrypsin was stabilized by covalent binding of a diisopropylphosphoryl residue to the active site, serine, no change in structure was observed at pressures of up to 3 kbar [68]. Because covalent bonds are highly incompressible, denatured proteins might be expected to have a low compressibility [69]. However, compressibility of the denatured protein is also influenced by solvation. As a result of solvation, the denatured form can be more compressible than the native form. Pressure can thus result in unfolded proteins with greater compressibility than the native form [69] due to increased solvation, but can also result in unfolded proteins with decreased compressibility due to loss of void volumes and solvent exclusion [38,70].

3. Intermolecular interactions

3.1. Protein–protein

In many cases relatively high pressure (1–2 kbar)

has been found to promote dissociation of oligomeric proteins. The dissociation of oligomeric proteins is typically accompanied by negative and relatively large ΔV values (−50 to −200 ml/mol) [18]. Oligomeric proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from yeast, lactate dehydrogenase (LDH), and malate dehydrogenase have been found to dissociate and reassociate reversibly upon application and release of hydrostatic pressure up to 2 kbar [34]. Pressure-dissociated monomers can also incur pressure-induced conformational changes, as was reported for dimeric Arc repressor [71,72]. Fig. 4 illustrates mechanisms believed to contribute to the pressure-induced dissociation and denaturation of multimeric proteins.

Hysteresis or conformational drift has been observed during pressure-mediated dissociation and association of many oligomeric proteins [2,34,73]. For example, in studies of the tetrameric proteins LDH [74] and GAPDH [75], reassociation of monomers to tetramer following pressure-induced dissociation was slow and sometimes incomplete. In a study of GAPDH from yeast by fluorescence spectroscopy, various conformations of oligomeric aggregates were observed depending on the protein concentration, temperature, pressure, and the presence of specific ligands [75]. The extent of hysteresis appears to increase as the number of protein subunit increases. According to a recent study by Panda et al. [76], pressure-dissociated subunits of tetradecameric GroEL did not readily reassociate or failed to refold at all.

Inactivation of oligomeric proteins by elevated pressure is not a universal phenomenon, especially under relatively moderate pressures (<1 kbar). There are many examples of pressure stabilizing thermophilic and hyperthermophilic proteins against thermal inactivation [7,9,24,25]. In particular, GAPDHs from thermophiles showed enhanced thermostability at 500 bar relative to 10 bar, in contrast to GAPDHs from mesophiles, which were destabilized by the elevated pressure [7]. Among the forces helping to maintain the structures of oligomeric proteins, electrostatic repulsion between subunits was found to play an important role in the pressure stabilization of glutamate dehydrogenase (GDH) [24,45]. Sun and co-workers proposed that destabilizing electrostatic repulsion in GDH could trigger

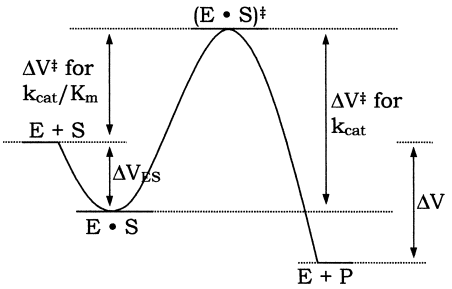

Fig. 5. Activation volumes and volume changes for an enzymatic reaction.

large conformational fluctuations of the entire hexamer. Studies of the wild-type and two single mutants of GDH from *T. litoralis* (D167T and T138E) indicated that the degree of pressure stabilization correlated with the magnitude of electrostatic repulsion created by charged residues at positions 138 and 167.

3.2. Enzyme–substrate

Binding of a substrate to the active site of an enzyme is typically accompanied by a small positive or negative volume change (ΔV_{ES} in Fig. 5) at atmospheric pressure [9]. Substrate binding stabilizes enzymes by shifting the unfolding equilibrium toward the native state [9]. Morita and co-workers showed that the activity of aspartase from *Escherichia coli* increased with pressure in the range of 1–1000 bar at temperatures above 45°C [77]. Above 53°C, the optimal activity occurred at 700 bar with a drop in activity at higher pressures. The increased activity was attributed to increased stability and was shown to require the presence of substrate to prevent initial heat inactivation before application of pressure. Pressure may also be a cause of pressure sensitivity for some reactions due to effects on substrate binding [9]. For example, studies of camphor analogues binding to cytochrome $P450_{camphor}$ under pressures up to 2.5 kbar showed that small substrates bind less tightly and with less efficient packing, increasing water accessibility and leading to increased likelihood of enzyme inactivation [3,78].

Due to the importance of ion pairs in substrate binding, electrostriction is one of the factors that may lead to weakened substrate binding under pressure. When water penetrates the binding site under

pressure, electrostriction can occur, resulting in disruption of ion pairs between the substrate and enzyme and inactivation of the enzyme. However, electrostriction of ion pairs in the binding site may also decrease the volume of the transition state, thus leading to a more negative activation volume and a pressure-activated reaction [79,80]. The activation volume, defined here as the volume difference between the transition state and ground state of the enzyme–substrate complex, will be discussed in a following section.

3.3. Protein–non-protein

Several molecular forces act in concert to effect the specific binding between proteins and DNA [81]. For example, the specificity of protein binding to DNA is known to change depending on the salt concentration. DNA is a polyelectrolyte with a high axial charge density, and the electrostatic potential is a function of the ionic strength due to counterions close to the poly-ion surface. Furthermore, water is commonly identified at specific binding sites in crystal structures of protein–DNA complexes, mediating hydrogen bonding between the protein and DNA. Hydrophobic interactions are also known to play an important role in sequence-specific protein–DNA interactions [82].

Hydrostatic pressure has been used to probe the role of water in interactions between DNA and restriction enzymes [2,83]. For example, the binding specificity of the restriction enzymes *Eco*RI, *Bam*HI, *Pvu*II, and *Eco*RV were investigated under hydrostatic and osmotic pressure [83,84]. Osmotic pressure was found to release water molecules from the protein–DNA complex, increasing the star activity of *Eco*RI ('star activity' refers to relaxed specificity of the restriction enzyme resulting in cleavage at sites different from the normal recognition sequences). In contrast to osmotic pressure, hydrostatic pressure rehydrated the complex, restoring the natural selectivity of *Eco*RI. From these experiments, it became evident that water molecules assist in the correct recognition of the DNA restriction site by *Eco*RI.

Binding to DNA has been reported to stabilize the Arc repressor dimer against pressure-induced denaturation. Arc repressor, a small DNA-binding dimeric protein (M_r 13 000), dissociates completely under a hydrostatic pressure of about 2 kbar [71]. However, upon binding to operator DNA, the Arc repressor became resistant to pressure denaturation at pressures up to 2 kbar. Formation of the Arc-operator DNA complex was presumably accompanied by burial of nonpolar side chains and weak hydrophobic interactions between the protein and bases on the DNA [82]. The pressure-stabilization effect of DNA is highly dependent on the specificity of binding for the DNA–Arc complex [85].

Molecular interactions between antigens and antibodies have also been investigated under hydrostatic pressure. Sundaram et al. [86] observed that the sensitivity of antigen–antibody complexes to pressure differed significantly depending on the nature of the complex, although the underlying mechanism for such differences was unclear. Surprisingly, studies of hen egg white lysozyme binding to two monoclonal antibodies showed that the complex with greater electrostatic interactions (three salt bridges at the interface) did not exhibit pressure sensitivity, in contrast to what would be expected from electrostriction. Moreover, the complex with greater hydrophobic interactions (six out of 13 contacts on the antibody are tyrosines) exhibited a strong sensitivity to pressure. In the case of digoxigenin and a monoclonal antibody, their association was promoted by hydrostatic pressure (2 kbar) with a ΔV of -30 ml/mol [87]. However, when several aromatic residues of the monoclonal antibody that contact digoxigenin were replaced with histidine, a significant decrease in the pressure-induced association was observed.

4. Implications for protein function and stability

4.1. Pressure-induced thermostabilization

Generally, pressure-induced stabilization has been discussed from the viewpoint of resisting thermal unfolding at elevated temperatures [7,9,42]. A major contributor to thermal unfolding are fluctuations that arise from higher temperatures [24,42]. Pressure-induced stabilization is not applicable to all proteins, in part because pressure can increase and not decrease fluctuations [29], as observed for proteins

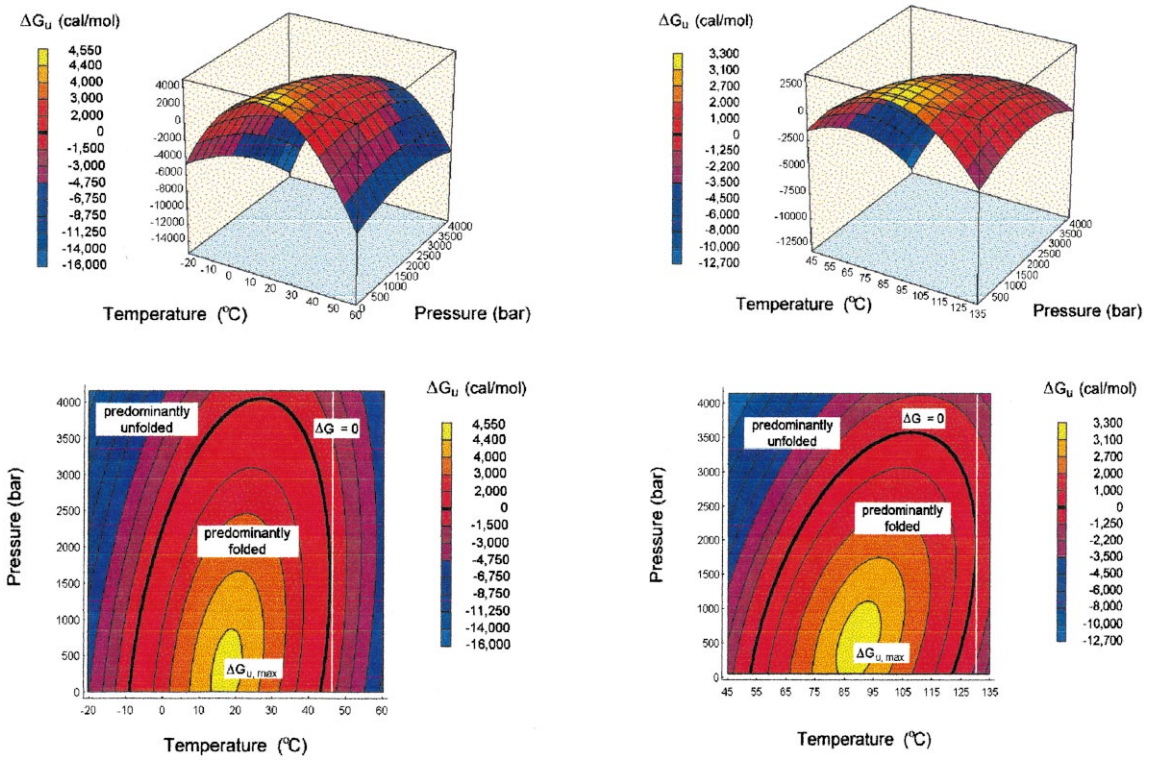

Fig. 6. Three- and two-dimensional free-energy diagrams for protein folding in P-T space: chymotrypsinogen (left) and *M. jannaschii* proteasome (right).

such as apomyoglobin, RNase T1, and phosphoglycerate kinase [28].

Some proteins are nonetheless stabilized against thermal inactivation within a limited pressure range (as illustrated by some of the examples already discussed in this review). When the protein-stability diagram was plotted for chymotrypsinogen in temperature–pressure space, it was found to have a curved boundary [9,88]. A curved protein-stability diagram has also been generated by Clark and co-workers for a putative protease from *M. jannaschii* (Fig. 6). As shown by these contour plots, there are temperatures at which each protein is stabilized by pressure, and a distinct pressure at which the concentration of folded protein is at a maximum. A limited range of pressure-induced stabilization was also shown by Rubens and co-workers, who found that β-galactosidases from *E. coli* and *Aspergillus oryzae* were stabilized maximally in the range of 2–3 kbar at 50°C and 65°C, respectively [89].

Notably, Hei and Clark reported the first examples of pressure-induced thermostabilization of proteins from extreme thermophiles [7]. Hydrogenases from *M. jannaschii* and *Methanococcus igneus* (both extreme thermophiles) were substantially stabilized by pressures of 500 bar, whereas hydrogenases from *Methanococcus thermolithotrophicus* (a moderate thermophile) and *Methanococcus maripaludis* (a mesophile) were destabilized by the same pressure at the selected inactivation temperatures. As mentioned previously, by comparing the hydrophobic s-loops of GAPDHs from various mesophiles and thermophiles, Hei and Clark found that increased thermostability correlated with increased hydrophobicity and postulated that pressure stabilization was also related to increased hydrophobicity at the intersubunit interface.

Several other proteins have also been shown tobe stabilized against thermal inactivation by pressure. The protein, α-chymotrypsin, was stabilized by pressures of 1.2 kbar against thermal inactivation at 50°C [42]. Clark and colleagues also showed that, at 125°C, a putative protease from *M. jannaschii* was stabilized by pressure. Raising the pressure from 1 bar to 500 bar increased the reaction rate 3.4-fold and the thermostability 2.7-fold [8]. Sun and co-

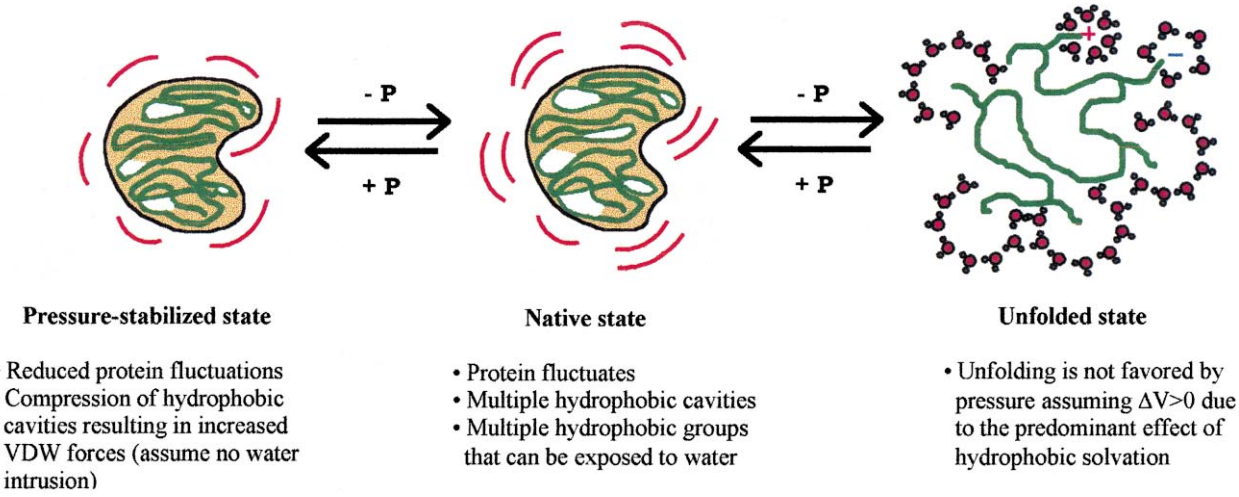

Fig. 7. Proposed mechanisms for pressure stabilization of enzymes. Interactions between the unfolded protein and water molecules are included for the unfolded protein.

workers have recently shown that glutamate dehydrogenases (GDHs) from *T. litoralis* and *P. furiosus* are stabilized against thermal inactivation in the range of 250–500 bar [24,45]. The half-life of the *P. furiosus* GDH as measured by remaining activity at 105°C increased from 13 min at 5 bar to 360 min at 500 bar [24]. Values of $\Delta\Delta G_p^{\neq}$ (the difference in the free energy of activation for inactivation) for the GDH from *T. litoralis* verified that the degree of pressure stabilization increased with temperature [45] (see Table 2).

Table 2
Effects of temperature on thermal half-lives of wild-type (WT) and two single mutants, D167T and T138E, of the *T. litoralis* GDH. Positive values of $\Delta\Delta G_p^{\neq}$ indicate stabilization by pressure ($\Delta\Delta G_p^{\neq} = \Delta G_{500atm}^{\neq} - \Delta G_{5atm}^{\neq}$). In general, the stabilizing effect of 500 atm increased with increasing temperature. Reproduced from Sun et al. [45]

Enzyme	Temp. (°C)	$t_{1/2}$ (5 atm) (min)	$\Delta\Delta G_p^{\neq}$ (kJ/mol)
D167T	101.5	80	3.4
D167T	102.9	67	3.4
D167T	104	32	3.9
WT	98.8	97	3.8
WT	100	79	4.9
WT	101.4	29	6.3
T138E	86	68	8.0
T138E	87	60	7.6
T138E	90	22	8.6
T138E	92	10	9.6

One reason for pressure-induced thermostabilization is that temperature and pressure are opposing forces in terms of entropy. Increasing temperature leads to disorder, while increasing pressure leads to order [42,90]. This is not always true, however, as increasing pressures may lead to hydration of protein interiors resulting in increased conformational fluctuations [27] and disorder. One example of pressure rigidification is the case of apoazurin. Below 1.5 kbar, pressure reduces the flexibility of apoazurin, leading to a more closely-packed, rigid structure and reducing thermal fluctuations caused by high temperatures [29,91]. For other proteins, i.e., lysozyme and ribonuclease A, pressure has been found (according to the H–^{2}H exchange for amide protons) to initially tighten the protein structure with an opposite effect at above 2.5 kbar [92,93]. Mozhaev et al. has proposed that pressure stabilization of chymotrypsin against heat inactivation may arise from protein interactions with water [42]. Hydration of charged groups by water molecules is favored by pressure due to solvent electrostriction. Temperature disrupts the highly ordered structure of electrostricted water and reduces the strongly destabilizing effect of pressure on ion pairs [9].

Sun and co-workers invoked the concept of different conformational substates (CS) to explain the pressure thermostabilization of the glutamate dehydrogenase (GDH) hexamer [45]. Pressure favors a compact, rigid structure where thermal fluctuations

are diminished, thus shifting the equilibrium toward the CS with the greatest reduction in volume. In the studies by Sun et al. [45], increased pressure stabilization was observed for a site-mutated GDH containing increased electrostatic repulsion. The apparent first-order inactivation of GDH was modeled by the following scheme:

$$H_{CS0} \rightarrow H_{CS1} \rightarrow H_U \rightarrow A$$

where H_{CS0} and H_{CS1} are CS that comprise the native-state ensemble of the GDH hexamer, and H_U is a partially-unfolded hexamer that is then able to aggregate to form the aggregated species A. Presumably, increased electrostatic repulsion in the protein leads to increased volume fluctuations, enabling pressure to stabilize GDH by shifting the equilibrium between H_{CS0} and H_{CS1} (the two CS that comprise the native-state ensemble of the GDH hexamer) towards H_{CS0}, the CS lower in energy and volume. Furthermore, amplification of pressure-induced stabilization at higher temperatures suggested the emergence of a new CS possessing even higher energies and volumes:

$$H_{CS0} \rightarrow H_{CS1} \rightarrow H_{CS2} \rightarrow H_U \rightarrow A$$

where H_{CS2} is a new CS that is populated to a significant extent only at higher temperatures. A general model for pressure-induced stabilization of proteins, based on evidence described in the aforementioned studies, is presented in Fig. 7.

4.2. Pressure-induced activation

The activation volume ($\Delta V^{\neq}$) for an enzymatic reaction is often defined as the overall molar volume difference between the transition state and ground state of the enzyme–substrate complex [9,94] (see Fig. 5). Volume changes include multiple contributions, notably volume changes in the reacting molecules themselves (including the enzyme), and volume changes arising from interactions between reacting molecules and the surrounding solvent [15].

Values of $\Delta V^{\neq}$ can be obtained by plotting $\ln(k)$ versus P where k is the enzymatic rate constant for formation of the transition state (either k_{cat} or k_{cat}/K_m depending on the reaction conditions and the particular definition of $\Delta V^{\neq}$) [66]. Such plots allow determination of the activation volume from the general relationship given by Eq. 2:

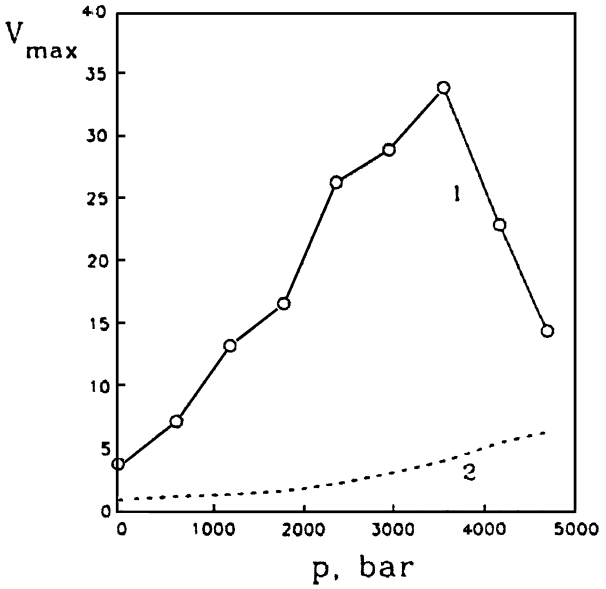

Fig. 8. Rate of hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide catalyzed by α-chymotrypsin in 50 mM Tris–HCl buffer (pH 8.0) at 50°C (curve 1) and 20°C (curve 2) as a function of pressure. V_{max} values are given in relation to rate at 1 atm and 20°C. Note the effect that temperature has on increasing enzyme activity and the decrease in activity at 50°C and pressures greater than about 3.5 kbar. Reproduced from Mozhaev et al. [100].

$$\Delta V^{\neq} = -RT\left(\frac{\partial \ln k}{\partial P}\right)_T \quad (2)$$

[9]. The plot may not be linear, however, if $\Delta V^{\neq}$ is pressure dependent (for example if the reactants or their activated states have different compressibilities [2]).

Even though activation volumes can be approximated using Eq. 2, prediction of activation volumes is quite complicated. In general, $\Delta V^{\neq}$ values for biological systems are difficult to interpret at the molecular level because of the many interactions involved [67]. Moreover, activation volumes for reactions catalyzed by enzymes with similar primary sequences or folding structures may be considerably different [95].

Eq. 2 illustrates that pressure accelerates reactions having negative activation volumes, and, the more negative the activation volume, the greater the rate acceleration with pressure. The pressure effect is especially strong for reactions accompanied by a significant change in the components' hydration state between the ground and transition state [42,96]. Pres-

sure acceleration of reactions with negative activation volumes is supported by evidence that, in the hydrolysis of an anilide substrate by chymotrypsin at 20°C, there was a 6.5-fold increase in activity at 4.7 kbar compared to 1 bar [42]. The activity increase was attributed to the negative activation volume for this reaction [97], which may result from electrostriction in the polar transition state of peptide-bond hydrolysis [98]. Another protease, thermolysin, also exhibited enhanced activity at pressures up to 1 kbar, with activation volumes for hydrolysis of 3-(2-furyl)acryloyl-blocked substrates ranging from −25 to −35 ml/mol [6].

Activation volumes can also vary with temperature. For example, Mozhaev et al. [42] (see Fig. 8) found that increasing the pressure from 1 bar to 3 kbar increased the rate of *N*-succinyl-L-phenylalanine-*p*-nitroanilide hydrolysis by α-chymotrypsin about 7-fold at 50°C, compared to about a 4-fold increase at 20°C. These results correspond to a $\Delta V^{\neq}$ of −25 ml/mol at 50°C, which is 2.5-fold more negative than at 20°C [42]. On the other hand, a further pressure increase to 4 kbar at 50°C led to a decrease in catalytic activity, which may have resulted from weakened enzyme–substrate interactions and/or enzyme denaturation. A temperature-dependent activation volume was also observed by Kunugi and co-workers, who showed that $\Delta V^{\neq}$ for the hydrolysis of a dipeptide amide substrate by thermolysin was −71 ml/mol at 25°C and −95 ml/mol at 45°C [99].

The dependence of activation volume on pressure and hydration creates the opportunity to manipulate enzymatic reactions. For example, enzymatic activity under pressure might be regulated by varying the water content of the system since the activation volume has been proposed to be dependent on water content [90,97]. In particular, the enzyme-catalyzed transformation of two substrates can be regulated if the activation volumes differ in sign [100]. This effect was demonstrated for the trypsin-catalyzed hydrolyses of *N*-benzoyl-L-arginine ethyl ester ($\Delta V^{\neq} = -2.4$ ml/mol) and *N*-benzoyl-L-arginine-*p*-nitroanilide ($\Delta V^{\neq} = 3$–6 ml/mol) [101]. This approach was also applied to chymotrypsin catalysis, whereby pressure was used to activate hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide ($-\Delta V^{\neq}$) while inhibiting hydrolysis of *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester ($+\Delta V^{\neq}$) [97].

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