Pressure-induced Structural and Hydration Changes of Proteins in Aqueous Solutions

Min ZHANG and Yuqing WU[†]

State Key Laboratory for Supramolecular Structure and Materials, Jilin University, No. 2699 Qianjin Street, Changchun 130012, P. R. China

The effects of elevated hydrostatic pressure on four representative proteins, lysozyme, human serum albumin, ubiquitin and RNase A, were investigated by using Fourier transform infrared (FTIR) spectroscopy, by principal component analysis (PCA) and by moving-window two-dimensional (MW2D) correlation analysis. In addition, we revealed the pressure-induced changes of secondary structure elements using curve fitting. With pressure increase, the amide I band shifted to lower wavenumbers, with a transition at 200 MPa, which was indicative of hydration enhancement. Moreover, the pressure-induced behavior of pure water was studied, similar transition pressure was observed with protein in aqueous solution, suggesting that structure change of water around 200 MPa caused a hydration enhancement of protein. Under pressure higher than 200 MPa, the structural changes of the four proteins were obviously different except for the common features shifting to lower wavenumbers with pressure, basically due to the distinct structural differences among them.

(Received August 17, 2011; Accepted September 2, 2011; Published November 10, 2011)

Introduction

The mechanisms of protein folding and unfolding have been investigated in intensive experimental and theoretical studies for several decades. Furthermore, a detailed understanding of the structure in the unfolded state of proteins has been a major research topic in protein chemistry and biophysics in recent years. Pressure as well as temperature induced effects in proteins are closely connected with the presence of water. Changes in the interaction with water (hydration) and the imperfect packing effects in the interior (void volume and cavities) contribute greatly to the structural and dynamic variations of proteins in aqueous solutions.¹⁻³

Pressure is able to change the shape of a protein, extremely high pressure can lead to unfolding of the protein, but lower pressures cause only elastic distortion of the secondary and tertiary structures.^{4,5} In order to clearly explain the relationship between structure changes of water and protein, we chose four typical proteins, lysozyme, human serum albumin (HSA), ubiquitin and RNase A as samples, these have different secondary structures and space conformations. Lysozyme mainly contains α -helices and subsidiary β -sheets.^{6,7} HSA mainly includes α -helices and minor random coils.⁸ Ubiquitin mainly contains β -sheets and subsidiary α -helices.⁹ RNase A with a β -sheet main structure, contains α -helices and turns.¹⁰ We investigated pressure-induced structure changes of four proteins in aqueous solution from 0.1 to 740 MPa by using infrared spectroscopy. Both the hydration and minor structural changes of proteins have been revealed clearly by principal component analysis (PCA) and moving-window two-dimensional (MW2D) correlation analysis.

Experimental

Reagents and chemicals

Lysozyme, HSA, ubiquitin, RNase A and deuterated Tris-DCI (Tris(hydroxymethyl)aminomethane) were purchased from Sigma Chemical Co.; these materials were used without further purification. Deuterium oxide (D₂O) was purchased from Cambridge Isotope Laboratory, Inc. The concentrations of proteins were 50 mg/ml in 50 mM Tris/D₂O, followed by a pD 7.8. Before the pressure scan, the samples were heated up to 45°C for 30 min and then cooled down to enable the complete H/D exchange of all solvent-accessible protons.¹¹

Apparatus and procedure

High pressure was generated in a diamond anvil cell (Diacell Lever DAC-Mini A65000), where the pressure was built up by means of a screw mechanism.¹² The BaSO₄ was the internal pressure calibrant, and the IR absorption band is 983 cm⁻¹.¹³ Infrared spectra were measured at room temperature on a Bruker Vertex 80v Fourier transform spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector. For each spectrum, 512 interferograms were co-added with a spectral resolution of 2 cm⁻¹. The obtained spectra were baseline corrected in the region of the amide I band.^{14,15} Curve fitting with Gaussian functions was performed using Origin software. PCA was performed with the treated spectra after mean-centering using the Unscrambler 5.0 program. MW2D correlation plots were computed using 2D Shige software.¹⁶

Results and Discussion

Pressure-induced structural changes of four proteins

The structural changes of lysozyme are displayed in Fig. 1A.

[†] To whom correspondence should be addressed.
E-mail: yqwu@jlu.edu.cn

Fig. 1 Pressure-induced IR spectra of (A) lysozyme, (B) HSA, (C) ubiquitin and (D) RNase A. Arrows represent the direction of change with increasing pressure.

Table 1 Fraction of secondary structure elements of four proteins in the initial and final state, respectively

Protein	P/MPa	Population of secondary structure elements			
		α -Helix, %	β -Sheet, %	β -Turn, %	Random coil, %
Lysozyme	0.1	47.7	18.5	15.7	18.2
	740	18.4	19.1	35.7	26.8
HSA	0.1	52.1 ^a , 29.3 ^b		8.1	6.8
	740	45.5 ^a , 41.0 ^b		5.6	5.5
Ubiquitin	0.1	21.4	45.9	21.5	11.1
	740	12.1	56.0	25.6	6.3
RNase A	0.1	20.0	46.1	28.6	5.3
	740	11.9	41.1	35.7	11.2

a. The solvent-inaccessible α -helix.

b. The solvent-exposed α -helix.

Under the initial pressure, the amide I band is dominated by 1656 and 1639 cm^{-1} , which are assigned to α -helix and the β -sheet, respectively.^{6,7} During the pressure increase, the band at 1656 cm^{-1} shifts to around 1643 cm^{-1} . The spectrum of the native HSA has a main peak at 1656 cm^{-1} , with shoulders at 1636 and 1673 cm^{-1} , the positions consistent with the crystallographic structure of HSA.^{13,17,18} As the pressure increases, the maximum gradually shifts to the lower wavenumbers. The band at 1639 cm^{-1} decreased with pressure in Fig. 1C, and shifted to lower wavenumbers. Under initial pressure, the amide I band of RNase A are dominated by the β -sheet at 1641 cm^{-1} and a turn at 1663 cm^{-1} . With pressure increase, weakening of the band at 1663 cm^{-1} and a shifting from 1641 cm^{-1} to lower wavenumbers occur; such band features can be observed more clearly in the second derivative spectra (Fig. S1, Supporting Information).

The curve fitting (Fig. S2, Supporting Information) was performed and is shown in Table 1. It clearly displays that the α -helix content in lysozyme decreases 29.3%, the turn and random coil increase 20.0 and 8.7%, respectively, but the β -sheet decreases slightly. In the HSA, the solvent inaccessible α -helix decreases 6.7% accompanied with an 11.8% increase of the solvent exposed α -helix, but the turn and random coil change slightly. The α -helix and random coil of ubiquitin

Fig. 2 Score plots of the first two principal components (■, PC1; □, PC2) for (A) lysozyme, (B) HSA, (C) ubiquitin and (D) RNase A.

decrease 9.3 and 4.8%, respectively, while the β -sheet and turn increase 14.4% in total. The main structure is β -sheet in RNase A, it decreases 4.9%, while the turn and random coil increase 13.0% in total.

The random coil increases with pressure in the lysozyme and RNase A, which is opposite to the HSA and ubiquitin cases, implying that a pressure induced structural change in proteins does not always transfer from a regular structure to a random one. In addition, the regular secondary structures in proteins like HSA and ubiquitin would rearrange from the original one under pressure, leading to a secondary structure, which is more stable than the native one under pressure.

Pressure induced dynamics of four proteins as revealed by PCA

PCA can evaluate some information in the directions of both the spectral variables (loading plot) and the perturbation (score plot) individually.¹⁹ Figure 2 shows score plots of the first two important PC factors, which account for nearly 100% of the total variance. The curve of PC1 in Fig. 2A is a plurality state process, with several transitions at about 200, 400 and 580 MPa. Further considering the scores of PC2, despite the fact that PC2 accounts for only 2% of the total variance, one can observe two significant transition pressures at 200 and 580 MPa, can be observed by PC2. The second transition ($P = 580$ MPa) in PC2 correlates well with that observed from the PC1, in agreement with the result of the band analysis (Fig. S3A, Supporting Information).

The two PCs of HSA is not a pure two-state process, several fluctuations appeared at around 200, 400, 500 and 600 MPa. Although PC2 accounts for only 5%, one significant change appears at 200 MPa. The PCA results of ubiquitin in Fig. 2C, similarly, for the curve of PC1-scores (89%) not a two-state process is evident. The PC2 (11%) value for ubiquitin is

Fig. 3 MW2D correlation spectra of (A) lysozyme, (B) HSA, (C) ubiquitin and (D) RNase A.

relatively higher than the value for other proteins, which can be attributed to the changes at around 200 and 680 MPa, respectively. Compared with lysozyme, HSA and ubiquitin, the PC1 curve (85%) of RNase A is more approximated by a two-state process, with a midpoint at $P_m = 400$ MPa. A slight fluctuation at around 200 MPa in the PC1 curve, combined with the 15% variance explaining PC2, illustrate the presence of two transitions at around 200 and 400 MPa.

Moving-window 2D correlation analysis reveals the hydration revealing of four proteins

We applied MW2D correlation analysis to these spectra.²⁰ As shown in Fig. 3A, a correlation band of 1650 cm^{-1} appears at around 200, 400, 600 MPa, respectively, while the intensity of correlation at 200 MPa is weaker than those at 400 and 600 MPa. A correlation band of 1626 cm^{-1} appears at around 600 MPa, but the intensity change is weaker than at 1650 cm^{-1} . This indicates that the stability of β -sheet is stronger than that of the α -helix in lysozyme upon pressure.

The weak correlation band at 1636 cm^{-1} appears at around 200 MPa for HSA, and two other correlation bands related to 1626 and 1673 cm^{-1} appear at around 400 MPa, while that of 1656 cm^{-1} appears near 450 MPa. This indicates that the stability of the solvent exposed α -helix (1636 cm^{-1}) is lower than that of the solvent inaccessible α -helix (1656 cm^{-1}), which is composed of short turn connecting structures. The correlation band of 1626 cm^{-1} has a broad region from 200 to 600 MPa in Fig. 3C. The strong band at 1639 cm^{-1} appeared at the center of 600 MPa, while a much weaker correlation band at 1673 cm^{-1} appeared at 400 MPa. It is sure that weak structural changes occurred at around 200 MPa. Figure 3D shows that the strong and broad correlation band positioned at 1639 cm^{-1} appears at around 200 MPa, and the weaker band at 1626 cm^{-1} comes into

Fig. 4 MW2D correlation spectra of deuterium oxide under pressure.

existence at around 500 MPa. The two correlation bands at 1639 and 1626 cm^{-1} can mainly be attributed to β -sheets. Notably, the pressure-induced structural transitions of all four proteins are polymorphism processes.

Water and structural changes of four proteins under pressure

Pressure-induced changes of D_2O , a band center at 1208 cm^{-1} can be clearly observed under atmospheric pressure; this center is assigned to OD bending,²¹ and gradual shifts to lower wavenumbers with a pressure increase (Fig. S4, Supporting Information). MW2D calculation was preformed and is recorded in Fig. 4. It must be noted that two correlation bands centered at 1194 cm^{-1} can be observed at 200 and 400 MPa, respectively; the one at 200 MPa is stronger than that at 400 MPa.

Zheng *et al.*^{22,23} investigated the absorbed band area change of water under pressure: they found that the structure change of water discontinues at 200 and 400 MPa, respectively. From the energy-dispersive X-ray diffraction technique, Okhulkov *et al.*²⁴ found that the average separation between the nearest molecules decreases with pressure up to ~ 200 MPa, but at higher pressures it begins to grow at 400 – 500 MPa. Raman scattering of the stretching band from water changes under pressure up to 600 MPa;²⁵ the result showed that the intensity decreased with increasing pressure initially and then reached a minimum at about 200 MPa, and increased again with the further increase about 400 MPa. These findings confirmed perfectly that local structure changes of water occurred at 200 and 400 MPa, in agreement with the current result of our MW2D analysis.

The above changes of water are highly consistent with the behaviors of four proteins under pressure. The water in the hydration shell of proteins has been proposed to play a crucial role in high pressure induced protein unfolding, and it was proposed that the penetration of water molecules into the hydrophobic core may induce unfolding of the system.²⁶ The water penetration model was also supported by high pressure molecular dynamics simulations.²⁷ Winter *et al.*²⁸ carried out a series of molecular dynamics computer simulations on SNase. Their results indicated that the hydration of the interfacial hydrophobic part and the water density of the protein increased under pressure. Notably, slight changes of the H-bond network are observed at 200 MPa, probably due to the rearrangement of the residues at the protein surface. In addition, the structure change of water in the hydration shell of proteins was also well documented by results from scattering experiments.²⁹ Those authors showed a wide range of non-random hydrogen bonding environments in the hydration shell of globular proteins. In addition to surface hydration, internal water molecules contribute

also to protein stability.³⁰ They provide the necessary flexibility for biological activity, act as a lubricant and render to the protein a certain level of plasticity. The presence of water molecules close to protein internal hydrogen bonds induces a lengthening of these hydrogen bonds.³¹ Therefore, it can be deduced that a structure change of water is an incentive that leads to changes of proteins when the pressure is up around 200 MPa.

In fact, similar phenomena also occur for other proteins as reported earlier. For example, high pressure NMR measurements of lysozyme indicated purely elastic deformation of the molecule in the 0.1 – 200 MPa.⁴ Marabotti *et al.*³² investigated pressure affects on the structure and the dynamics of D-galactose/D-glucose-binding protein: a slope can be observed at 200 MPa for the plot of wavenumber *versus* pressure, which was also observed in deconvoluted spectra of GGBP. Pfeiffer *et al.*³³ reported an FTIR study on the pressure induced unfolding of lysozyme in pure D₂O, being close to the conditions of non-excess hydration. A similar phenomenon was also observed but it was not pointed out.

Conclusions

Pressure-induced structure changes of protein have been investigated by FTIR spectroscopy. The combination of the analysis of curve fitting, PCA and especially MW2D revealed information on structure transitions induced by pressure.

With regard to the pressure induced structure changes of lysozyme, HSA, ubiquitin, and RNase A, it became obvious that the characterized amide I band of protein shifted to lower wavenumbers with a common transition at 200 MPa, indicating that pressure induced a hydration increase at the lower pressure region. This is similar to the pressure induced structure change of pure water, and it is possible that the structure change in the proteins studied is caused by the structure change of water. Moreover, the change processes for the proteins were found not to be two-state processes, as several fluctuations along the pressure change were observed according to the analytical results of the PCA and MW2D analyses.

Acknowledgements

The authors are grateful for the financial supports of the Natural Science Foundation of China (Nos. 20934002 and 20973073), the National Basic Research Program (2007CB808006), Jilin Province Natural Science Foundation (20070926-01), the Programs for New Century Excellent Talents in University (NCET) and the 111 Project (B06009).

Supporting Information

The second derivative spectra, curve fitting, plots of position of amide I band *versus* pressure, and the pressure-induced IR spectra of deuterium oxide are available. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

References

1. K. Heremans and L. Smeller, *Biochim. Biophys. Acta*, **1998**, 1386, 353.
2. H. Imamura and M. Kato, *Proteins: Struct., Funct., Bioinf.*, **2009**, 75, 911.
3. S. Chodankar, V. K. Aswal, J. Kohlbrecher, R. Vavrin, and A. G. Wagh, *Phys. Rev. E*, **2008**, 77, 031901.
4. Y. O. Kamatari, H. Yamada, K. Akasaka, J. A. Jones, C. M. Dobson, and L. J. Smith, *Eur. J. Biochem.*, **2001**, 268, 1782.
5. K. Suzuki, Y. Miyosawa, and C. Suzuki, *Arch. Biochem. Biophys.*, **1963**, 101, 225.
6. D. W. Goddette, C. Paech, S. S. Yang, J. R. Mielenz, C. Bystroff, M. E. Wilke, and R. J. Fletterick, *J. Mol. Biol.*, **1992**, 228, 580.
7. M. A. Walsh, T. R. Schneider, L. C. Sieker, Z. Dauter, V. S. Lamzin, and K. S. Wilson, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, **1998**, 54, 522.
8. S. Sugio, A. Kashima, S. Mochizuki, M. Noda, and K. Kobayashi, *Protein Eng., Des. Sel.*, **1999**, 12, 439.
9. S. Vijay-Kumar, C. E. Bugg, and W. J. Cook, *J. Mol. Biol.*, **1987**, 194, 531.
10. A. Wlodawer, N. Borkakoti, D. S. Moss, and B. Howlin, *Acta Crystallogr., Sect. B: Struct. Sci.*, **1986**, 42, 379.
11. Y. Wu, K. Murayama, and Y. Ozaki, *J. Phys. Chem. B*, **2001**, 105, 6251.
12. Y. Wu, F. Meersman, and Y. Ozaki, *Macromolecules*, **2006**, 39, 1182.
13. P. T. T. Wong and D. J. Moffatt, *Appl. Spectrosc.*, **1989**, 43, 1279.
14. B. Czarnik-Matusiewicz, K. Murayama, Y. Wu, and Y. Ozaki, *J. Phys. Chem. B*, **2000**, 104, 7803.
15. K. Murayama, Y. Wu, B. Czarnik-Matusiewicz, R. Tsenkova, and Y. Ozaki, *J. Phys. Chem. B*, **2001**, 105, 4763.
16. M. Unger, S. Morita, H. Sato, Y. Ozaki, and H. W. Siesler, *Appl. Spectrosc.*, **2009**, 63, 1027.
17. P. I. Harris and D. Chapman, *Biopolymers*, **1995**, 37, 251.
18. F. Meersman, L. Smeller, and K. Heremans, *Biophys. J.*, **2002**, 82, 2635.
19. K. R. Beebe, R. J. Pell, and M. B. Seasholtz, “*Chemometrics: A Practical Guide*”, **1998**, Wiley-Interscience, New York.
20. S. Morita, H. Shinzawa, R. Tsenkova, I. Noda, and Y. Ozaki, *J. Mol. Struct.*, **2006**, 799, 111.
21. S. Y. Venyaminov and F. G. Prendergast, *Anal. Biochem.*, **1997**, 248, 234.
22. H. F. Zheng, A. Shen, M. Zhang, Q. Sun, and J. Y. Chen, *Prog. Nat. Sci.*, **2002**, 12, 662.
23. L. R. Murphy, N. Matubayasi, V. A. Payne, and R. M. Levy, *Fold. Des.*, **1998**, 3, 105.
24. A. V. Okhulkov, Y. N. Demianets, and Y. E. Gorbaty, *J. Chem. Phys.*, **1994**, 100, 1578.
25. Q. Sun, H. Zheng, J. Xu, H. Xie, and E. Hines, *Chin. Phys. Lett.*, **2003**, 20, 445.
26. A. Paliwal, D. Asthagiri, D. P. Bossev, and M. E. Paulaitis, *Biophys. J.*, **2004**, 87, 3479.
27. G. Hummer, S. Garde, A. E. Garcia, M. E. Paulaitis, and L. R. Pratt, *Proc. Natl. Acad. Sci. U. S. A.*, **1998**, 95, 1552.
28. N. Smolin and R. Winter, *Biochim. Biophys. Acta*, **2006**, 1764, 522.
29. V. Makarov, B. M. Pettitt, and M. Feig, *Acc. Chem. Res.*, **2002**, 35, 376.
30. K. Takano, J. Funahashi, Y. Yamagata, S. Fujii, and K. Yutani, *J. Mol. Biol.*, **1997**, 274, 132.
31. S. Fischer and C. S. Verma, *Proc. Natl. Acad. Sci. U. S. A.*, **1999**, 96, 9613.
32. A. Marabotti, A. Ausili, M. Staiano, A. Scirè, F. Tanfani, A. Parracino, A. Varriale, M. Rossi, and S. D'Auria, *Biochemistry*, **2006**, 45, 11885.
33. H. Pfeiffer, K. Heremans, and M. Wevers, *Chem. Phys. Lett.*, **2009**, 469, 195.