Possible Mechanism for Cold Denaturation of Proteins at High Pressure

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We study cold denaturation of proteins at high pressures. Using multicanonical Monte Carlo simulations of a model protein in a water bath, we investigate the effect of water density fluctuations on protein stability. We find that above the pressure where water freezes to the dense ice phase (≈ 2 kbars) the mechanism for cold denaturation with decreasing temperature is the loss of local low-density water structure. We find our results in agreement with data from bovine pancreatic ribonuclease A.

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Some proteins become thermodynamically unstable at low temperatures, a phenomenon called cold denaturation [1–3]. This phenomenon has been mainly observed at high pressures, ranging from approximately 200 to 700 MPa [4]. An explanation of the P-T phase diagram of a protein with cold denaturation has been proposed [5], but a microscopic understanding of the mechanisms leading to cold denaturation has yet to be developed, due in part to the complexity of protein-solvent interactions.

Existing theories of folding and unfolding of diluted proteins consider hydrophobicity as the driving force of protein stability [6–10]. In the case of apolar macromolecules, hydrophobicity has been identified with the assembly and segregation of the macromolecule to minimize the disruption of hydrogen bonds among water molecules [6,10,11]. Water tends to be removed from the surface of apolar molecules, forming a cage composed of highly organized water molecules around the molecule, where the disruption of hydrogen bonds is minimized [12]. The simplest hydrophobic model features an effective attraction between hydrophobic molecules [13], but does not reproduce cold denaturation. In order to obtain cold denaturation with this model, new studies [14,15] had to insert a temperature-dependent attraction derived from experimental observations at ambient pressure [16]. An explicit account of water around the hydrophobic molecules has also been considered in order to understand the cold denaturation process with temperature-independent interactions. Theoretical attempts modeled the effective water-protein interactions with the free energy cost of excluding the solvent around the nonpolar molecule [11,17]. Numerical simulations based on these attempts have been applied to study the pressure denaturation found in proteins [9].

Not until recently has cold denaturation been studied at the microscopic level. Simplified models [18], based on a bimodal description of the energy of water in the shell around the hydrophobic molecule [19], predicted cold denaturation. Similar results were obtained using a lattice model of a random hydrophobic-hydrophilic heteropolymer interacting with the solvent [20]. Several models mimicking the interaction between water molecules and nonpolar monomers have also been applied to the study of cold denaturation [21]. One possible reason for the inability of the previous models to capture both the molecular details of cold denaturation and the effect of pressure is the neglect of (i) correlations among water molecules near the freezing point and (ii) the density anomaly due to the tetrahedral structure of the hydrogen bonded network. Here, we implement a two-dimensional lattice model of water that captures the above mentioned water properties [22]. In the model, the possible orientations of water molecules are set by the allowed values of a q-state Potts variable $\sigma_i$. Only when two neighbor molecules $\langle i, j \rangle$ are in the correct orientation ($\sigma_i = \sigma_j$) does a hydrogen bond (HB), that increases the volume of the system by $\Delta V$, form. This interaction mimics the increment of volume due to the incipient formation of a tetrahedral structure. If the two neighbor molecules $\langle i, j \rangle$ are not in the correct orientation, the interaction of the particles does not imply any increment in volume. The Hamiltonian for our model of water-water interaction may be written as [22]

$$H_{\text{HB}} = -J \sum_{\langle i,j \rangle} \delta_{\sigma_i \sigma_j} \tag{1}$$

where $J > 0$ is the scale of the interaction between water molecules upon tetrahedral network formation. The total volume of the system is given by $V = V_0 + N_{\text{HB}} \Delta V$, where $N_{\text{HB}} = \sum_{\langle i,j \rangle} \delta_{\sigma_i \sigma_j}$ is the total number of hydrogen bonds with $\Delta V > 0$ in the system. The sum $\sum_{\langle i,j \rangle}$ extends only to nearest neighbors, implying that two water molecules cannot form a hydrogen bond with $\Delta V > 0$ if they are separated by one residue of the protein. Note that a lattice site cannot be occupied by both a solvent molecule and a monomer. The enthalpy of the system ($H_{\text{HB}} + PV$) is given by

$$H_{\text{HB}} + PV = -(J - P \Delta V) \sum_{\langle i,j \rangle} \delta_{\sigma_i \sigma_j} \tag{2}$$

where $P$ is the pressure applied to the system.
Our model solvent features a limiting pressure \( P_c = \frac{J}{\Delta V} \). Above \( P_c \), we find that \( N_{\text{HB}} \) decreases as we decrease the temperature, and the water model undergoes a transition to a state where all hydrogen bonds with \( \Delta V > 0 \) are broken. Below \( P_c \), we find that \( N_{\text{HB}} \) increases as we decrease the temperature, and the water model undergoes a sharp transition, at \( T = T_c = (J - P\Delta V)/(\ln(1 + \sqrt{q})) \) [22], to a state where all hydrogen bonds with \( \Delta V > 0 \) are formed. Thus, our water model reproduces the freezing of water to low- and high-density ice, since for \( P < P_c \) (\( P_c \approx 200 \text{ MPa} \) in real water) water freezes to the low-density ice Ih, and for \( P > P_c \), water freezes to the high-density ice II [23]. A relation between these two phases of ice and protein folding has already been suggested from a thermodynamic point of view [24].

We model the protein as a self-avoiding random walk embedded in a water bath. For simplicity, we consider a nonpolar homopolymer that interacts with water via the partial ordering of water molecules, forming hydrogen bonded structures around the protein. We mimic the interaction using the Hamiltonian

\[
\mathcal{H}_p = J_p n_{\text{HB}} \left( n_{\text{max}} - \sum \langle n_i n_j \rangle \right),
\]

where the parameter \( J_p > 0 \) is the strength of the repulsive interaction and \( n_{\text{HB}} = N_{\text{HB}}/N_{\text{water}} \) is the number density of hydrogen bonds with \( \Delta V > 0 \), \( N_{\text{water}} \) is the number of water molecules. The water-protein repulsion increases as the water molecules tend to form the tetrahedral, low-density, hydrogen bonded network, where an unfolded apolar macromolecule is unlikely to be embedded. \( n_{\text{max}} \) is the maximum number of residue-residue contacts and \( \sum \langle n_i n_j \rangle \) is the number of residue-residue contacts, where \( n_i = 1 \) if the lattice position \( i \) is occupied with a residue, and zero otherwise. Therefore, \( n_{\text{max}} = \sum \langle n_i n_j \rangle \) is a measure of protein compactness, and equals zero when the protein is maximally compact. Thus, Eq. (3) states that the hydrophobic repulsion driving the protein to a compact state is equal to zero when the protein is maximally compact (\( \sum \langle n_i n_j \rangle \approx n_{\text{max}} \)) or when the water forms the high-density bond network (\( n_{\text{HB}} = 0 \)).

We hypothesize that the inability of water molecules to arrange in the low-density icelike structures is the principal mechanism responsible for protein cold denaturation. At low pressures (\( P < P_c \)) and low temperatures, water molecules form a low-density hydrogen bonded network, so the protein is forced to adopt a compact state. At high pressures (\( P > P_c \)), the water is not able to form the low-density network and forms a more dense state. In this case the effective repulsion between the residues and the solvent decreases, and water molecules penetrate into the protein core, unfolding the compact state. Our hypothesis is supported by the experimental observations [4] that cold denaturation exists mainly at high pressures (of the order of kbars), where water freezes only in the dense ice II phase [25].

Next we demonstrate that our model of a protein embedded in a water network with an enthalpy given by

\[
\mathcal{W} = \mathcal{H}_p + \mathcal{H}_{\text{HB}} + PV
\]

(4)
gives rise to both cold and warm denaturation of the protein and agrees with experimentally observed protein denaturation at high pressures. Since the energy landscape of the protein interacting with the water network is characterized by a multitude of local minima, we perform multicanonical Monte Carlo simulations to avoid transient trapping of our water-protein system in local energy minima at low temperatures. Specifically, we use the multiple-range random walk algorithm [28] to calculate the density of states. We adopt the algorithm in order to embed the self-avoiding protein into the lattice, and to calculate the two-parameter density of states \( g(N_{\text{HB}}, N_c) \), where \( N_c = \sum \langle n_i n_j \rangle \) is the number of residue-residue contacts. From the density of states we calculate the temperature and pressure dependence of the average number of residue-residue contacts

\[
\bar{N}_c = \sum_{N_{\text{HB}}} \sum_{N_c} N_c g(N_{\text{HB}}, N_c) e^{-\mathcal{W}(N_{\text{HB}}, N_c)/T} Z,
\]

(5)

where \( Z \) is the partition function. We perform Monte Carlo simulations of a system of 383 water molecules and a protein consisting of 17 nonpolar residues with periodic boundary conditions. Figure 1 shows the

**FIG. 1.** Normalized number of residue-residue contacts versus temperature for pressure values \( PV_0/J = 0, 0.25, 0.5, 0.75, 1, 1.1, 1.125, 1.15, 1.175, 1.2, 1.25, 1.4, 1.5, 2. \) A magnified region where the cold denaturation takes place is shown in the inset. The dotted line corresponds to \( N_{\text{HB}}/n_{\text{max}} > 0.95 \). We represent the curve corresponding to \( PV_0/J = 0 \) by a bold line. The values of the parameters used are \( J = 1, J_p = 10, \Delta V = 1, \) and \( q = 10. \)
dependence of $\overline{N}_c/n_{\text{max}}$ on temperature for different values of the pressure both above and below $P_c$. The calculated density of states $g(N_{\text{HB}}, N_c)$ converges to the true value with an accuracy of the order of $10^{-5}$. The value of $\overline{N}_c/n_{\text{max}}$ ranges from 1 (maximally compact protein) to approximately $0.71$ (which is the average number of residue-residue contacts found at high temperatures). Only when $P > P_c$ do we observe the cold denaturation of the protein. Above $P_c$, $n_{\text{HB}}$ decreases monotonically with decreasing temperature; thus the repulsion term $J_r/N_{\text{HB}}$ also decreases monotonically. We find then that a reentrant transition, as the one found in cold denaturation, does not require a nonmonotonic function of the temperature for the polymer-water repulsion.

We also reconstruct the phase diagram of the water-protein system in the $P$-$T$ plane (Fig. 2). We consider the protein to be in the collapsed state if $96\%$ of all possible contacts are formed, i.e., if $\overline{N}_c/n_{\text{max}} > 0.96$. For each pressure value, the freezing lines of water shown in Fig. 2 are given by the temperature at which we observe a maximum in the specific heat of the water bath. We compare our findings to experimental observations [29] for bovine pancreatic ribonuclease A studied by $^1\text{H}$NMR spectroscopy. We find remarkable qualitative agreement between the experimental and numerical $P$-$T$ phase diagrams. In both experimental and numerical $P$-$T$ phase diagrams, we observe that cold denaturation occurs at high pressures and, as we lower the temperature, close to the water-ice II freezing line and in the region where water molecules are not capable of forming low-density icelike structures. In addition to the study of ribonuclease A [29], cold denaturation at very high pressures in the kbar range has also been observed in chymotrypsinogen [5], myoglobin [5], and staphylococcal nuclease [30], and has been proposed as the principal mechanism for the observed pressure inactivation of bacteria, such as Escherichia coli [31].

Not all proteins behave equally as we decrease temperature at high pressure. In particular, there are some proteins that do not exhibit cold denaturation. We reproduce the variability of protein dynamics at high pressure and low temperature by varying the hydrophobic parameter $J_r$ to lower values, effectively impeding a stable compact state for pressures above the $P = P_c$ line. In Fig. 3 we present the phase diagrams obtained for different values of $J_r$, ranging from 2 to 20. The shape of the phase diagram changes as we increase the value of the repulsive interaction $J_r$, allowing stabilization of the compact state and cold denaturation above the $P = P_c$ line.

Within the framework of our model, we reproduce the experimentally observed thermodynamics of cold denaturation, but we cannot address the kinetics of this process. It could be also feasible to investigate in future work the dynamics with a more sophisticated model, where we consider not just the average number of hydrogen bonds with $\Delta V > 0$, but the actual numbers for each water molecule that is a neighbor to a residue.

Recent computer simulations studies [32] with all-atom models have studied the effect of pressure and average density on the hydrophobic effect. The authors performed simulations of two Lennard-Jones (LJ) particles in the four point transferable intermolecular potential (TIP4P) water model [33]. At constant temperature, they found that the aggregation of the two particles is favored with a moderate increase of pressure,
or analogously, with a moderate increase of water density. However, this effect is reversed for pressures in the kbar range, so that aggregation becomes unstable with increasing water density. These studies support our results of a critical pressure above which an increase of pressure leads both to an increase of water density (because of the reduction of the number of hydrogen bonds with \( \Delta V > 0 \)) and to destabilization of the model protein. Following these results and based on previous studies [10], Shimizu et al. [15] have shown that three-body interactions have a destabilization effect on the aggregation of three LJ particles. However, the inclusion of these interactions into a model of a more complex protein did not lead to significant changes. Finally, all-atom simulations recently addressed the pressure denaturation of proteins [34], but they have not provided conclusive evidence.

We conclude that the effect of pressure on water density is key for understanding cold denaturation of proteins. The density anomaly of water arises from the low-density hydrogen bonded structures responsible for the hydrophobic effect, driving the protein to a compact state \([2,8,10,12]\). At extreme pressures above \( P_c \), lowering the temperature implies an increasing free energy cost to form a hydrogen bond with \( \Delta V > 0 \), so the density anomaly disappears. In this scenario, the hydrophobic effect decreases and cold denaturation occurs. Our model supports this mechanism. Also, a specific arrangement of amino acids in the protein structure, determined by amino acid interactions, dictates the dynamics of proteins at low temperature and high pressure, thus making some proteins more stable than others at these \( P-T \) conditions.

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[25] High pressure is not the only mechanism promoting cold denaturation. Alterations in the solvent conditions, such as variations in the pH [26] or the addition of chemical denaturants [27], may weaken the hydrophobic interactions, inducing cold denaturation of selected proteins at ambient pressures.