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Review Article

A molecular perspective on the limits of life: Enzymes under pressure

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From a purely operational standpoint, the existence of microbes that can grow under extreme conditions, or "extremophiles", leads to the question of how the molecules making up these microbes can maintain both their structure and function. While microbes that live under extremes of temperature have been heavily studied, those that live under extremes of pressure have been neglected, in part due to the difficulty of collecting samples and performing experiments under the ambient conditions of the microbe. However, thermodynamic arguments imply that the effects of pressure might lead to different organismal solutions than from the effects of temperature. Observationally, some of these solutions might be in the condensed matter properties of the intracellular milieu in addition to genetic modifications of the macromolecules or repair mechanisms for the macromolecules. Here, the effects of pressure on enzymes, which are proteins essential for the growth and reproduction of an organism, and some adaptations against these effects are reviewed and amplified by the results from molecular dynamics simulations. The aim is to provide biological background for soft matter studies of these systems under pressure.

Key words: *enzymes, hydrostatic pressure, intracellular environment*

PACS: *87.14.ej, 87.15.La, 87.15.B-*

1. Introduction

The discoveries of "extremophilic" microbes and even higher organisms that thrive under extremes of many conditions such as temperature, pressure, salinity, pH, etc., raise many questions on how life can exist under such conditions and what the limiting conditions are, i.e., the "limits of life" [1, 2]. Fundamentally, determining the adaptations for extreme conditions leads to a greater understanding of all life at a molecular level. In addition, understanding these adaptations can guide the search for life in extreme environments such as beneath the continental and oceanic surface or even extraterrestrially. Practically, understanding these adaptations is also important for methods for sterilization and food preservation by extreme conditions, which play critical roles in human health and welfare. In addition, extremophilic microbes and macromolecules from these organisms can play roles in biotechnology, such as *Thermophilus aquaticus* DNA polymerase in polymerase chain reaction (PCR) techniques [3]. While there are clearly many types of adaptations, one basic question is how the macromolecules making up extremophiles can maintain their functional structure under conditions that would destroy their counterparts in mesophiles (moderate-loving).

Among the best-understood extremophiles are thermophiles (hot-loving) and psychrophiles (cold-loving). Studies indicate that these extremophiles employ both "molecule-specific" and "global" adaptive strategies to protect their macromolecules against extremes of temperature. "Molecule specific" adaptive strategies involve utilizing variations of molecules found in mesophiles so that the molecules themselves are adapted for the extreme, such as changes in the lipid composition of membranes [4, 5] or genetic mod-

ifications of the amino acid sequence of proteins [6]. By contrast, "global" adaptive mechanisms protect general classes of molecules in the organism against the extreme. These include heat-shock and cold-shock proteins, many of which apparently function by assisting in folding new proteins or refolding the damaged proteins. Additionally, cryoprotectors and antifreeze proteins [7, 8] are global mechanisms that protect against cold by making changes in the physical properties of the intracellular environment. Since the temperature limits where microbial communities have been found range from -20 to 122°C [2], this type of protection can be considered as ways the microbe find to "cheat" two phase transitions of water, freezing and boiling, beyond the simple colligative properties of freezing point depression and boiling point elevation.

While the effects of temperature are heavily studied, pressure is an underappreciated physical and thermodynamic parameter that has influenced the evolution and distribution of life [9–11]. High-pressure environments are the largest part of the biosphere and include the deep sea, the sub-seafloor and the continental subsurface [2, 12]. This represents $\sim 10^{30}$ microbial cells, a large fraction of total organism numbers, biomass, and evolutionary history [13, 14]. Microbes that grow best under pressures greater than atmospheric pressure are termed piezophiles [15]. From the wide spread phylogenetic distribution of piezophiles, it is apparent that piezophilicity has evolved multiple times. However, piezophiles are among the least understood extremophiles, in part because of the difficulty in collecting samples and performing experiments at high pressures. In addition, while freezing and boiling of water are everyday phenomena, the upper limit of pressure at which microbes have been found correspond to pressures of ~ 1.4 kbar (1 bar = 0.1 MPa $\approx$ 1 atm) [16, 17], which is near the ultimate compressive strength of bone [18]. This is far beyond everyday experience, which is why physical intuition fails.

So far, the most studied piezophiles are mainly from deep ocean environments. However, since deep ocean environments are mostly cold, it is difficult to distinguish adaptations for high pressure versus low temperatures. Because of this, more studies on the combined effects of temperature and pressure, as well as salinity, on microbes such as has been done for four species of *Halomonas* [19] are warranted. Molecule specific strategies have been found for the lipid composition of membranes [2], although there is a debate over whether there is genetic adaptation of proteins to pressure [20]. In addition, a few piezophiles have been shown to preferentially accumulate certain osmolytes in response to pressure, namely β-hydroxybutarate [21] and glutamate [22]. This indicates they might be "piezolytes" that protect against hydrostatic pressure much like cryoprotectors protect against freezing by making changes in the physical properties of the intracellular environment. In addition, while the accumulation of osmolytes may indicate that piezophilicity might be connected to resistance to osmotic pressure, the preferential accumulation of only certain osmolytes indicates that this may be too much of a simplification. Of course, other global mechanisms are likely to be important as well.

Understanding the pressure resistance of mesophilic pathogenic microbes is also important. High-pressure preservation of food (called pascalization analogous to pasteurization), which relies on killing microbes using pressures of 6 to 8 kbar, is becoming popular since it does not greatly affect the nutritional value, taste, texture, or appearance and does not involve chemical preservatives [1]. In addition, high-pressure treatments may become important in sterilization, especially with the increase in drug-resistant bacteria. Disturbingly, a pioneering study indicated that some mesophilic microbes are capable of surviving pressures above 1 GPa (10 kbar) [23]. Although originally met with skepticism [24], 'directed evolution' experiments have shown that while the maximum survival temperature could only be extended a few degrees, the maximum survival pressure was extended to the GPa range [25, 26], although whether it was due to changes in gene expression or some other biochemical response, or selection of a small collection of survivors is not clear [26]. While many strategies are likely to be involved, a clue about a global mechanism microbes might use to survive pressure comes from the observation that a mesophile has been shown to accumulate sucrose and fructose at high pressures [27], which may increase the intracellular viscosity. In addition, the halophilic (salt-loving) *Halobacterium salinarum* NRC-1, which accumulates high intracellular concentrations of ~ 4 M KCl, normally lives at atmospheric pressure, but has been shown to survive pressures up to at least 4 kbar [28]. This indicates that vitrification of the intracellular environment may also play a role since 4 M KCl in aqueous solution at 1 GPa is near freezing even at 298 K [29, 30] and its viscosity can be estimated as almost 2 mPa-s compared to 0.89 mPa-s for pure water at 1 bar based on pressure-temperature data for pure water [31] and aqueous salt solutions [32, 33].

The above-mentioned studies of deep ocean piezophiles and of pathogenic mesophiles point out that

there are actually two limits of life: (1) the limits of growth, or being capable of thriving and reproducing under the extreme, and (2) the limits of survival (or viability), or being capable of enduring the extreme and thriving again when conditions become more hospitable. These limits are important in determining the conditions for discovering new microbial communities and for killing pathogenic microbes, respectively, and may also involve different timescales. For instance, microbial communities may take millennia to adapt to a specific habitat so that the entire genome may be evolved for the habitat, including multiple molecule-specific changes in each protein sequence involving hydrogen bonds, hydrophobic interactions, void volumes, etc., although non-harmful traits or mechanisms may also be retained from their original habitat. Conversely, molecule-specific changes may be hard to evolve in every protein of a pathogenic mesophilic microbe during the time frame of developing resistance, and are less likely to be preserved from more ancient "extreme" conditions if they are deleterious for mesophilic conditions. Instead, a microbe might adapt or resurrect specific parts of the genome for global mechanisms to help preserve the entire proteome. For pressure, ~ 1.4 kbar [[34] and Bartlett et al., unpublished results] is currently an upper limit for growth based mainly on observations of piezophiles from the cold deep ocean trenches, and ~ 8 to 9 kbar [35, 36] is currently an upper limit for survival based mainly on the observation of the pressures where even the hardiest pathogenic microbes are killed.

When pressure is applied to a microbe, the pressure is transmitted into the intracellular domain [figure 1 (a)]. Thus, the biomembranes and also the macromolecules inside the cell feel the effects of pressure. While there are many complex mechanisms involved in the overall growth and survival of a microbe under pressure, there must also be a connection between how macromolecules behave under pressure and how microbes live under pressure. As mentioned above, microbes can alter the chemical composition of the lipids in their membranes and also the amino acid sequences of their proteins through evolution, but can also respond more rapidly by changing the composition of the intracellular co-solvent environment. However, the effects of changes in the intracellular environment on macromolecules under pressure are relatively unknown, and it is not even clear if they are favorable for all types of biological macromolecules. For instance, a piezolyte may favor the membrane fluidity but disfavor the enzyme activity. While typical biochemical and biophysical studies of biological macromolecules are carried out *in vitro*, with perhaps salts and buffers added to the aqueous solution of the protein, the intracellular environment *in vivo* is a complex concentrated mixture of other macromolecules, small organic molecules, salts, and water [figure 1 (b)]. This leads to large differences between the *in vitro* and *in vivo* environments, including in the hydrogen bonding, hydrophobic effects, and crowding, which are important for

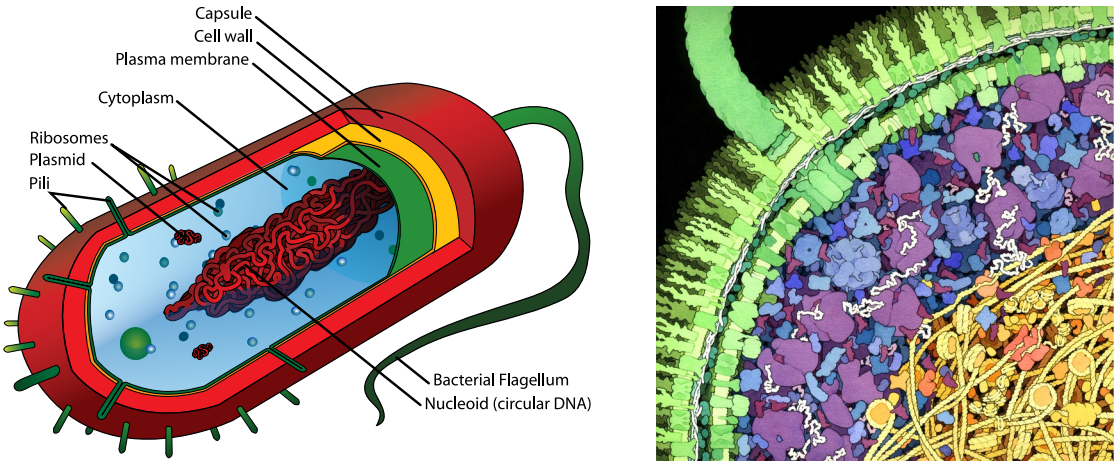


Figure 1. (Color online) (a) Adapted from "average prokaryote cell-en" by Mariana Ruiz Villarreal, Wikipedia. (b) A cross-section of a small portion of an *Escherichia coli* cell. The cytoplasmic area is colored blue and purple. The large purple molecules are ribosomes and the small, L-shaped maroon molecules are tRNA, and the white strands are mRNA. Enzymes are shown in blue. The cell wall is shown in green and the nucleoid region is shown in yellow and orange. Copyright David S. Goodsell, 1999.

protein function.

At a molecular level, the range of conditions where macromolecules will maintain activity by maintaining their *functional* structure can help define the limits for growth, while the range where they will regain activity upon return to growth conditions by maintaining their *stable* but perhaps not functional structure can help define the limits for survival. Since protecting the functional structure against pressure has requirements different from protecting stable structure, it is not clear if either the molecule-specific or global protective strategies are the same at the molecular level for both limits. In addition, *by understanding the molecular mechanisms that organisms use to withstand pressure, determining both limits could be made more predictive rather than observational.*

The main focus here is on how enzyme activity, which is essential for growth of an organism, responds to pressure. Interestingly, the "material" requirements, or structural characteristics, for enzymes may be opposite for growth under high pressure and survival after high pressure. Both the structural requirements of the protein for activity and how piezolytes could affect these requirements are considered. Results from two sets of molecular dynamics simulations using CHARMM36 [37, 38] at pressures between 1 bar and 10 kbar are used to illustrate certain points: one set is of 24 ns simulations of GB1, the B1 domain of protein G, in TIP3P [39] water [Huang, Rodgers, and Ichiye, unpublished results], and the other set is of 2 μs simulations of *Clostridium acidurici* ferredoxin in TIP4P-Ew [40] water [Tran and Ichiye, unpublished results].

2. Effects of pressure on proteins

Major effects of pressure on proteins are compression, making them more compact and/or distorted, and unfolding (figure 2), as noted by Bridgman [41]. Since pressure-induced protein unfolding has been studied extensively by many groups including Royer and co-workers (i.e., reference [42]), a brief but by no means complete background is given here first, followed by a more thorough discussion on the effects of pressure on proteins that are relevant to enzyme activity.

2.1. The limits of survival: protein unfolding and oligomer dissociation

From a molecular perspective, the disruption of protein structure to the extent that normal structure cannot be regained in the intracellular milieu upon the release of pressure may be a factor in the limits of survival. For instance, complete unfolding of a protein would most likely lead to non-specific aggregation within the cell upon release of pressure so that refolding to the active state is not possible.

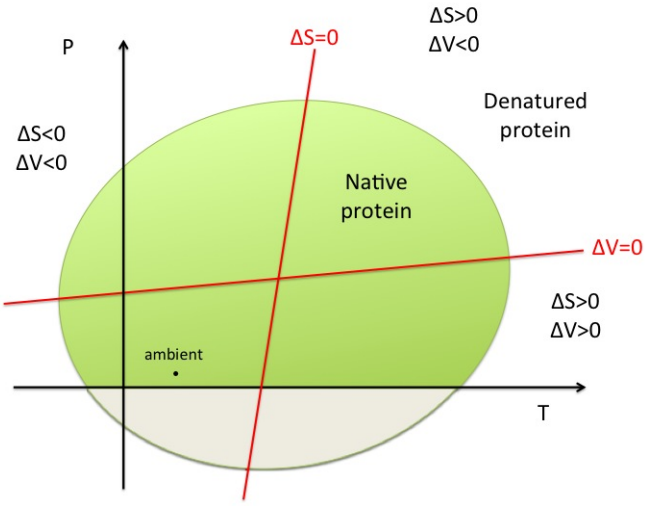


Figure 2. (Color online) Schematic of a pressure-temperature stability diagram for proteins.

In addition, since enzyme activity often depends on being in the correct oligomeric state, dissociation of oligomeric enzymes might also lead to non-specific association with other molecules in the cytoplasm so that reassociation to the functional oligomer is not possible.

Many studies of the sensitivity of proteins to pressure have focused on the *unfolding* of mesophilic proteins at high pressures, which occurs between 4 to 8 kbar *in vitro* [2]. By using pressure as another perturbant in addition to temperature and chemical denaturants, important insights can be gained in understanding the protein folding and stability. To take advantage of high-pressure instrumentation, studies have often utilized either mutants of staphylococcal nuclease or T4 lysozyme, which unfold at unusually low pressures without chemical denaturants. Although seemingly contrary to the reduction of volume due to pressure, pressure unfolding is driven by the reduction of volume of the entire system, which appears to be due to changes in interactions between the polypeptide chain and water. Although many specific effects have been proposed, it appears to occur due to the loss of internal void volume in the protein upon unfolding [43], or the “destruction of voids”.

Studies of mutants of staphylococcal nuclease have given a compelling evidence that larger changes in internal volume due to larger internal cavities lead to lower unfolding pressures [42]. In addition, complementary structural studies using X-ray crystallography, NMR solution studies, and molecular dynamics simulations have supported these results, although these techniques tend to give more information on the folded state. For instance, a crystallographic study of a T4 lysozyme L99A mutant at increasing pressures showed up to four water molecules inside a highly hydrophobic internal cavity created by the L99A mutation starting at 1 to 2 kbar [44], suggestive of initial stages in pressure-induced unfolding. However, the

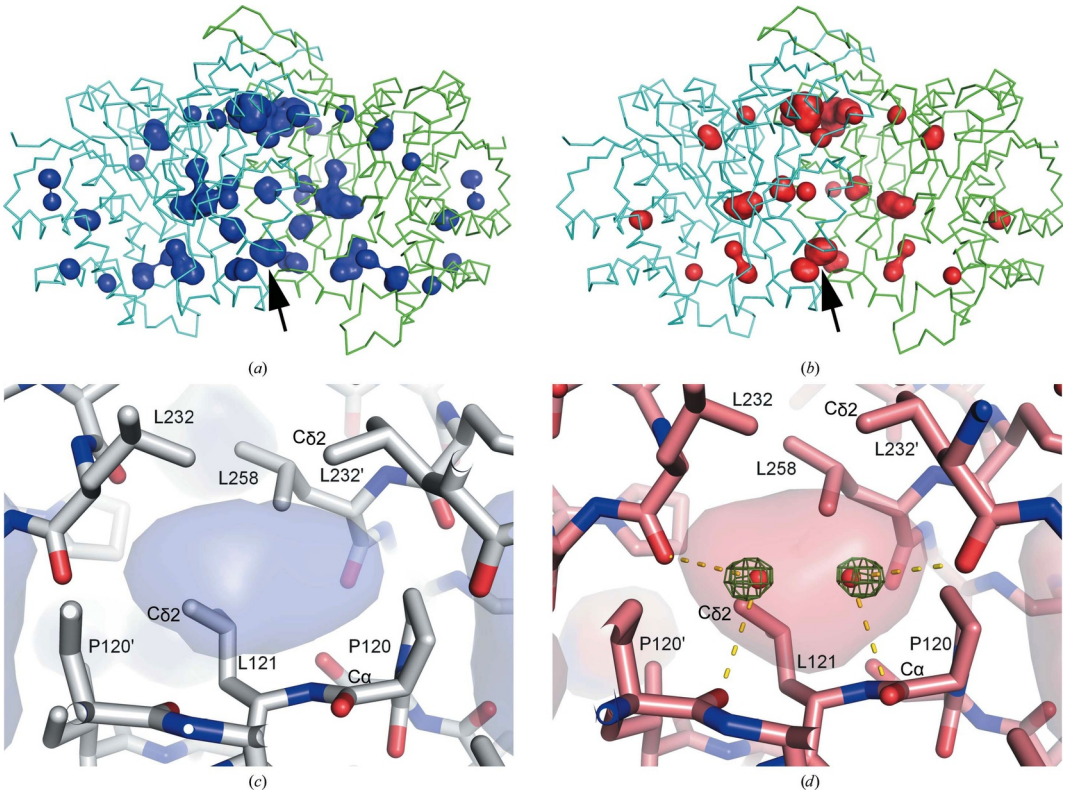


Figure 3. (Color online) Internal cavities of the *Shewanella oneidensis* IPMDH dimer and observed water penetration. Internal cavities of the dimer are shown as surface representations at (a) 1 and (b) 5.8 kbar; the cavity with the volume increase with increased pressure is indicated by an arrow. In magnified views, water inside this cavity, defined by transparent surfaces, is shown at (c) 1 and (d) 5.8 kbar. Figures from Nagae T., Kawamura T., Chavas L.M.G., Niwa K., Hasegawa M., Kato C., Watanabe N., Acta Crystallogr. D, 2012, **68**, 300. Reproduced with permission of the International Union of Crystallography. (http://journals.iucr.org).

protein remained folded up to 6.5 kbar even though fluorescence and small-angle X-ray scattering studies indicate that the protein is unfolded at these pressures [45]. High-pressure NMR studies of T4 lysozyme provided further support for the "destruction of voids" mechanism by showing that in the L99A mutant, the domain with the mutation unfolds with increasing pressure while a "wild-type-like" T4 lysozyme with no cavity and a L99A mutant with benzene in the cavity do not unfold [46]. In addition, by comparing unconfined proteins with proteins that were confined in reverse micelles that prevented unfolding, these studies showed that the volume reduction from pressured-induced unfolding in the unconfined proteins was translated to increasing incorporation of water into the cavity in the confined proteins, much like the crystallographic experiments [46]. This points to the importance of crowding effects in the intracellular environment [figure 1 (b)].

Overall, compared to thermal or denaturant unfolding, two important differences have been emerging: the pressure-induced unfolded state appears to be more compact than the thermally unfolded state [47] and pressure unfolding appears to involve extensive hydration in the interior of the protein rather than exposure of the inner hydrophobic core to the bulk solvent as in thermal unfolding [48]. However, care must be taken about interpreting the latter as a dynamic picture of water being pushed inside the protein with increasing pressure, since atomic fluctuations that would allow water to "penetrate" also decrease with increasing pressure. Instead, a better interpretation may be a thermodynamic picture of a shifting equilibrium of the populations of protein states towards states with greater numbers of water molecules inside the cavities with increasing pressure.

In addition, while unfolding of the entire protein by 8 kbar would certainly limit the survival of a microbe, other less drastic effects on proteins at lower pressures could also limit survival. For instance, dissociation of oligomeric enzymes, which occurs below 3 kbar *in vitro* [49], will disrupt their activity, so it may be a better determinant of the limits of survival than complete unfolding. However, the intracellular milieu may have two opposing effects on dissociation. In particular, while reassociation of oligomers upon the release of pressure would be made more difficult by non-specific association in the heterogeneous environment, it might also be made easier since the crowded intracellular environment might prevent oligomers from completely dissociating. As in pressure induced protein unfolding, water may play a role in oligomer dissociation by pressure due to a "destruction of voids" mechanism. For instance, crystallographic studies at different pressures of the dimeric (~ 340 residues/monomer) enzyme 3-isopropylmalate dehydrogenase (IPMDH), a pressure sensitive enzyme in the biosynthesis pathway of leucine, from the mesophilic *Shewanella oneidensis* [50] shows water inside a cavity at the interface of the two monomers between 4.1 to 5.8 kbar while no water is present at 1 bar (figure 3). This cavity may be a pressure sensitive point for dimer dissociation.

2.2. Limits of growth: compaction and conformational changes of proteins

From a molecular perspective, the perturbation of protein structure to the extent that enzymes are no longer active should be a factor in the limits of growth. For instance, the activity of *Escherichia coli* dihydrofolate reductase (DHFR) at 1 bar was reduced to 65% at 1 kbar [51], which indicates that pressure can affect the enzyme activity. These perturbations can be grouped into compaction and conformational changes.

The compaction of domains of protein has been demonstrated using various structural methods. For instance, high-pressure NMR solution studies of GB1, a small folding domain of protein G, show that the domain compacts by ~ 1% between 30 bar and 2 kbar [52]. Compaction is illustrated by the changes in the radius of gyration, R_{gyr}, of *C. acidurici* ferredoxin with pressure in 2 μs molecular dynamics simulations [Tran and Ichiye, unpublished results] [figure 4 (a)], which also show a ~ 1% compaction between 1 bar and 2 kbar. The results from these simulations also indicate that μs simulations are needed to evaluate the changes in structural properties at different pressures since very low frequency motions are apparent. High-pressure crystallographic studies of monomeric and dimeric proteins have been used to estimate the compressibility to be between 4 to 6 Mbar^{-1} [50, 53, 54], and internal cavities within the monomers of IPMDH have been shown to be compressed monotonously up to 6.5 kbar [50], indicating that the cavities allow the protein to be more compressible.

More important to enzyme function, compaction results in reduced atomic fluctuations, which have often been noted as important for enzyme activity. Reduction in atomic fluctuations with pressure is il-

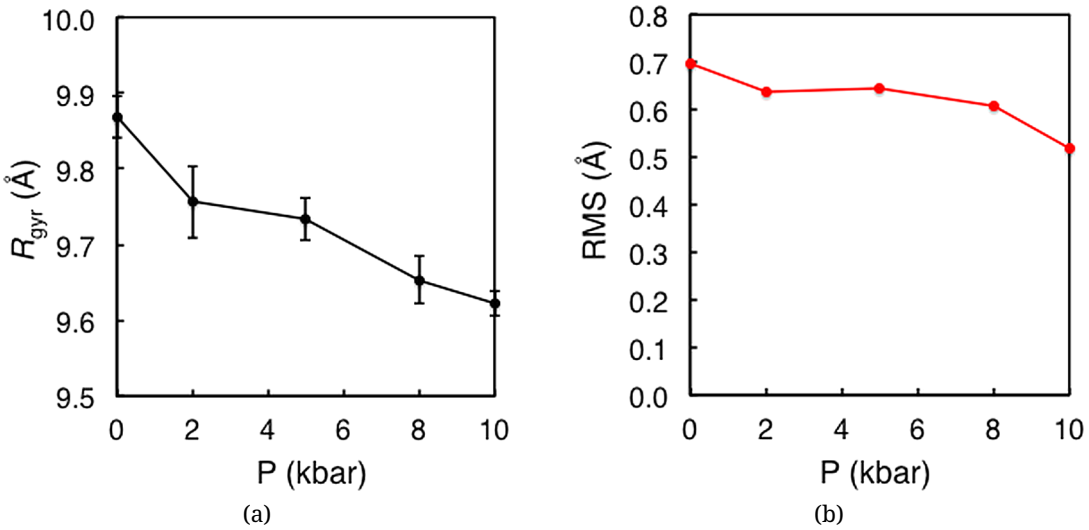


Figure 4. (Color online) (a) Radius of gyration and (b) root mean-square fluctuations of protein atoms as a function of pressure in 2 μs molecular dynamics simulations of *Clostridrium acidurici* ferredoxin [Tran and Ichiye, unpublished results]. The error bars for the radius of gyration correspond to standard deviations to demonstrate the size of fluctuations.

lustrated by the root mean-square fluctuations of all protein atoms in *C. acidurici* ferredoxin from the 2 μs simulations [Tran and Ichiye, unpublished results] [figure 4 (b)], which show an average reduction over the entire protein of about 10% between 1 bar and 2 kbar. Both the R_{gyr} and the atomic fluctuations show a transition in behavior around 2 to 4 kbar. Additionally, an analysis of the pressure dependence of fluctuations in staphylococcal nuclease [55] and lysozyme [56] using multiple short simulations shows a similar transition around 4 kbar, which was attributed to the loss of large amplitude, collective modes and restriction of large-scale solvent translational modes. Since these large-scale modes have often been implicated in the functional activity of enzymes, the loss of such motions may be important in determining the upper limit of growth.

In addition, compaction may lead to small perturbations of active sites, including deformation since the compressibility of a protein molecule is inhomogeneous, possibly leading to a decrease or cessation of activity in an enzyme. In crystallographic studies of yellow fluorescent protein (citrine) at pressures up to 5 kbar, a shift in the fluorescence spectra between 1 and 2.8 kbar is attributed to the progressive deformation of its chromophore by up to 0.8 Å [57]. On the other hand, biochemical studies of *E. coli* DHFR indicated that pressure did not affect the hydride transfer, a chemical step, which indicates that no active site distortion occurred [58]. This suggests that it may be very enzyme dependent how much distortion occurs with pressure and how much will cause inactivation.

Finally, pressure could induce conformational changes, or subtly, shift populations of conformers that may play roles in different stages of enzyme activity. While these could be the direct result of compressive pressures stresses such as the loss of large amplitude modes described above, the stable conformation may be determined by the “destruction of voids” mechanism. In fact, the observed changes seem to fall in the latter category, since more open structures, which would be easier to solvate, seem to be preferred at higher pressures. For instance, the small monomeric (~ 200 residues) enzyme adenylate kinase (AK), which catalyzes the reversible conversion between AMP/ATP and two ADP important to cellular energy homeostasis, has large domain motion upon substrate binding at atmospheric pressure based on X-ray crystallography [59]. *E. coli* DHFR shows large conformational changes between 1.3 to 2.5 kbar in fluorescence studies and although enzyme activity was not measured during pressure treatment, it is presumed that the activity is destroyed by conformational changes [60]. Perhaps more subtly, pressure can shift populations of conformations that correspond to different steps of the catalytic mechanism. For instance, DHFR has three conformations of the M20 (or Met20) loop over the nicotinamide ring binding pocket based on X-ray [61] and NMR [62] data that all appear to play a role in its mechanism [63]. While

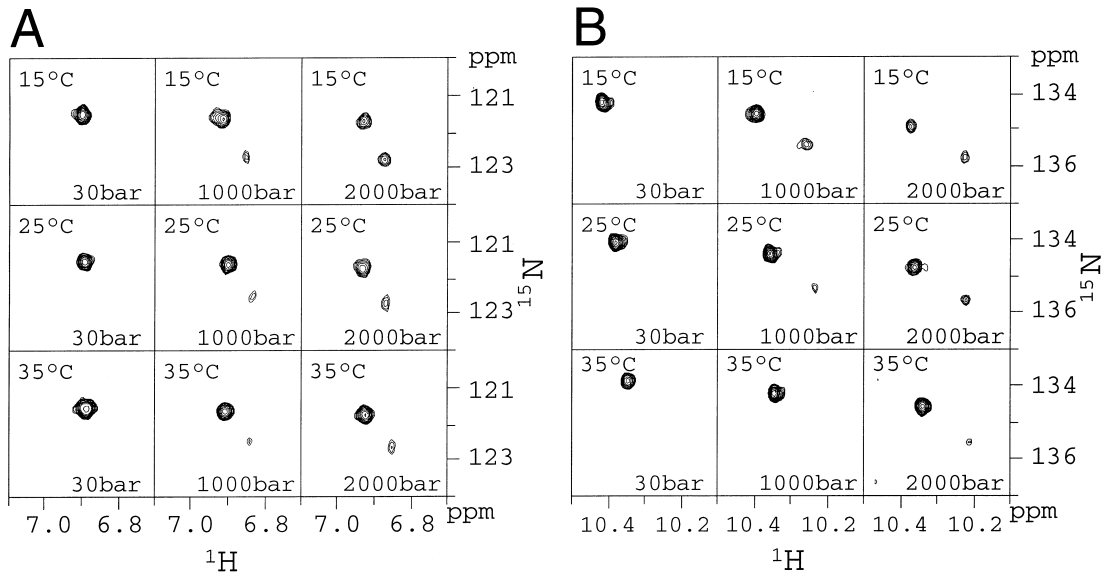


Figure 5. The Val12-H and Trp22-NεH cross-peaks in the ^{15}N/^{1}H HSQC spectra of *Escherichia coli* dihydrofolate reductase at various pressures and temperatures. Reprinted with permission from Kitahara R., Sareth S., Yamada H., Ohmae E., Gekko K., Akasaka K., Biochem., 2000, **39**, 12789. Copyright American Chemical Society (2000).

the *E. coli* DHFR-THF binary complex is in the occluded state at atmospheric pressure, high-pressure NMR studies show increasing populations of the open state at pressures above 500 bar (figure 5) [51, 63], which may interfere with the reactive cycle. Also, high-pressure NMR studies of ubiquitin indicate that pressure induces a transition from a closed to an open conformation suitable for enzyme recognition [64]. Finally, IPMDH from the mesophile *S. oneidensis* has a sharp drop in activity above 50 kbar [65], which appears to be due to the pressure induced closure of the entrance to the active site that occurs simultaneously with the opening of the groove of the active site within the monomers of IPMDH [66].

2.3. Protein stability under pressure

The simulation results also illustrate why pressure induced unfolding should be viewed as a thermodynamic rather than a dynamic process. Analysis of the short 24 ns simulations of GB1 indicate that water can readily enter and leave at 1 bar up to 2 kbar, but above 2 kbar, does not enter if no water is initially present. Even after 2 μs, water does not start penetrating *C. acidurici* ferredoxin above 2 kbar up to 10 kbar even though the latter is well above the unfolding pressures for typical proteins *in vitro* (4 to 8 kbar) [2]. This might seem contrary to the above-mentioned observations of water inside cavities of a protein only at high pressures in crystallographic studies of T4 lysozyme [44] and *S. oneidensis* IPMDH [50] unless the thermodynamic and dynamic viewpoints are considered simultaneously.

The thermodynamic picture is that the change in free energy for folding a protein at constant T and P is

$$\Delta G = \Delta H - T\Delta S = \Delta U + P\Delta V - T\Delta S. \tag{1}$$

Even though pressure is associated with forces, there is no special force pushing water into the protein at high pressure just as there is no hydrophobic force pushing the protein into an unfolded state at high temperature. Instead, the protein molecules with water inside them become more favorable as pressure is increased according to equation (1), since water fills the voids and thus reduces the system volume in the unfolded state.

However, water appears to find pathways into the protein in the high-pressure crystal structures. At a molecular level, a simple picture consistent with the simulation results and thermodynamics is as follows. For a given cavity in the protein, a water molecule can enter the cavity from bulk water by going

over a barrier that is lower than the barrier in a hypothetical rigid protein due to the atomic fluctuations of the protein. At 1 atm, the atomic fluctuations lower the barrier enough so that water can readily enter and readily leave, as seen in the 20 ns GB1 simulations at 1 bar to 2 kbar. However, since the cavity has a low probability of having water inside it thermodynamically [equation (1)], the cavity is mostly empty at low pressures. As pressure increases, the probability of water being in the cavity increases thermodynamically [equation (1)], since the overall volume of the system is less when water is inside. However, the atomic fluctuations of *C. acidurici* ferredoxin decrease with pressure, as seen in the 2 μs simulations, which implies that the barriers for water penetration are larger. Thus, while the equilibrium would be shifted toward water being inside as pressure increases, it becomes a rare event for an individual protein to reach that equilibrium state. Since the simulations of *C. acidurici* ferredoxin begin with a single protein with no water inside (the most favored state at 1 bar), the probability of finding a water inside the protein at 10 kbar is very small even after 2 μs. Furthermore, the picture of empty cavities in the protein waiting to be filled is simplistic in that some of the cavities only appear due to atomic fluctuations, which are certainly reduced at high pressures. Altogether, the slow approach to equilibrium indicates that very high pressures for a short duration could be less disruptive to a protein than moderately high pressures for a longer duration.

2.4. Summary

Of various effects of pressure on proteins, maintaining flexibility and populations of loop conformations appear important in maintaining enzyme activity under pressure. From a physical viewpoint, the effects of pressure on general flexibility as measured by atomic fluctuations are a reflection of the overall compressibility of a protein, a material science problem, while the effects of pressure on populations of loop conformations may involve the volume differences between the conformations.

3. Protection against pressure

While many mechanisms are most likely involved in protection against pressure, the focus here is on the possible mechanisms that could directly protect the enzyme activity in microbes by changes in physical-chemical properties rather than on repair mechanisms. As found in other extremophiles, a “molecule-specific” mechanism is that the sequence of the enzyme could make operation of the enzyme more favorable under pressure. Additionally, a “global” mechanism is that the composition of the intracellular environment could influence the enzyme activity under pressure.

3.1. Molecule specific modifications of enzymes

One type of a protective mechanism can be found by comparing homologous proteins from extremophiles and mesophiles, which will identify evolutionary timescale “molecule-specific” mechanisms involving genetic mutations to protect each protein in the proteome. For temperature, the “flexibility-matching” strategy has been noted by comparing homologous proteins from a psychrophile (low-temperature loving) and a thermophile (high-temperature loving) with a mesophile [67]. In this strategy, the flexibility of the proteins from the extremophiles at their growth temperature matches the flexibility of the protein from the mesophile at standard temperatures and pressures. In addition, comparisons of homologous proteins from thermophiles and mesophiles indicate two different ways of achieving flexibility matching, thermophilic archaea generally have more hydrogen bonds while thermophilic bacteria generally have a few more salt-bridges [68]. Since archaea are thought to have evolved first in high temperature environments while bacteria are thought to have evolved at lower temperatures but became adapted to some high temperature environments, the observed adaptations are consistent [68]. In particular, it is easier to lose thermophilicity by losing multiple hydrogen bonds than it is to gain it by adding multiple hydrogen bonds, and it is easier to gain thermophilicity by adding a few salt-bridges than by adding multiple hydrogen bonds.

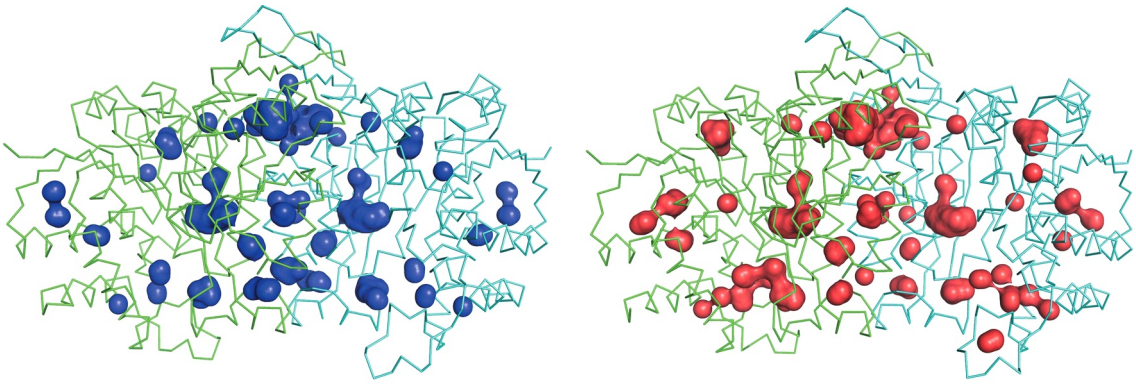
Moreover, the need for flexibility matching has led to contrary requirements for proteins from psychrophiles: while increasing atomic fluctuations by weaker intramolecular interactions leads to an in-

creasing flexibility needed for functioning at low temperatures, they also lead to less stable proteins that are more readily unfolded. In fact, it has been noted that the cold-induced unfolding temperature, T_u, for enzymes from psychrophiles is actually higher for the homologous enzymes from mesophiles, which has led to an activity-stability-flexibility hypothesis for enzyme function for psychrophiles [69]. In short, it appears that maintaining flexibility is more important than stability, as long as the enzyme is stable enough to maintain sufficient structure.

Since an increasing pressure or a decreasing temperature can be expected to reduce atomic fluctuations, this indicates that piezophiles may also adapt by increasing the flexibility of their proteins so that their fluctuations at high pressure are similar to the fluctuations of proteins from mesophiles at atmospheric pressure. In addition, an interesting correlation has been made between cold and pressure unfolding of proteins [70]. However, since most piezophiles that have been studied are from cold (but not freezing) deep ocean environments, it is difficult to separate the effects of low temperature and high pressure. Thus, there is a debate over whether proteins are actually adapted to high pressure [20, 71]. Intriguingly, there are also piezophiles that are thermophilic [2], so more studies of proteins from these organisms would be of great interest.

The question regarding the relative balance between an increased flexibility for activity over a decreased flexibility for stability for enzymes from piezophiles can be examined by comparing homologous enzymes from piezophiles and mesophiles. Crystallographic studies of IPMDH from the obligate piezophile *Shewanella benthica*, with the growth pressures of 0.7 to 1 kbar [72], and the mesophile *S. oneidensis* have been performed [66]. The piezophile IPMDH has a more open structure with a larger internal cavity volume than the mesophile IPMDH (figure 6), which would seemingly make it more susceptible to pressure unfolding. Instead, a larger internal cavity volume was proposed to make the protein more compressible and less subject to pressure-induced distortion, thus allowing it to remain active at higher pressures. In particular, since the piezophile IPMDH retains almost the same k_{cat} up to 2 kbar while that from the mesophile drops sharply above 50 bar [65] and there are no other significant differences in the crystal structures, the larger void volume may help maintain protein flexibility at a higher pressure. However, it must be noted that *S. oneidensis* MR1 may not be a typical mesophile since it was isolated from Lake Oneida in New York State, which is a shallow freshwater lake that freezes over completely in winter [73]. Thus, it is capable of growing over a wide range of temperatures including near 0°C so that the differences between *S. benthica* and *S. oneidensis* may not be purely reflective of pressure adaptation.

In another comparison, the facultative psychropiezophile *M. profunda* DHFR (55% sequence identity with *E. coli* DHRF) was found to display an increased activity followed by a decreased activity as a function of an increasing pressure, a feature found in some piezophiles [figure 7 (a)] [74] but the cause is not



(a) (b)

Figure 6. (Color online) The internal cavities of (a) *S. oneidensis* IPMDH (blue) and (b) *S. benthica* IPMDH (red) dimers. The wire representation shows the overall structure of the IPMDHs (each subunit is drawn in green and cyan). Figures from Nagae T., Kato C., Watanabe N., Acta Crystallogr. F, 2012, **68**, 265. Reproduced with permission of the International Union of Crystallography. (http://journals.iucr.org).

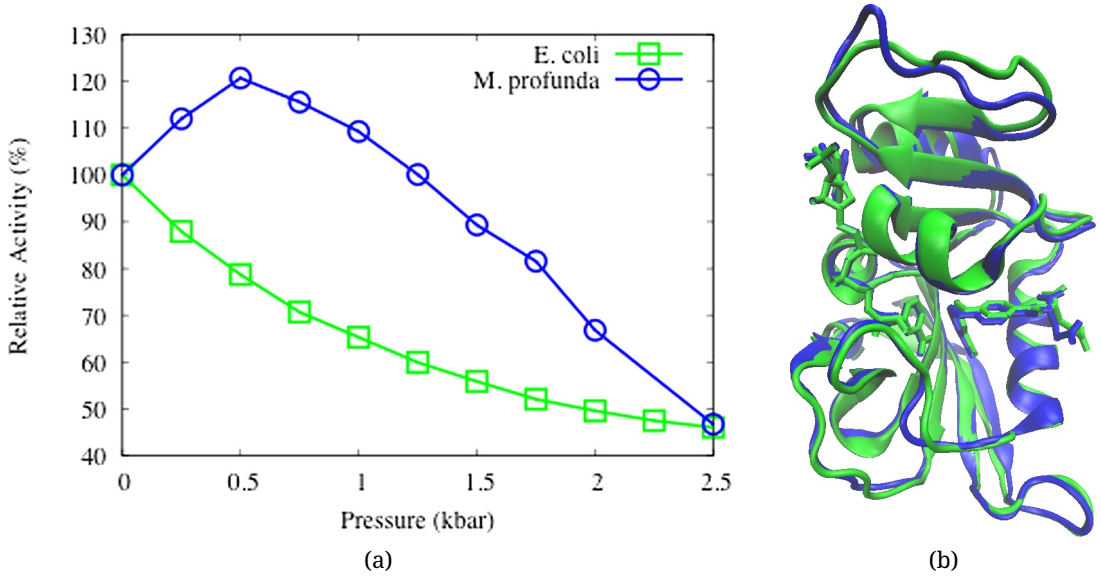


Figure 7. (Color online) (a) Pressure dependence of relative enzyme activity of *E. coli* DHFR (filled circle) and *M. profunda* DHFR (open circle) at 25°C and pH = 7. Figure redrawn from Ohmae E., Murakami C., Tate S.-i., Gekko K., Hata K., Akasaka K., Kato C., Biochim. Biophys. Acta, Proteins Proteomics, 2012, **1824**, 511. Copyright with permission from Elsevier, 2012. (a) Crystal structures of DHFR with NADP+ and folate from *E. coli* (green, PDB ID: 1RX2) and *M. profunda* (blue, PDB ID: 2ZZA).

apparent in the crystal structures of *E. coli* and *M. profunda* DHFR [figure 7 (b)] [20]. Since the solution conditions were the same for mesophile and piezophile DHFR activity studies, a molecule-specific modification could be responsible for the initial increased activity observed in the piezophile. For instance, *M. profunda* DHFR is more flexible than *E. coli* DHFR at atmospheric pressures [75], indicating a flexibility matching mechanism for protection. Specifically, *M. profunda* DHFR may have a reduced activity at 1 atm because it is too flexible and reaches both optimal flexibility and activity at ~ 0.5 kbar. In addition, the subsequent decrease in the activity of *M. profunda* DHFR is most likely due to a similar mechanism responsible for the decrease in *E. coli* DHFR. For instance, the opening of the M20 loop with pressure noted in *E. coli* DHFR may occur in *M. profunda* DHFR, since the open state also has been seen in crystal structures of *M. profunda* DHFR [58]. The M20 loop opening was noted above as a "destruction of voids" mechanism. Interestingly, the unfolding pressure for *M. profunda* DHFR (0.66 to 0.73 kbar between 15.6 and 28.8°C) is much lower than for *E. coli* DHFR (2.58 to 2.72 kbar between 15.2 and 27.0°C) [74], indicating a similar trend as the activity-stability-flexibility hypothesis for psychrophiles. However, it should be noted that an activity maximum at higher pressures is not always associated with piezophiles, as noted in a study of six homologous DHFR from different species of *Shewanella* bacteria [76]. The lack of a maximum at high pressure for a piezophile could reflect that the absolute (rather than relative) activity is sufficient at its growth pressure, or that other factors such as the intracellular environment enhance its activity.

3.2. Global changes in the intracellular milieu

At least circumstantial evidence exists that accumulation of certain co-solutes is a response to pressure. In particular, β-hydroxybutyrate monomers and oligomers accumulate in the deep-sea bacterium *P. profundum* SS9, which grows optimally at 15°C and 0.28 kbar [77] and glutamate accumulates in the hydrothermal vent bacterium *Desulfovibrio hydrothermalis* sp. nov., which grows optimally at 35°C and better at 0.26 kbar than 1 bar [78]. In addition, the mesophilic *Lactococcus lactis* has been shown to accumulate sucrose and fructose at high pressures [27] and *H. salinarum* NRC-1, which accumulates high intracellular concentrations of K^+ and Cl^- at similar molarity to hypersaline environments (~ 4 M NaCl) [79], normally lives at atmospheric pressure but can survive pressures up to at least 4 kbar [28]. How-

ever, it is not clear that the protective mechanisms for piezophiles and microbes that normally live at atmospheric pressure are the same at a molecular level, or whether they should be. For instance, an increased viscosity of the environment probably protects proteins against pressure-induced unfolding in a mesophile to survive high pressure while it is not clear whether it is more important to protect the stability or the flexibility of proteins in a piezophile. However, it may be possible that piezophiles have proteins with genetic modifications for flexibility but use piezolytes to stabilize them against unfolding. In addition, it is not even clear if the accumulation of these co-solutes is protective of, deleterious to, or neutral for protein function, as they probably protect other parts of the microbe.

So far, there have been a few studies of the effects of osmolytes and kosmotropic/chaotropic salts on pressure-induced unfolding of proteins, which indicate the effects consistent with the effects of small solutes on unfolding by other means [80, 81]. For instance, an FT-IR study of staphylococcal nuclease indicates that kosmotropic salts protect against pressure-induced unfolding in the volumetric contribution while polyhydric alcohols and sugars stabilize against pressure-induced unfolding in the energetic or entropic rather than volumetric contribution, possibly due to preferential hydration of the protein [80]. In addition, the 24 ns molecular dynamics simulations of GB1 [Huang, Rodgers and Ichiye, unpublished results] were performed in 0.15 M and 3 M KCl, to examine the effects of salt. Since the diffusion constant of water, D_w, around GB1 decreases slightly with pressure in 0.15 M KCl indicating an increase in solvent viscosity, and decreases even more in 3 M KCl (figure 8), the effect of salt may also be in the viscosity of the environment, although the magnitude of the effects may be exaggerated due to the water model used in these studies. This may protect the protein structure at higher pressures, consistent with the observation that a halophile with high intracellular salt concentration that normally lives at atmospheric pressures is capable of surviving pressures up to at least 4 kbar [28]. The latter suggests that further studies might be necessary by examining the protein stability in different salts and sugars at pressures beyond 8 kbar in order to determine the pressure limits of microbial survival.

However, studies of the effects of osmolytes on the pressure sensitivity of enzymatic activity have been even more rare. Studies of co-solvents including glycerol, ethylene glycol, sucrose, and methanol on *M. profunda* DHFR indicate that the activity generally decreases with an increasing concentration, with strong dependence on the dielectric constant and a weak dependence on the viscosity [75], although the effects of sucrose are weak. However, the decrease in the dielectric constant is reflective not only of the decreased dielectric shielding but also of the decreased long-range hydrogen-bonded order in the liquid [82] so care must be made in interpreting these results. In addition, simple salts inhibit the activity of *E. coli* DHFR [83]. Altogether, these studies suggest that although sugars and kosmotropic salts increase the protein stability, they decrease the enzyme activity perhaps by suppressing the protein flexibility by the effects such as an increased viscosity, although further investigation is warranted.

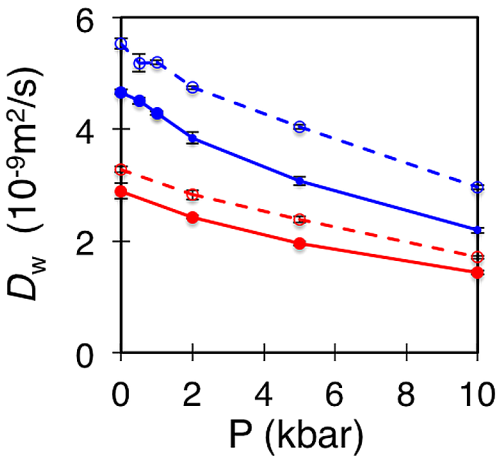


Figure 8. (Color online) Diffusion coefficients of water as a function of pressure in 24 ns molecular dynamics simulations with GB1 (filled, connected by solid lines) and without GB1 (open, connected by dash lines) at 0.15 M (blue) and 3 M (red) KCl. The error bars indicate uncertainty amongst 4 ns blocks within each simulation.

Of natural piezolytes, it has been well demonstrated that the levels of the compatible solute trimethylamine-N-oxide (TMAO) in deep-sea metazoans correlate well with their depth of capture and presumably their pressure tolerance [84] and that it stabilizes the proteins against pressure [85]. The effects of TMAO in stabilizing the proteins and in inducing the folding under a variety of denaturing conditions is well established [86]. However, the effects of purported piezolytes found in microbes on the enzyme activity, or even water, have not been studied. Moreover, since the need to maintain flexibility over stability appears important for enzyme activity, the protection mechanisms of piezolytes might be different from sugars and kosmotropic salts implicated

for protein stability, or if they are the same, would seem to indicate the necessity of extra flexibility of the enzyme. The moderate piezophile *P. profundum*, which preferentially accumulates β-hydroxybutarate under high pressure, was isolated at ~ 260 bar and can withstand pressures up to 900 bar, yet the activity of its DHFR is reduced to ~ 20% of its atmospheric pressure value *in vitro* at 1000 bar [87]. This suggests that other factors *in vivo* such as a piezolyte may enhance its activity at high pressures.

3.3. Summary

Microbes may use both genetic modifications of proteins as well as changes in the intracellular milieu to protect against pressure. Genetic modifications of proteins could involve changes in its material properties or its volumetric properties. However, how changes in the intracellular milieu affect the pressure effects on proteins and especially the enzyme activity is much less understood, and could involve many possible factors such as viscosity, water activity, changes in hydrogen bonding of water, and crowding.

4. Conclusions

The capability of microbes to function at a variety of extreme conditions, or at least to preserve a sufficient structure to survive the extreme conditions, appears to be a combination of the chemical composition of biological macromolecules making up a microbe, the intracellular environment in which they reside, and biochemical pathways for a repair of the damage due to the extreme. Understanding the adaptations against pressure is still in its infancy, but appears to involve more than adaptation by repair mechanisms. In particular, adaptations for high-pressure environments that modify the physical properties of the intracellular environment may play a significant role by affecting the soft matter properties of the macromolecules comprising the cell.

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Молекулярні перспективи для границь життя: ензими під тиском

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З чисто функціональної точки зору існування мікробів, що можуть рости в екстремальних умовах, чи “екстремофілів”, приводить до питання як молекули, з яких створені ці мікроби, можуть підтримувати їх структуру і функцію. Тоді як мікроби, що живуть при екстремумах температури, були докладно досліджені, то ті, що живуть при екстремумах тиску, нехтувались, частково через труднощі в збиранні зразків та проведенні експериментів при нормальних умовах для мікроба. Однак, термодинамічні аргументи передбачають, що ефекти тиску могли б привести до різних організмових варіантів ніж ефекти температури. Очевидно, деякі з варіантів моли б бути серед властивостей конденсованої речовини у внутріклітинній рідині на додаток до генних модифікацій макромолекул чи відновлювальних механізмів для макромолекул. В даному огляді ефекти тиску на ензими, які є важливими протеїнам для росту і репродукції організму, та деякі аргументи проти цих ефектів аналізуються та доповнюються результатами моделювання методом молекулярної динаміки. Метою огляду є закласти біологічну основу для досліджень цих систем під тиском з точки зору м’якої речовини.

Ключові слова: *ензими, гідростатичний тиск, внутріклітинне середовище*