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Preserving Proteins Under High Pressure and Low Temperature

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1. Introduction

A promising method of preserving aqueous protein solutions is to subject them to high pressure and low temperature. However, two significant issues can arise under these conditions: (1) ice nucleation in aqueous solution at 273 K and 1.0 GPa, and (2) structural changes to the protein (Kunugi & Tanaka, 2002; Frank, 1995; Heremans & Smeller, 1998; Vandekooi, 1998).

The first issue can be avoided by addition of a freezing-protection agent such as sugar or salt. For example, Miyazaki *et al*. reported that myoglobin in a highly aqueous NaCl solution forms a glassy state during quenching and annealing processes (Miyazaki et al., 1993, 2000).

Our group has a continuing interest in devising new freezing-protection agents. To this end, we have systematically investigated the structure of aqueous electrolyte salt solutions and room-temperature ionic liquids (RTILs), which consist of the cations and anions. We found that such water-mixed solutions remain liquid even at 1.0 GPa and 77 K (achieved by rapid cooling at 500 K/min) (Imai et al., 2010; Takekiyo et al., 2006a, 2006b; Yoshimura et al., 2009, 2011) and that water-soluble RTILs such as 1-butyl-3-methylimidazolium tetrafluoroborate remain liquid even at 2.0 GPa (Imai et al., 2011, Takekiyo et al., 2011). These results suggest that high pressure and low temperature by the use of RTILs-water mixtures might be useful for the freezing preservation of aqueous protein solutions.

The second issue can best be addressed by developing a better understanding of the structural stability of aqueous protein solutions under conditions of high pressure and low temperature.

Pressure–temperature (*P*–*T*) phase diagrams provide valuable information about the structural stability of proteins. Such diagrams have been obtained by thermodynamic analysis of the pressure and temperature dependencies of structural stability by various experimental methods including NMR, UV–VIS, and vibrational spectroscopy (Brandts et al., 1970; Hawley, 1971; Lassale et al. 2000; Panick et al., 2005; Taniguchi & Suzuli, 1983; Yamaguchi et al., 1995; Zip & Kauzman, 1973). They show that, for many proteins, pressures of >0.5 GPa induce pressure unfolding and temperatures of <253 K induce cold unfolding.

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With respect to pressure unfolding, many proteins—such as ribonuclease A(RNase A), lysozyme, myoglobin, and trypsin—pressure unfold at >0.5 GPa (Meersman et al, 2002, 2003;Ruan et al., 1999; Smeller et al., 2006), whereas other proteins such as cytochrome *c* and *trp* repressor do not pressure unfold under high pressure. Dewa *et al*. and Dubins *et al*. showed that cytochrome *c* undergoes pressure-induced recovery (Dewa et al., 1998, Dubins et al., 2003). Moreover, Desai *et al*. showed that the population of the solvated α-helical structure of *trp* repressor increases from approximately 20% to 40% with increasing pressure, whereas the population of the buried α-helical structure, located in the interior of the protein, decreases from approximately 60% to 30% (Desai, et al., 1999).

With respect to cold unfolding, Nash and Jonas suggest that, for some proteins, the cold-unfolded state might resemble an early folding intermediate (Nash & Jonas, 1997). Zhang *et al*. showed that the cold-unfolded state retains a native-like core structure (Zhang et al., 1995). Building on their results, Meersman *et al*. reported that cold unfolding results in the formation of a partially unfolded state in which some secondary contacts are still present (Meersman et al, 2002a).

These results suggest the following: Under high pressure, helical proteins such as myoglobin and cytochrome *c* exhibit different structural stabilities, and α/β- and β-proteins such as RNase A, lysozyme, and trypsin tend to unfold in water. At low temperature, protein denaturation induces partial unfolding.

Thus, two important questions arise: What dominant factor accounts for the different structural stabilities of α-proteins under high pressure? And what is the nature of the structural changes induced in proteins at low temperature? Our goal, in investigating the feasibility of protein preservation under high pressure and low temperature, is to answer these questions. To this end, we report herein the systematic investigation of pressure- and temperature-induced changes in the secondary structures of oligopeptides and helical proteins by means of FTIR and circular dichroism (CD) spectroscopy combined with density functional theory (DFT) and geometric volume calculations.

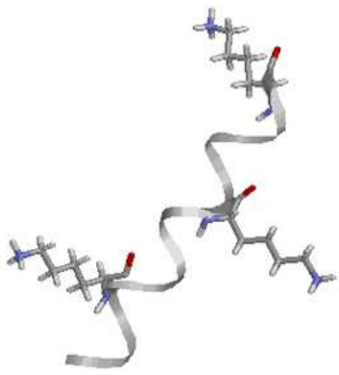
2. Experimental methodology

2.1 Samples

For peptide samples, Ac-YGAA$(KAAAA)_2$KA-NH_2(AK peptide) (Shimizu Laboratory, Soka University) and poly-$_L$-glutamic acid (PLGA) sodium salts (MW = 8000; Peptide Inc.) were used without further purification. For protein samples, bovine serum albumin (BSA) and horse-heart cytochrome *c* (Sigma-Aldrich Co.) were used without further purification. Figure 1 shows the three-dimensional structures of these peptide and proteins.

Sample solutions were prepared as follows. For FTIR spectral measurements, solutions were prepared at concentrations of 20 mg/mL in two different buffer solutions (20 mM MES, pD 5.0; 50 mM tris-DCl buffer, pD 7.6). All exchangeable backbone amide protons in the peptides and proteins were deuterated by incubating in a D_2O solution at 298 K. Completion of hydrogen–deuterium exchange was confirmed by the cessation of shifts in the amide II band. This band, in the frequency region around 1550 cm^{-1}, is known to shift to around 1450 cm^{-1} on deuteration of the backbone amide protons. For CD spectral measurements, solutions were prepared at concentrations of 0.1 mg/mL (~0.06 mM) in a buffer solution

(pD 6.7). Sample concentrations were determined by UV absorption at 280 nm of Tyr and Trp (ε = 1197 M^{-1} cm^{-1} for Tyr and 5559 M^{-1} cm^{-1} for Trp).



(a) AK peptide

(b) Serum Albumin (1AO6) (c) Cytochrome *c* (1HRC)

Fig. 1. Three-dimension structures of (a)AK peptide, (b) serum albumin (1AO6), and (c) cytochrome *c* (1HRC). The structure of AK peptide is built using the Gauss view program.

2.2 High-pressure and low-temperature experiments

FTIR and CD spectroscopy are powerful tools for investigating changes in the secondary structures of peptides and proteins. For our purposes, the amide I′ (the deuterium peptide groups) vibrational mode, which consists of C=O stretching, C–N stretching, and C–C–N deformation in the region 1620–1690 cm^{-1} (Figure 2), is highly sensitive to the secondary structures of peptides and proteins, and thus serves as an indicator of α-helical and/or β-sheet structures (Bandekar, 1992; Krimm & Bandekar, 1986). Similarly, CD spectroscopy enables estimation of the helical content of proteins. CD-ellipticity at 208 and 222 nm, which originates from n–π* excitation of the peptide backbone, correlates strongly with helical content (Chen et al., 1974; Greenfield et al., 1969; Kelly et al., 2005). We previously reported the relevant details of FTIR and CD spectroscopy (Takekiyo et al., 2006c, 2009).

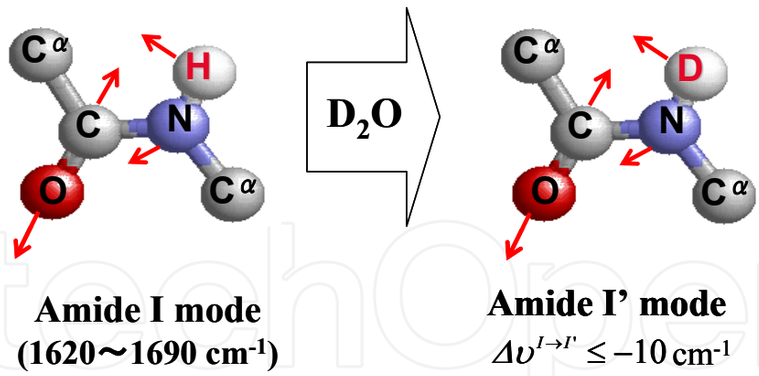


Fig. 2. Relationship between the amide I and I′ modes of protein.

For high-pressure experiments, we used a diamond anvil cell (DAC) (Figure 3a). Sample solution was placed together with a small amount of α-quartz for a pressure marker (Siminovitch, et al., 1987; Wong, et al., 1985) in a Hasteloy C-276 gasket (φ = 1.0 mm, t = 0.05 mm) mounted in the DAC (Figure 3b). Pressure was measured to a precision of ±0.05 GPa. The infrared beam was condensed by a zinc selenide lens system onto the sample in the DAC.

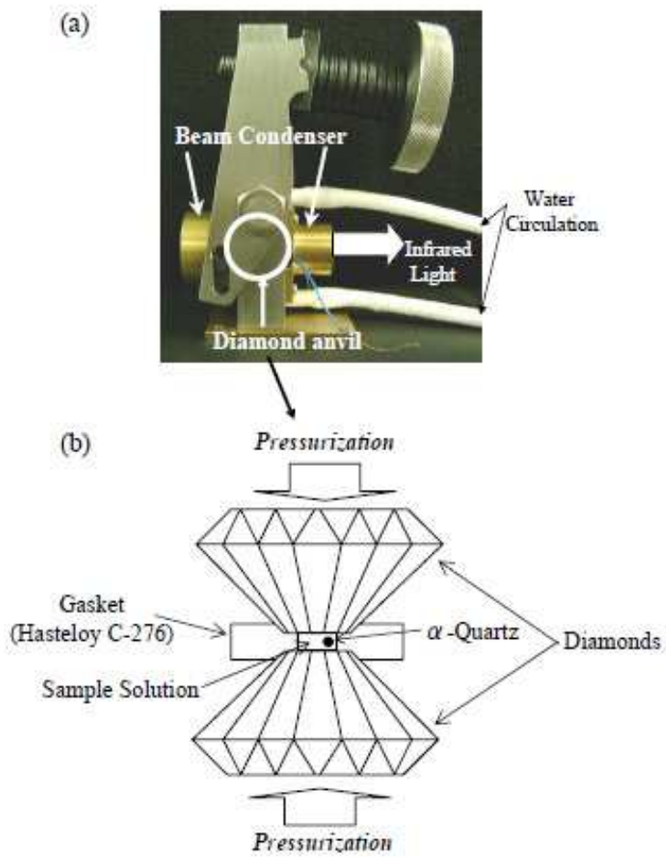


Fig. 3. (a) Schematic picture of diamond anvil cell (DAC) and (b) Schematic diagram of diamond anvils with a gasket and α-quartz.

For low-temperature experiments, we used a cryostat chamber (Figure 4a). The outer windows of the chamber were made of CaF_2. A Teflon spacer (100 μm) was placed between two CaF_2 windows in the transmission cell (Figure 4b), the cell was set in the chamber, and the chamber was filled with liquid N_2 at 0.1 MPa. Temperature was controlled with a mass flow controller. The cooling speed was 30 K/min.

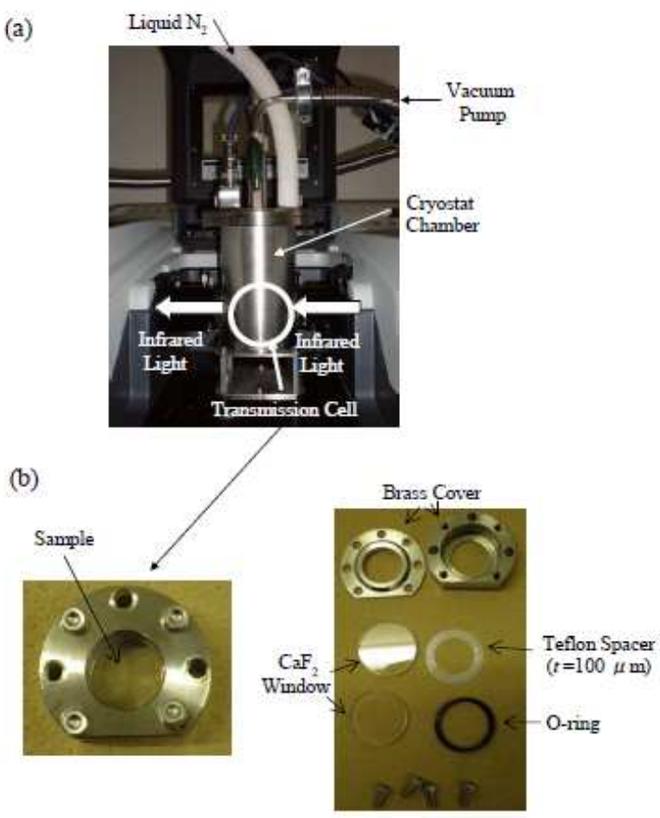


Fig. 4. Schematic picture of (a) cryostat stetted up with FTIR spectrophotometer and (b) transmission cell.

2.3 Density Functional Theory (DFT) and geometric volume calculations

Density functional theory (DFT) calculations have been used as the investigation methodology for the intermolecular interaction between the biomolecules and water molecules (Ham et al., 2003; Kubelka & Keiderling, 2001; Nemukhin et al., 2002). The present DFT calculations were performed using the GAUSSIAN03 program (Frish et al., 2003). For our present calculations, we performed geometry-optimization and frequency calculations at the B3LYP/6-311G++(d,p) level (Becke, 1998, 1998; Lee et al., 1998), which includes the solvent effect, using the polarized continuum model (PCM) (Wiberg & Tomasi, 1982).

Geometric volume calculation has been conducted as the analysis methodology for obtaining the information on a relationship between the partial molar volume (PMV) and

the structure of a biomolecule (Imai et al., 2005a, 2005b; Ling et al., 1998). The geometric volume calculations of peptides and proteins were carried out using the alpha-shapes program (Edelsbrunner et al., 1995; Ling et al., 1998). Geometric volume component of peptides and proteins enables the calculation of the solvent accessibility of the molecular surface, assuming that a water molecule is a hard-sphere probe (Edelsbrunner et al., 1995; Imai et al., 2005a, 2005b; Ling et al., 1998). In the present study, we calculated both van der Waals volume (V_w) and molecular volume (V_M). The latter, V_M, consists of V_w plus void volume (V_v), which is the volume of the structural void within the solvent-inaccessible core of the solute molecule. Hence, V_v can be obtained by subtracting V_w from V_M. That is, $V_v = V_M - V_w$. The diameter of water molecule was taken as 2.928 Å, again assuming that a water molecule is a hard-sphere (Imai et al., 2005a). Figure 5 shows schematic definitions of these various volume contributions.

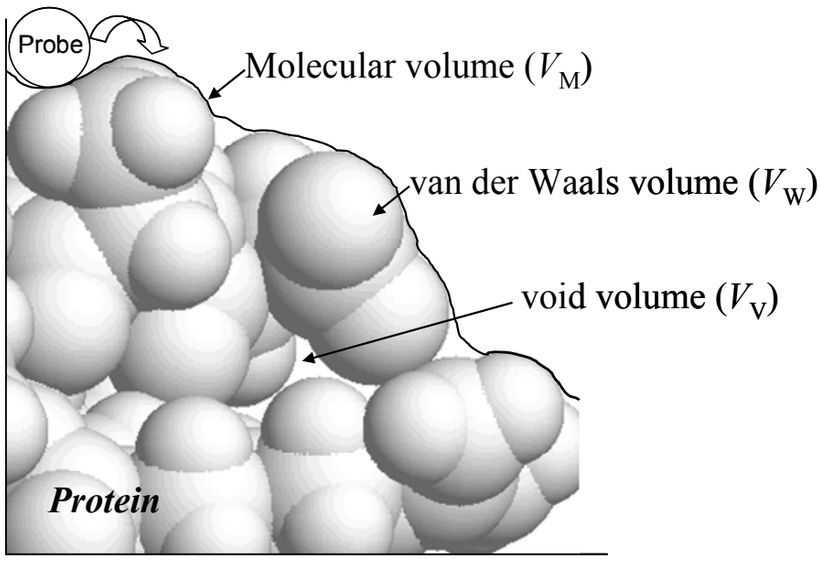


Fig. 5. Definition of geometric volume contributions.

3. Results and discussion

3.1 Pressure-induced structural stability of proteins

Figure 6 shows FTIR and second-derivative spectra as a function of pressure in the amide I′ region for AK peptide and PLGA. The spectra show three peaks at 1635, 1645, and 1672 cm^{-1} for AK peptide and three peaks at 1637, 1648, and 1670 cm^{-1} for PLGA. In a previous FTIR study (Manas et al., 2000; Reisdorf et al., 1996; Silva et al., 2002, Walsh et al., 2003), two peaks at 1635 and 1637 cm^{-1} for AK peptide and PLGA were assigned to the solvated α-helical structure, as shown in the same figure. The peak frequency for a solvated α-helical structure is lower than the characteristic frequency for a buried α-helical structure (~1650 cm^{-1}) due to the hydrogen bond between the α-helix and water molecule as shown in Fig. 7. The peak at ~1645 cm^{-1} for both peptides is thus assigned to a random-coil structure. The peaks at 1672 cm^{-1} for AK peptide are assigned to the asymmetric carboxylate stretching of trifluoroacetic acid ion remained after dialysis (Williams et al., 1996). The peak at 1670 cm^{-1} for PLGA is assigned to a turn structure.

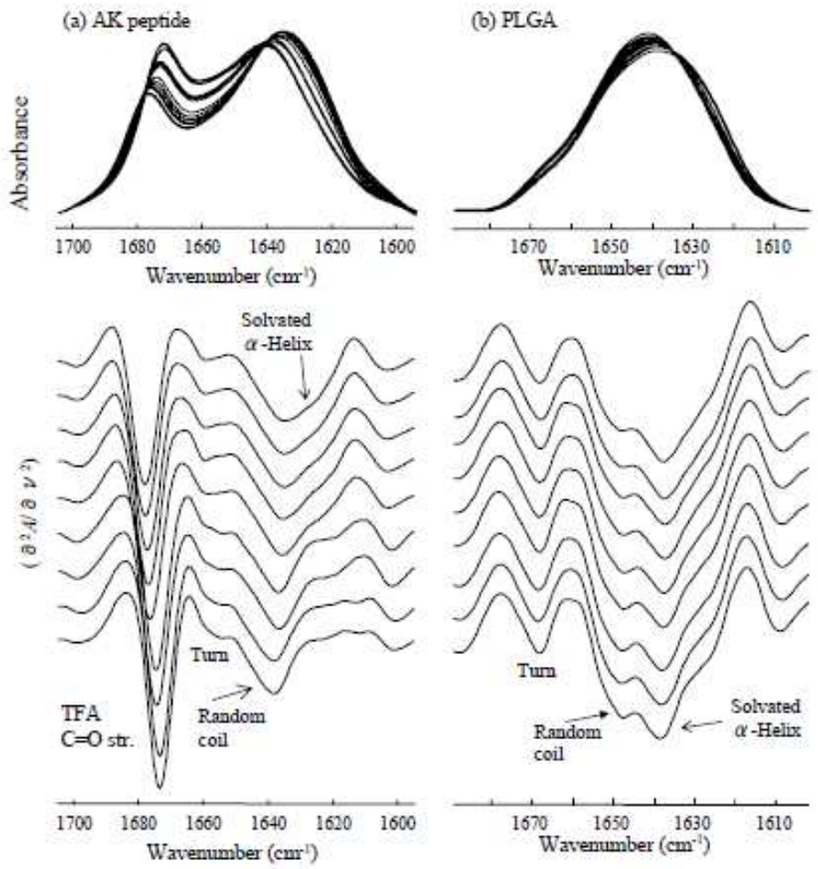


Fig. 6. FTIR (upper) and second derivative (lower) spectra in the amide I′ region of (a) AK peptide and (b) PLGA in aqueous solution as a function of pressure.

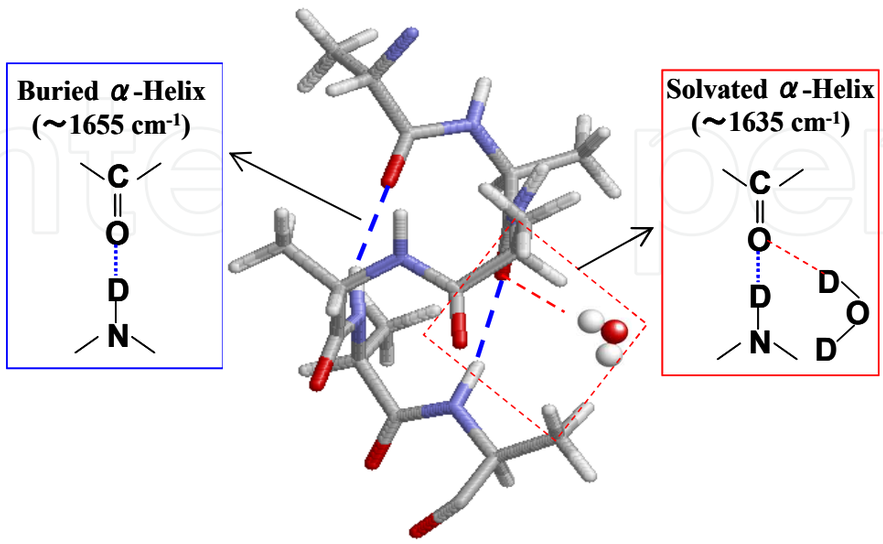


Fig. 7. Scheme of the buried α-helix and solvated α-helix in proteins.

Significantly, for AK peptide and PLGA, the solvated α-helical structures increase with pressure but the random-coil structures decrease from the second derivative spectra. Thus, the solvated α-helical structures of AK peptide and PLGA are clearly stabilized at high pressure. Similar observations have previously been reported. For example, Desai *et al*. reported that the solvated α-helical structure of *trp* repressor is maintained at 0.8 GPa (Desai et al., 1999). A recent FTIR study showed that the solvated α-helical structures of alanine-based peptide (Imamura et al., 2009), four-helix bundle protein $(\alpha\text{-l-}\alpha)_2$ (Takekiyo et al., 2006c) and GCN4-p1 (Imamura et al., 2010) do not unfold even at >1.0 GPa; rather, with increasing pressure, the populations of the buried α-helical structure decrease and those of the solvated α-helical structure increase, implying that the solvated α-helical structures of $(\alpha\text{-l-}\alpha)_2$ and GCN4-p1 hydrate strongly under high pressure. In contrast, however, Meersman *et al*. reported that the α-helical structure of myoglobin begins to break down at >0.5 GPa and denatures completely at 0.7 GPa (Meersman et al., 2002), and Wojciech *et al*. reported that apo and holo types of α-lactalbumin unfold completely at 0.5 GPa (Wojciech et al., 1999). Thus, from the viewpoint of the pressure-induced unfolding of helical proteins, the structural stabilities of even similar helical proteins can differ from one another under high pressure.

3.2 Pressure effect on amide I' frequency shifts of proteins: Interpretation from dft calculations

What, then, is the dominant factor affecting the structural stability of helical proteins under high pressure? We investigated this question by examining the amide I′ frequency shift and geometric volume. The pressure-induced amide I′ frequency shift of a protein is clearly related to environmental changes around the protein.

Recent FTIR studies of helical peptides and proteins have shown that, for $(\alpha\text{-l-}\alpha)_2$ and GCN4-p1, with increasing pressure, the amide I′ frequency of the solvated α-helical structures (~1635 cm^{-1}) shift to lower frequency (Takekiyo et al., 2006c; Imamura et al., 2010). Our current results are consistent with these findings. Figure 8 shows that, for AK peptide and PLGA, with increasing pressure, both amide I′ frequencies of the solvated α-helical structures shift to lower frequency.

To clarify the nature of this pressure-induced amide I′ frequency shift for the α-helical structures of proteins, we focus on the hydrogen bond (H-bond): the intermolecular H-bond between the α-helical structure and water molecules. We then explain the pressure-induced shift to lower frequency by speculating that, with increasing pressure, the intermolecular H-bond between the C=O group of the peptide bond and water molecules strengthens, the C=O force constant weakens, and the contribution of C=O bond unharmonicity increases.

As a model system of solvated α-helical structure, we selected *N*-methylacetamide (NMA; $CH_3CONHCH_3$) dimer + D_2O complex. NMA is the simplest model compound for investigating the structural and physical properties of the peptide group (Ham et al. 2003; Kubelka & Keiderling, 2001). For our present calculations for the NMA dimer + D_2O complex, we examine the two peaks at 1632 and 1656 cm^{-1}, which are amide I′ peaks arising from the NMA–D_2O H-bond and NMA H-bond free, respectively (Figure 9). The former H-bond pattern corresponds to the solvated α-helical structure.

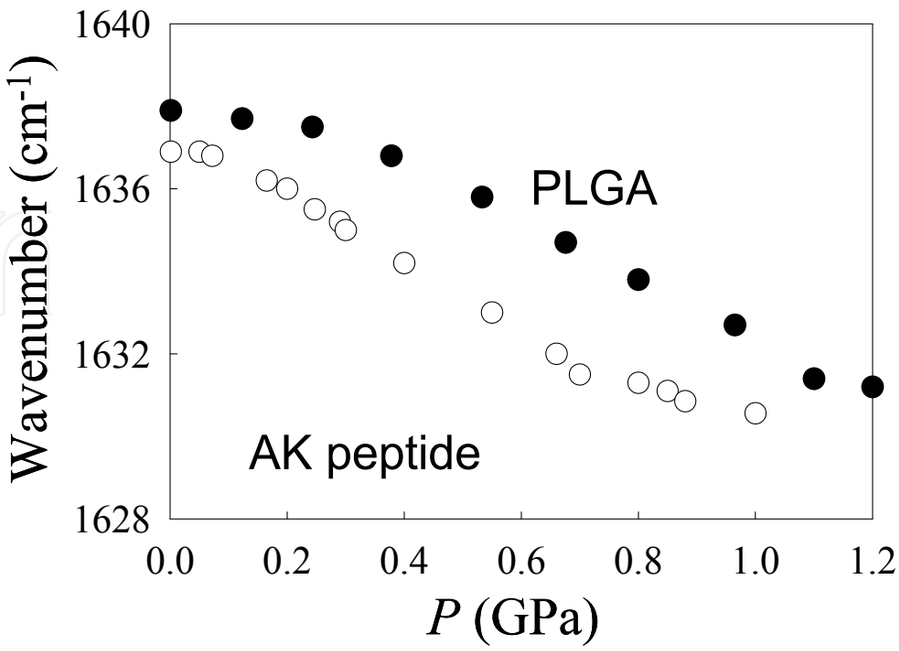


Fig. 8. The peak wavenumber of the solvated α-helical structure of AK peptide and PLGA in aqueous solution as a function of pressure. The closed and open circles represent PLGA and AK peptide, respectively.

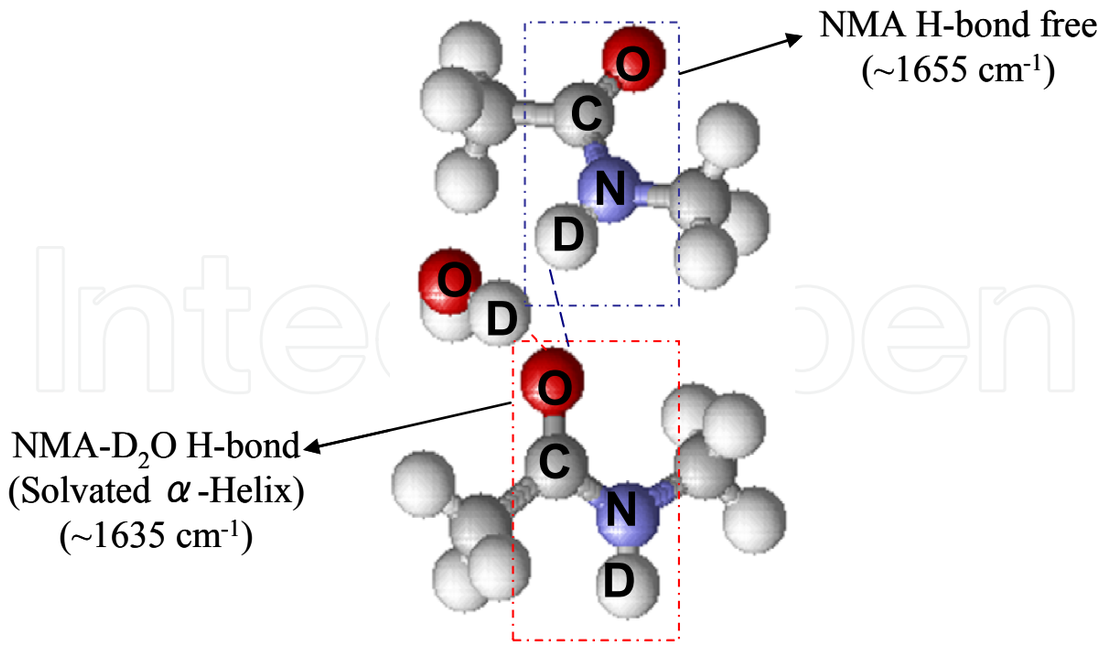


Fig. 9. Amide I′ frequency and hydrogen bond of the peptide bond in the NMA-dimer + D_2O complex. The red and blue long-dashed lines represent the H-bond between the NMA–D_2O and between the NMA–NMA, respectively.

The change in the intermolecular H-bond distance ($d_{O\cdots O}$) between the NMA dimer's C=O group and the water molecule's O–D group is related to pressure-induced changes of the intermolecular H-bond distance in the α-helical structure. We calculated the $d_{O\cdots O}$ dependence of the amide I′ frequencies of NMA dimer + D_2O (Figure 10). With decreasing $d_{O\cdots O}$ (i.e., increasing pressure), the peak at 1632 cm^{-1} (•) shifts to lower frequency. Below $d_{O\cdots O}$ = 2.36 Å, the frequency shift at 1632 cm^{-1} (●) increases significantly. Thus, with decreasing $d_{O\cdots O}$, the intermolecular H-bond between the NMA dimer's C=O group and the water molecule's O–D group strengthens. Because the peak at 1632 cm^{-1} shifts to a slightly extent than that at 1656 cm^{-1}, the average amide I′ frequency shift for NMA dimer + D_2O below $d_{O\cdots O}$ = 2.36 Å is dominated by the peak at 1632 cm^{-1}. This lower amide I′ frequency shift with decreasing $d_{O\cdots O}$ (below $d_{O\cdots O}$ = 2.36 Å) for NMA dimer + D_2O is similar to that for the solvated α-helical structure. Thus, our results indicate that pressure-induced shorting distance of the intermolecular H-bond between the peptide's C=O group and the water molecule's O–D group causes shifts of the amide I′ mode to lower frequency for the solvated α-helical structure. Paschek *et al.*, in a recent IR simulation study (Paschek et al., 2005), proposed that the pressure-induced shift of AK peptide's amide I′ mode to lower frequency is due to hydration effects between the peptide and water molecules rather than structural changes in the peptide. Our results support this suggestion. The amide I′ frequency shifts for the solvated α-helical structure strongly correlate with changes in $d_{C=O\ldots D-O}$.

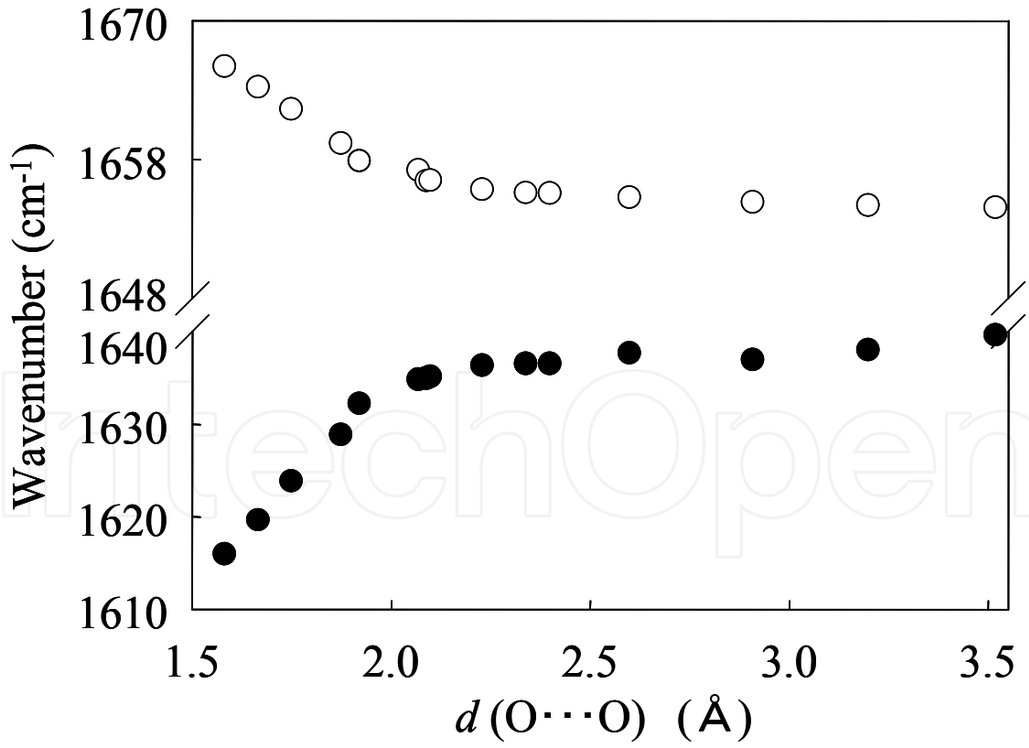


Fig. 10. Changes in the amide I′ frequency of NMA in the NMA-dimer + D_2O complex as a function of the bond distance ($d_{O\cdots O}$) between the C=O group of the peptide bond of NMA dimmer and the O-D group of water calculated at B3LYP/6-311+(d,p) basis set.

3.3 Origin of pressure stability of proteins: Interpretation from geometric volume calculations

We now consider the pressure stability of proteins in terms of geometric volume. PMV is a fundamental thermodynamic quantity that characterizes protein conformation (Chalikian, 2003) and is of principal importance in the analysis of pressure-induced denaturation of protein by Le Chatelier's law (Balny et al., 2002; Royer, 2002). Assuming that the protein unfolding process is a two-state transition (folded (F) ↔ unfolded (U)), the relationship between the pressure dependence of the equilibrium constant (K) and PMV is as follows:

$$(\partial \ln K/\partial p)_T = -\Delta V/\mathrm{RT}$$

where K is the equilibrium constant, where ΔV is the difference in PMV between the protein's folded and unfolded states. From this relationship, it is evident that increasing pressure causes the protein structure to change so as to minimize PMV ($\Delta V < 0$). Therefore, analysis of PMV differences associated with pressure unfolding can improve our understanding of the structural stability of proteins under high pressure.

Drawing upon the PMV analysis reported by Chalikian and Breslauer (Chalikian & Breslauer, 1996), we now systematically consider PMV differences between the folded and unfolded conformers of helical peptides and proteins. The PMV difference between the folded and unfolded states can be decomposed into three terms as follows:

$$\Delta V = \Delta V_{\mathrm{w}} + \Delta V_{\mathrm{v}} + \Delta V_{\mathrm{Hyd}},$$

where ΔV_{w} and ΔV_{V} are the van der Waals and void-volume differences, respectively, and ΔV_{Hyd} is the hydration volume difference caused by changes in intermolecular interactions between the solvent water and solute.

We suggest that protein void space may be the most significant factor affecting the pressure stability of proteins. Lopez *et al*. have speculated similarly (Lopes et al., 2004). They investigated the pressure dependence of the volume contribution of the hydrophobic internal cavity inside three-helix bundle mutant proteins and showed that a large cavity space induces a large change in volume. Therefore, we focus herein on the void volume of various helical peptides and proteins to elucidate the relationship between the pressure stability and volume properties of proteins.

We systematically calculated the geometric volumes of various peptides and proteins (probe size 2.98 Å). Table 1 shows the volume properties of various peptides and proteins. Figure 11(a) shows plots of V_{w}, V_{M}, and V_{v} as a function of molecular weight (MW). V_{w}, V_{M}, and V_{v} all increase linearly with MW. Imai *et al*. recently calculated the MW dependence of the molecular volume of various proteins and showed that V_{v} correlates well with MW (Imai et al., 2005a); our results are consistent with these. Figure 11(b) shows plots of V_{v}/MW as a function of MW. V_{v}/MW increases up to MW = ~20000 and remains constant thereafter.

The pressure stability of a protein structure is thought to correlate with the adiabatic compressibility (β) of the folded protein (Taulier & Chalikian, 2002; Gekko, 2004). That is, in a folded protein, increasing V_{v} causes an increase in β. The β values for the well-known helical proteins cytochrome *c* (Kamiyama et al., 1999) and myoglobin (Gekko & Noguchi, 1974) are 3.60 and 8.98 Mbar^{-1}, respectively. The former is clearly smaller than the latter.

Similarly, the V_v/MW values for cytochrome *c* and myoglobin are 0.153 and 0.223 $cm^3 \cdot mol^{-1} \cdot MW^{-1}$, respectively. Thus, for these two proteins, the difference in relationship of V_v/MW values correlates with the difference in β values.

Protein	PDB ID	$N_{residue}$*	MW**	V_w	V_M	V_v	V_v/MW
Helical peptide	1DJF	15	1586	955.6	1067.1	111.6	0.070
GCN4-p1	2TZA	68	7958	4550.2	5900.4	1353.3	0.170
Cytochrome *c*	1HRC	105	11688	7126.1	8913.9	1787.7	0.153
Four helix bundle	1MFT	106	12898	6855.8	8691.9	1836.2	0.142
Myohemerythrin	2MHR	118	13761	9679.0	12495.1	2816.0	0.204
Myoglobin	101M	154	17266	11134.3	14987.6	3853.3	0.223
Phospholipase c	1AH7	245	28373	17438.2	23448.4	6010.2	0.211
Hemerythrin (deoxy)	1HMD	452	53328	31758.1	42452.6	10694.5	0.200
Human Hemoglobin	1A3N	574	61922	39184.1	53289.1	14105.1	0.227

*$N_{residue}$: Amino acid residue
** MW: Molecular weight

Table 1. Volume contributions (cm^3/mol) of proteins by geometric volume calculation.

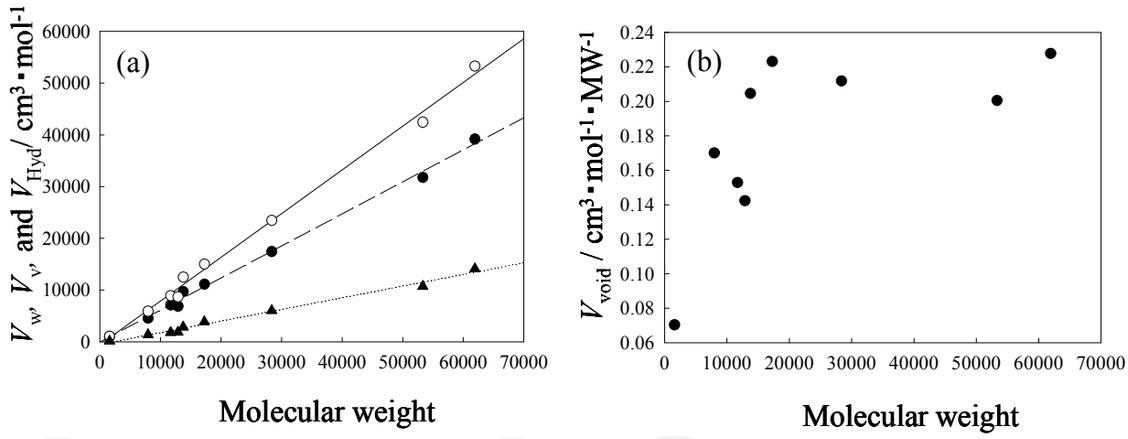


Fig. 11. (a) Fig. 11. (a) The changes in the van der Waals volume (V_w)(●), molecular volume (V_M)(○), and void volume (V_V)(▲) of various helical peptide and proteins as a function of molecular weight. The straight lines are the results of the least-squares analysis. The correlation coefficient values (*R*) of the least-square fit are 0.9736 for V_w, 0.9769 for V_M, and 0.9398 for V_V, respectively. (b) Change in the V_v per molecular weight of helical peptides and proteins as a function of molecular weight.

Next, we consider the pressure stability of proteins obtained by our present and previous results. The helical structures of AK peptide (15 residues), helix bundle proteins (64 residues) (Takekiyo et al., 2006c), and GCN4-P1 (68 residues) (Imamura et al., 2010) do not unfold even at ~1.0 GPa. The V_v/MW values for these helical peptides and proteins are all <0.2 $cm^3 \cdot mol^{-1} \cdot MW^{-1}$. In contrast, the helical structures of myoglobin (153 residues) (Meersman et al., 2002) and α-lactalbumin unfold completely at 0.7 GPa (Wojciech et al., 1999). The V_v/MW value for myoglobin is 0.223 $cm^3 \cdot mol^{-1} \cdot MW^{-1}$. From Figure 11 (b), we see that the

structures of helical peptides and proteins for which $V_v/MW < 0.2$ $cm^3 \cdot mol^{-1} \cdot MW^{-1}$ exhibit small pressure stability, and those for which $V_v/MW > 0.2$ $cm^3 \cdot mol^{-1} \cdot MW^{-1}$ exhibit large pressure stability. Thus, our calculation result is in qualitatively good agreement with previous experimental results for the pressure stability of helical peptides and proteins, and both the examined proteins do indeed exhibit large pressure stability. On the basis of these calculations, we suggest that helical proteins having a small void space are stabilized at high pressure.

Our findings so far suggest that, for successful preservation under high pressure, two conditions must be met: (1) the α-helical structure must be sufficiently hydrated with water molecules, and (2) the protein must have a small void space.

3.4 Comparisons of the structural stability of proteins at low temperature

We have just discussed the structural stability of proteins under high pressure. We now focus on the structural stability of proteins at low temperature (liquid N_2, 77 K).

Figure 12 shows the FTIR (upper) and CD (lower) spectra of PLGA, BSA, and cytochrome *c* in aqueous solution at 298 K and 77 K, both at 0.1 MPa. The FTIR spectrum of PLGA shows an intriguing change: the peak at 1638 cm^{-1} increases in intensity with decreasing temperature from 298 to 77 K. This peak position is close to that assigned to the solvated α-helical structure, despite the fact that bulk water is crystallized at 77 K. If low temperature induces the solvated α-helical structure of PLGA, the associated CD spectrum at low temperature should show double negative bands at 208 and 222 nm, characteristic of an α-helical structure. Indeed, the CD spectra of PLGA show, at 298 K, a negative band at 195 nm, characteristic of a random-coil structure, the CD spectra show the characteristic of α-helical structure. Thus, low temperature induces the transition of PLGA from coil to helix and then stabilizes the resulting solvated α-helical structure.

What is the α-helical structure of proteins at 77 K? In Figure 12, the peaks for BSA and cytochrome *c* at ~1635 cm^{-1} increase with decreasing temperature from 298 to 77 K. The CD spectra show negative bands at 208 nm and 222 nm even at 77 K, albeit at changed intensity. Thus, the solvated α-helical structure of PLGA, BSA, and cytochrome *c* is clearly stabilized at 77 K despite the crystallization of bulk water. Moreover, these spectral changes are completely reversible. Thus, the low-temperature-induced stabilities of the solvated α-helical structure of helical proteins are very similar to the pressure-induced stabilities.

Previous FTIR studies by Manas *et al*. and Walsh *et al*. showed that the solvated α-helical structure of α_3D (a *de novo* designed three-helix bundle protein), GCN4-p1, and parvalbumin in glycerol–D_2O mixed solution does not unfold at low temperature (10 K) but rather is stabilized (Manas et al., 2000; Walsh et al., 2003). Generally, addition of glycerol to a protein solution is known to induce incremental structural stability in the protein (Zelent et al., 2004; Wright et al., 2003). Our present results show that the solvated α-helical structures of PLGA, BSA, and cytochrome *c* are stabilized at 77 K without the addition of glycerol. On the other hand, the secondary structures of RNase A, lysozyme, and trypsin having a β-sheet structure show an increase in the ratio of unfolded structure to β-sheet structure at 77 K (data not shown). Based on these results, we found the difference of the structural stability of proteins at 77 K from the viewpoint of two secondary structure types (the α-helix and β-sheet). A general rule is that the solvated α-helical structure is hydrophilic and the β-sheet

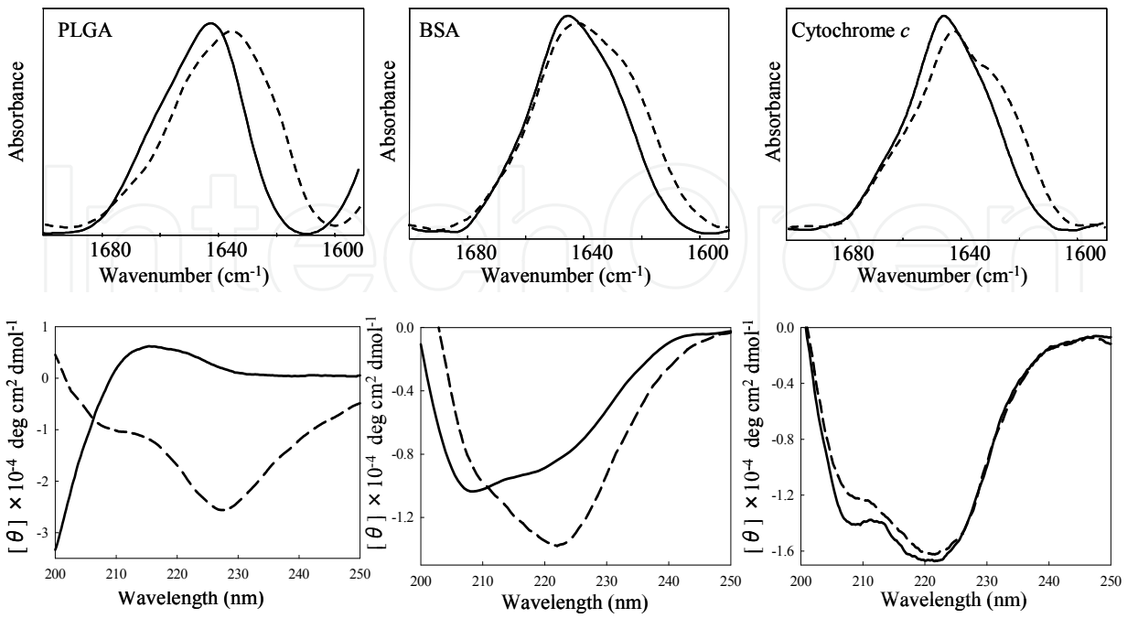


Fig. 12. FTIR (upper) and CD (lower) spectra of PLGA, BSA, and cytochrome *c* in water at 298 (solid line) and 77 K (long-dashed line).

structure is hydrophobic. Typically, the stability of β-sheet cores in proteins requires the exclusion of water since the hydrogen bonding is much sparser between the β-sheets (between individual strands) than between the turn or α-helix. If the bulk water is frozen it is impossible for the water exclusion effects to act and no reduction in free energy is achieved by formation of β-sheet structure. In contrast, as stated above, α-helical structures are formed by H-bonds between successive loops in the helix. These H-bonds would come more stable (relative to thermal fluctuations) at low temperature. Hence, lowering the temperature may even induce α-helix formation such as PLGA and helical proteins.

In this section, our findings so far suggest that, for successful preservation under low pressure, one condition must be met: the α-helical structure must be sufficiently hydrated with water molecules as in the case of high pressure.

3.5 Feasibility of protein preservation under high pressure and low temperature

As mentioned in Section 1, a necessary condition for protein preservation under high pressure and low temperature is that ice formation must be avoided, and this can be accomplished by addition of a freezing-protection agent such as sugar or salt. For example, Yamamoto *et al*. recently reported compression recovery of the secondary structure of RNase A in a sucrose–D_2O mixed solution (Yamamoto, et al. 2010). Manas *et al*. and Walsh *et al*. reported, by FTIR study, that the secondary structures of α_3D, GCN4-p1, and parvalbumin in a glycerol–D_2O mixed solution do not unfold at low temperature (10 K), suggesting that the solvated α-helical structure hydrated with water molecules is stabilized under this condition (Manas et al., 2000; Walsh et al., 2003). Moreover, Thus, under high

pressure and low temperature, sugar solutions can induce retention of the secondary structures of proteins.

Given that the protein structure can be retained with addition of a freezing-protection agent, can it also be retained without addition of such an agent? Our present and previous results (Meersman et al, 2002, 2003; Ruan et al., 1999; Smeller et al., 2006) show that proteins having a β-sheet structure unfold at high pressure and show an increase in the ratio of unfolded structure to β-sheet structure at 77 K. Therefore, protein preservation under high pressure and low temperature is difficult without the use of a freezing-protection agent. However, our present results show that the secondary structures of helical proteins having a small void space do not unfold at high pressure and low temperature. Thus, helical proteins may resist low–temperature- and high-pressure-induced protein unfolding, and their preservation under high-pressure and low-temperature conditions may thus be feasible.

4. Summary and conclusions

Protein preservation under high pressure and low temperature can be hampered by two issues: (1) conversion of bulk water to ice, and (2) structural instability of the protein by pressure unfolding (which occurs at pressures of >0.5 GPa) and cold unfolding (which occurs at temperatures of <243 K). The first issue can be solved by use of a freezing-protection agent. Our present results show that the second issue is not a concern for helical proteins having a small void space because these proteins are clearly stabilized at high pressure (~1.0 GPa) or low temperature (77 K). Thus, proteins having a high degree of solvated α-helicity have a high feasibility of preservation under high pressure and low temperature, even without the use of a freezing-protection agent.

Many previous studies have reported the structural stability of proteins in aqueous sugar or salt solutions at low temperature, but only a few have done so at high pressure. However, both pressure and temperature play critical roles in regulating the structures and properties of proteins, and both are important tools for exploring new methods of protein preservation. Our present results demonstrate the feasibility of protein preservation under conditions of both high pressure and low temperature.

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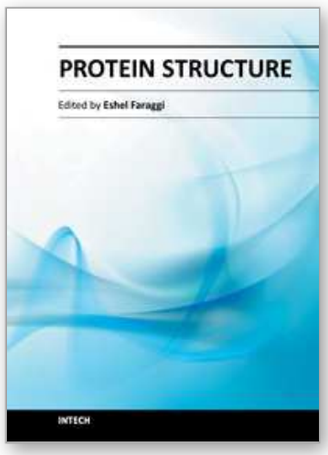

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Since the dawn of recorded history, and probably even before, men and women have been grasping at the mechanisms by which they themselves exist. Only relatively recently, did this grasp yield anything of substance, and only within the last several decades did the proteins play a pivotal role in this existence. In this expose on the topic of protein structure some of the current issues in this scientific field are discussed. The aim is that a non-expert can gain some appreciation for the intricacies involved, and in the current state of affairs. The expert meanwhile, we hope, can gain a deeper understanding of the topic.

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