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2006 J. Phys.: Condens. Matter 18 L107

(http://iopscience.iop.org/0953-8984/18/7/L01)

INSTITUTE OF PHYSICS PUBLISHING JOURNAL OF PHYSICS: CONDENSED MATTER

J. Phys.: Condens. Matter **18** (2006) L107–L113 doi:10.1088/0953-8984/18/7/L01

LETTER TO THE EDITOR

On the physics of pressure denaturation of proteins

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Received 2 November 2005, in final form 23 January 2006
Published 2 February 2006
Online at stacks.iop.org/JPhysCM/18/L107

Abstract

We show that the entropic effect originating from the translational movement of water molecules plays critical roles in the pressure-induced denaturation of proteins. In our statistical–mechanical method, the partial molar volume governing the pressure dependence of the structural stability is expressed in terms of the two geometric measures of each protein structure, the excluded volume and the accessible surface area for water molecules, and a parameter related to the water-density profile entropically formed near its surface. An unfolded structure, which is shown to turn more stable than the native one at an elevated pressure, successfully features the experimentally observed denaturation.

A protein, a long polypeptide chain, spontaneously folds into a unique native structure in aqueous solution under physiological conditions [1]. However, the native structure is unfolded by various perturbations such as the addition of chemical substances, the change in the temperature or pH, and the application of a high pressure [2–6]. Investigating those processes will provide physical insights into the folding/unfolding transition of proteins. Above all, pressure-induced unfolding is one of the most interesting subjects. Recent experimental studies indicate that the ensemble of unfolding pathways for pressure denaturation is inherently different from those for heat or chemical denaturation [3, 4]. The conformational entropy of the polypeptide chain for an unfolded state is much larger than that for a folded state, and the difference increases further at a higher temperature, leading to the heat denaturation [7]. Since the denaturants have high affinity with the protein surface, an unfolded structure with a larger exposed-surface area is more favoured, causing the chemical denaturation [8]. In the native structure of a protein, the backbone and side chains are closely packed with little space in the interior [9, 10], and one might think that such a structure is even more stabilized by applying a higher pressure. However, this thought completely contradicts the experimentally known fact that a protein unfolds at an elevated pressure. Compared to heat or chemical denaturation, the pressure effect appears to be considerably more difficult to understand. It

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is definite that pressure-induced unfolding takes place only through the solvent effect, and the pressure dependence of solvation properties of a protein is a key factor to be investigated.

It is stated in literature [11, 12] that heat denaturation corresponds to the transfer of nonpolar residues from the protein interior into water, whereas pressure denaturation can be studied in terms of the free energy of water transfer to the hydrophobic core of the protein. For methane–methane interaction in water, the stability of the contact pair relative to the solvent-separated one is shown to decrease with rising pressure [11]. Computer simulation studies [12, 13] indicate that nonpolar side chains are more separated in a pressure-denatured structure with water molecules penetrating its core than in the native one. This indication is suggestive that the weakening of the hydrophobic interactions between nonpolar side chains is the major cause of pressure denaturation. However, the physical origin of the weakening is not clear. For a complete interpretation of the protein denaturation, it is necessary to treat a protein itself immersed in water rather than to deduce the mechanism from the behaviour of small molecules or protein subunits. Recently, we have shown that the entropic effect, which promotes an increase in the translational entropy of water, is a major driving force in protein folding toward the native structure [14, 15]. At least within the framework of classical mechanics, the potential energies among the atoms constituting the protein–water system do not change against rising pressure. The conformational entropy of an unfolded state is much higher than that of a folded state, but the difference in this quantity between the two states should be almost independent of the pressure. By contrast, the entropic effect for water is strongly dependent on the pressure [14, 15] and likely to play dominant roles in pressure denaturation. Although one might imagine that the native structure is further stabilized through the entropic effect by applying a higher pressure, the behaviour of the water entropy is not that simple. In this letter, we employ an elaborate statistical–mechanical theory for liquids and show that pressure denaturation can certainly be elucidated in terms of the purely entropic effect.

To concentrate on the entropic effect in our analysis, the solvent molecules are modelled as hard spheres with diameter $d = 0.28$ nm that is the size of water molecules, and a protein molecule is treated as a set of fused hard spheres. The diameter of each atom is set at the Lennard-Jones sigma of AMBER 99. In such a system, all the allowed configurations share the same energy, and the system behaviour becomes purely entropic in origin. The solvation free energy (SFE), which is attributable to the entropic effect alone in our model, governs the structural stability of a protein. Figure 1 illustrates a schematic relation between $\Delta\mu$ and the pressure P for three different structures including the native structure and the pressure-denatured one. The slope is the partial molar volume (PMV) Δv defined by

$$\Delta v = (\partial \Delta\mu / \partial P)_T, \tag{1}$$

where T is the absolute temperature. For the denaturation to occur, $\Delta\mu$ of the denatured structure minus $\Delta\mu$ of the native structure must be a decreasing function of P, requiring that the PMV of the denatured structure be smaller than that of the native one. As a striking case, although the SFE of the denatured structure is higher than that of the native one in the low-pressure region, the inversion in the SFE occurs at a sufficiently high pressure as shown in figure 1. We show in this letter that the inversion actually occurs through the solvent-entropy change. Even when there is a structure (structure X in figure 1) whose PMV is smaller than that of the native one, however, such inversion can hardly occur if its SFE is much higher. Therefore, the SFE of the denatured structure needs to be sufficiently low even at low pressures.

We employ the three-dimensional (3D) integral equation theory [16, 17] to account for the complex polyatomic structure of a protein. The details of this theory and the numerical procedure for solving the basic equations are described in earlier publications [14–17]. The PMV and SFE are considered for a prescribed structure. The SFE is calculated by simple

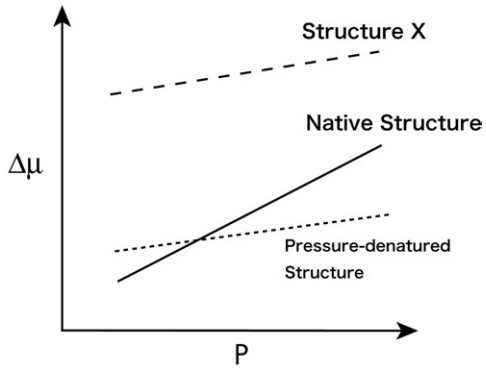

Figure 1. Schematic relation between the solvation free energy and the pressure for three different structures of a protein. The slope represents the partial molar volume (PMV). Though the straight lines are drawn for simplicity, the actual PMV is not constant (see figure 2(b)).

integrations of the protein–solvent correlation functions [18, 19]. The PMV is calculated in accordance with the formulation [20],

$$\Delta v = \chi_T k_B T + \int\int\int \{1 - g(x, y, z)\}\, dx\, dy\, dz, \tag{2}$$

where χ_T is the isothermal compressibility of pure solvent, k_B is Boltzmann's constant, and $g(x, y, z)$ has the physical meaning that the number of solvent molecules within the volume element $dx\, dy\, dz$ is given by $\rho g(x, y, z)\, dx\, dy\, dz$ (ρ is the solvent number density in the bulk). The first term in the right-hand side of equation (2) is relatively much smaller and can be neglected. The second term can be divided into the integrations inside and outside the core region. Inside the core region, due to the overlap of the protein and solvent, the protein–solvent potential is infinitely large and $g = 0$. It follows that the integration inside the core region equals the excluded volume (EV) which the centres of solvent molecules cannot enter (the EV is denoted by v_{ex}). The integration outside the core region takes a negative value because a layer within which the solvent is denser than in the bulk is formed near the protein surface due to the packing force arising from the translational movement of solvent molecules. Further, the integration outside the core region is roughly in proportion to the accessible surface area (ASA) denoted by a. Thus, we can write

$$\Delta v \sim v_{ex} - \xi a, \qquad \xi > 0. \tag{3}$$

Since the pressure-denatured structure is characterized by small Δv, it should possess considerably large a despite sufficiently small v_{ex}. It was suggested that the small cavities or void spaces in the protein interior make a significant contribution to the PMV [21]. We remark that the effects of the presence of such small cavities or void spaces are completely incorporated in the EV defined above.

In the real system, the average value of the water density within the layer formed near a hydrophobic group is lower than the value calculated for the hard-sphere solvent while that near a hydrophilic group is higher. Since the exposed surface of a protein comprises both hydrophobic and hydrophilic groups [10, 14, 15], the PMV of a protein in aqueous solutions can be reproduced fairly accurately by our method even in a quantitative sense. For example, the calculated value for the PMV of lysozyme (the Protein Data Bank (PDB) code: 1HEL) is 11 600 $cm^3\ mol^{-1}$ (the solvent number density is set at the value for water at 298 K), which is in good agreement with the average of the experimentally measured values [22]

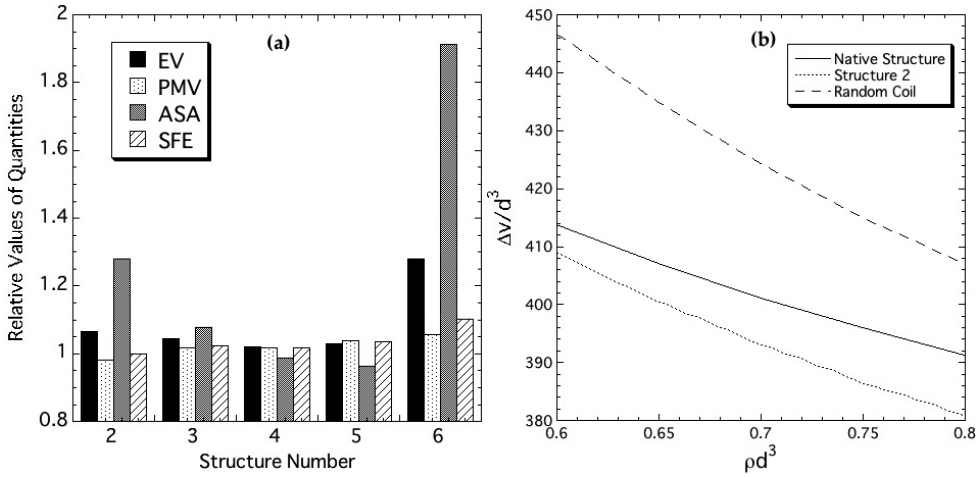

Figure 2. (a) Values of the excluded volume (EV), partial molar volume (PMV), accessible surface area (ASA), and solvation free energy (SFE) for five representative structures (structures 2–6) of protein G. They are divided by the values for the native structure (structure 1), respectively, and the quotients are referred to as 'relative values'. Structures 2–5 are much more compact than the random coil named structure 6. (b) PMV scaled by d^3 plotted against ρd^3 corresponding to P. Structures 1, 2, and 6 are considered.

10 100 $cm^3\ mol^{-1}$. Further, our method is capable of capturing the high sensitivity of the PMV to the atomic details of protein structure. Two structures at low (PDB code: 1GXV) and high (PDB code: 1GXX) pressures, respectively, have been reported for lysozyme [23]. These structures (not shown here) are almost indistinguishable by sight because the pressure is not high enough to cause appreciable denaturation. Nevertheless, the PMV calculated for the high-pressure structure is smaller than that for the low-pressure one (by 110 $cm^3\ mol^{-1}$) at the same solvent density, which is physically reasonable.

We consider four different structures of protein G in addition to its native structure and a random coil. The native structure is taken from the Protein Data Bank (PDB code: 2GB1). The random-coil structure is generated in accordance with the procedure described in our earlier publication [15]. The other four structures are taken from the local-minimum states of the energy function in computer simulations using all-atom potentials [24]. Figure 2(a) compares the values of the EV, PMV, ASA, and SFE for the five structures divided by the values for the native structure, respectively ($\rho d^3 = 0.7$, where ρd^3 denotes the dimensionless number density of the solvent and corresponds to P). Structure 1 is the native structure and structure 6 is the random coil. The EV and ASA are calculated using an analytical method [25]. It is observed that the EV and ASA vary greatly from structure to structure while the variation of the PMV and SFE is much smaller. There is only one structure, structure 2, the PMV of which is smaller than that of the native structure. Hereafter, we concentrate on structures 1, 2, and 6. In figure 2(b) the PMV scaled by d^3 is plotted against ρd^3. The solvent layer near the protein surface becomes even denser as P increases, and the parameter ξ is a monotonically increasing function of P. Hence, the slope given by

$$(\partial \Delta v/\partial P)_T \sim -(\partial \xi/\partial P)_T a = -\{\partial \xi/\partial(\rho d^3)\}_T \{\partial(\rho d^3)/\partial P\}_T a, \qquad \{\partial(\rho d^3)/\partial P\}_T > 0 \tag{4}$$

is negative and steeper for a structure with larger ASA ($\{\partial(\rho d^3)/\partial P\}_T$ is independent of the protein structure). A significant point is that the PMV of the random coil is larger than that

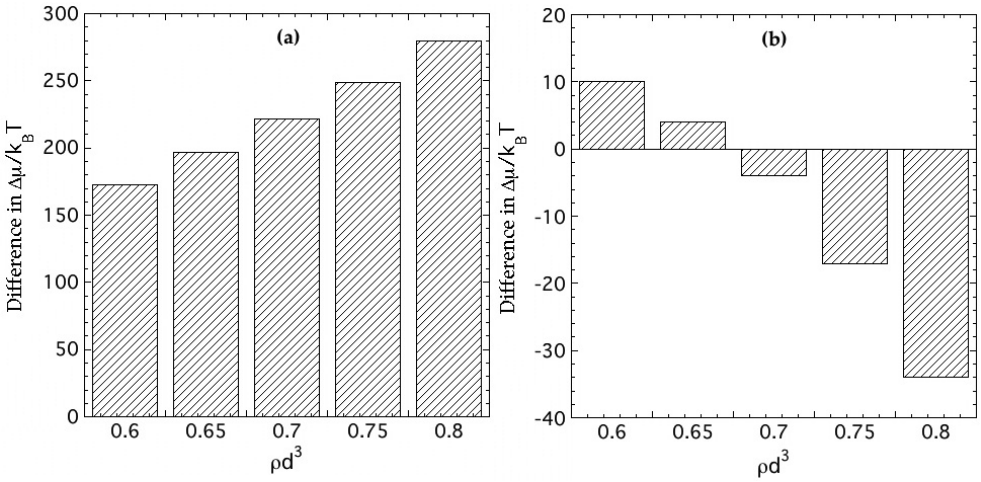

Figure 3. (a) Solvation free energy scaled by $k_{\mathrm{B}}T(\Delta\mu/k_{\mathrm{B}}T)$ of the random coil minus that of the native structure plotted against ρd^3 corresponding to P. (b) $\Delta\mu/k_{\mathrm{B}}T$ of structure 2 minus that of the native structure plotted against ρd^3.

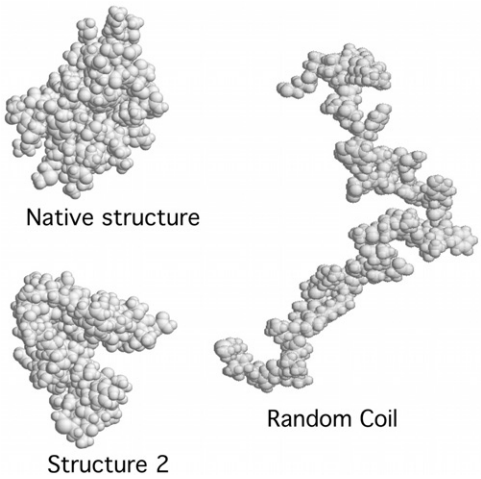

Figure 4. Space-filled representation of the native structure, structure 2, and the random coil of protein G.

of the native structure while the PMV of structure 2 is the smallest. Figure 3(a) shows the SFE scaled by $k_{\mathrm{B}}T(\Delta\mu/k_{\mathrm{B}}T)$ of the random coil minus that of the native structure plotted against ρd^3 corresponding to P. As P increases, the difference becomes larger and the random coil is relatively more destabilized. By contrast, as shown in figure 3(b), where $(\Delta\mu/k_{\mathrm{B}}T)$ of structure 2 minus that of the native structure is plotted against ρd^3, the inversion of the SFE occurs at a sufficiently high pressure. It is remarkable that structure 2 thus becomes more stable than the native structure. This result is in accord with the physical picture illustrated in figure 1 and thermodynamically consistent.

The three structures are compared in figure 4. The random coil, which is often referred to as the heat-denatured structure, has quite large ASA. However, its EV is extremely large

and the PMV remains relatively larger. The difference between the random coil and the native structure in terms of the stability becomes increasingly larger with rising pressure, and the pressure-denatured structure can never be like the random coil. Structure 2 has a large cleft allowing the penetration of water molecules. This penetration enlarges the ASA though the structure itself maintains small EV. These features are not found in structures 3–5 (not shown here). It is interesting that the water penetration (and the presence of a cleft) is not allowed at a low pressure, whereas it is more favoured at a sufficiently high pressure. The pressure-denatured structure should be qualitatively similar to structure 2. It is only moderately less compact than the native structure, nevertheless its ASA is much larger. These results are in good accord with the experimental observations [2–6, 12] indicating the swelling, water penetration into the interior, and only a moderate reduction of the compactness. They are also consistent with the results of the theoretical and computer simulation studies [11–13], though the physics is made considerably clearer in the present analysis. We note that the presence of a water molecule generates an excluded volume for the other water molecules. This water crowding is serious when the pressure becomes very high. The only compromise is the penetration of water molecules into the protein interior for repacking protein atoms with these water molecules even more closely. This facilitates the translational movement of the water molecules outside the protein while that of the water molecules in the interior is largely restricted. The former effect dominates at a sufficiently high pressure: the total entropy of water becomes all the higher when the protein is denatured.

We have chosen several representative structures and analysed the stability of each structure at different pressures. For a complete argument, structural ensembles of protein states should be considered. However, the qualitative aspects of the conclusions are not altered: we have verified the following. A total of ten different random coils are tested but they all share qualitatively the same characteristics, and structure 2 definitely represents one of the most probable structures in the ensemble of pressure-denatured states. More details will be presented in a future publication.

The partial molar compressibility (PMC) β_T of a protein is defined by

$$\beta_T = -(\partial \Delta v/\partial P)_T/\Delta v \sim (\partial \xi/\partial P)_T a/\Delta v = \{\partial \xi/\partial(\rho d^3)\}_T\{\partial(\rho d^3)/\partial P\}_T a/\Delta v. \qquad (5)$$

We find that β_T for structure 2 is larger than that for the native structure by $\sim$29%. According to the experimental measurement [26], upon pressure denaturation β_T increases by $\sim$27% for ribonuclease A and by $\sim$23% for chymotrypsinogen. It is remarkable that these numbers are in quantitatively good agreement with one another. This agreement implies that not only the parameter ξ but also its pressure derivative can reasonably be estimated using our method and that the PMC increase is ascribed to the difference in solvation properties between the native and denatured structures rather than to the difference in the conformational flexibility.

It is definite that the entropic effect arising from the translational movement of water molecules plays crucially important roles in a variety of processes occurring in biological systems [14, 15, 17–19, 27, 28]. As a striking example, we have treated the protein-folding problem and shown that pressure denaturation of proteins can be elucidated in terms of the entropic effect. In our statistical–mechanical method, the protein structure is characterized by the EV for the solvent molecules and the ASA. The PMV, the pressure derivative of the SFE, is expressed in terms of the EV, ASA, and parameter ξ related to the solvent-density profile entropically formed near the protein surface. By a thermodynamic argument combined with our method, structures with only moderately larger EV and much larger ASA as compared to the native structure are shown to turn more stable at an elevated pressure. It is quite interesting that the entropic effect, which greatly stabilizes the native structure at a low pressure, drives the protein to be denatured at a sufficiently high pressure. In the Asakura–Oosawa

theory [29, 30], a conventional theory for considering the entropic effect, the formation of the solvent microstructure near a protein is neglected, with the result that the PMV always equals the EV. The native structure is even more stabilized than any less compact one with larger EV as the pressure increases. By contrast, this drawback is not inherent in the 3D integral equation theory [14–17] we have employed. The theory, which also allows us to analyse the interaction entropically induced between biomolecules [18, 19], provides the possibility to study the pressure effects on various physicochemical processes occurring in aqueous solutions and living systems. For example, high pressure can reverse the aggregation of protein-folding intermediates [31] and dissociate amyloid fibrils [32] or multiprotein virus assemblies [33], which is to be tackled in future studies.

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (No. 15076203) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by NAREGI Nanoscience Project.

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