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Cavity hydration as a gateway to unfolding: An NMR study of hen lysozyme at high pressure and low temperature

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ABSTRACT

We have used low temperatures (down to −20 °C) and high pressures (up to 2000 bar) to populate low-lying excited state conformers of hen lysozyme, and have analyzed their structures site-specifically using $^{15}N/^{1}H$ two-dimensional HSQC NMR spectroscopy. The resonances of a number of residues were found to be selectively broadened, as the temperature was lowered at a pressure of 2000 bar. The resulting disappearance of cross-peaks includes those of residues in the β-domain of the protein and the cleft between the β- and α-domains, both located close to water-containing cavities. The results indicate that low-lying excited state conformers of hen lysozyme are characterized by slowly fluctuating local conformations around these cavities, attributed to the opportunities for water molecules to penetrate into the cavities. Furthermore, we have found that these water-containing cavities are conserved in similar positions in lysozymes from a range of different biological species, indicating that they are a common evolutionary feature of this family of enzymes.

1. Introduction

The folding and dynamics of proteins are central features of molecular biology [1–5]. The thermodynamics of protein conformational stability is strongly influenced by the enthalpy and entropy of the solvent water that is in contact with the polypeptide chain [6–9]. In this regard, protein unfolding and folding are associated with the solvation and de-solvation of the polypeptide chain. Most globular proteins undergo apparent two-state transitions as the temperature is raised [10], but as the pressure is changed they often disclose a multiplicity of transitions [11–13]. This means that the solvation of the polypeptide chain can occur in a series of discrete steps, that are strongly pressure-dependent as each step is accompanied by a change in the partial molar volume of the protein [14]. Exploration of the multiple conformations that can be adopted by a protein therefore allows the thermally accessible excited state conformers; they are likely to be essential for function [15].

In the case of enzymes, however, the structural deviations are usually rather small because the active site of an enzyme is usually composed of a number of side chains distant in the primary structure that must be well-defined to perform a catalytic reaction. This requirement makes the conformational fluctuations rather subtle, namely from the stable folded conformer N to a low-lying excited state conformer N′, making the spectroscopic detection of conformer N′ difficult.

In this article, we report the detection and characterization of low-lying excited states in hen lysozyme [1,2,16,17] at high pressure and low temperature. Although equilibrium experiments using calorimetry, high pressure fluorescence, and other spectroscopic analyses have shown a rather clear "two-state" folding behavior of this protein [18–20], kinetic folding studies provide evidence for a degree of step-wise formation of the folded structure [19,21,22]. Previous studies of amide hydrogen/deuterium exchange reaction also showed a distribution of protection factors, suggesting heterogeneity in the conformational properties of this protein [15,23–25]. In this connection, Tsuda et al. found the NMR structure of hen lysozyme significantly different at 5 °C compared to that at 35 °C in the β-domain and the helix D [26].

High pressure NMR spectroscopy is a generally applicable technique for detecting equilibrium conformations of proteins in a wide range of conformational space [11–13,27–29]. Under isothermal conditions, pressure shifts the conformational equilibrium from the highest volume conformer (usually the "native" conformer N) to lower volume conformers (non-native conformers, e.g., N′).

Our previous high pressure NMR study of hen lysozyme at 30–2000 bar at 25 °C revealed fluctuations localized around water containing cavities within the ensemble of folded conformers [30]. For technical reasons, however, we could not apply sufficiently high pressure (>4–6 kbar) to enable low-lying excited states of this protein to be resolved.

Abbreviations: N, the native state; N′, low-lying excited state; HSQC, heteronuclear single-quantum correlation; ppm, parts per million.

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Nevertheless, along with a number of previous reports [31–33], our recent high-pressure study of tryptophan fluorescence carried out over a wide range of temperature (between 50 °C and −10 °C) and pressure (between 1 bar and 7000 bar) has clearly shown that, at low enough temperatures the folded conformational ensemble becomes highly unstable, and the protein is close to the onset of cold denaturation [20]. In the present study, we utilize ^{1}H one-dimensional and ^{15}N/^{1}H two-dimensional NMR spectroscopy at pressures of 2000 bar and temperature as low as −20 °C by exploiting the fact that the freezing point of water is −21 °C at this pressure [34], and examine the structures of low-lying excited states of hen lysozyme prior to unfolding.

2. Materials and methods

2.1. Protein samples

Uniformly ^{15}N-labeled hen egg white lysozyme was expressed in *Aspergillus niger* using $^{15}NH_4Cl$ as the sole nitrogen source and was purified from a filtered culture medium as described previously [35]. For the NMR studies the sample contained 1.7 mM protein in 100 mM formic acid buffer (volume change on proton dissociation, $\Delta V = -8.5$ ml/mol) at pH 3.8. Sodium 3-(trimethylsilyl) propionate (TSP) was used as internal chemical shift reference.

2.2. High pressure NMR apparatus

High resolution-high pressure NMR measurements were performed on a Bruker DMX-750 spectrometer equipped with an on-line cell made of synthetic quartz with an inner diameter of ~1 mm and an outer diameter of ~3 mm [27,36]. The spectrometer operates at a ^{1}H frequency of 750.13 MHz and a ^{15}N frequency of 76.01 MHz. The protein solution in the cell was separated from the pressure mediator (a mixture of kerosene and machine oil) by a small frictionless piston (Teflon) in a separator cylinder made of BeCu. The pressure in the cell was varied between 1 and 2000 bar (1 bar = 10^5 Pa = 0.9869 atm) with a hand-pump located remotely from the 17.6 Tesla magnet (Japan Magnet Technology). A commercial 5 mm ^{1}H-detection inverse probe with xyz-field gradient coils (Bruker) was used for all measurements.

2.3. NMR measurements and data analysis

The temperature dependences of resonances in one-dimensional ^{1}H NMR and two-dimensional ^{15}N/^{1}H HSQC spectra of ^{15}N-uniformly labeled hen lysozyme (1.7 mM, pH 3.8) were measured at pressures between 30 and 2000 bar and at various temperatures between 25 °C and −20 °C. The ^{15}N/^{1}H HSQC spectra [37–39] were recorded as echo anti-echo gradient-selected sensitivity enhanced experiments, in which the ^{15}N dimension was acquired with 256 increments covering 3125 Hz; ^{15}N decoupling during acquisition was achieved with GARP (globally-optimized alternating-phase rectangular pulses) [40]. In the ^{1}H dimension, 2048 complex points were collected with the offset of the ^{1}H frequency set at the residual water signal. The NMR data were processed using Felix 2.3 (Biosym Technologies) and Felix 97 (Molecular Simulation) on a SUN workstation, nmrPipe [41] on Linux and MacOSX, and UXNMR (Bruker) on a Silicon Graphics workstation. Spectra were zero-filled to give final matrices of 4096×512 real data points and were apodized with a sine-squared-bell window function in both dimensions.

2.4. Identification of hydrogen bonds and cavities in folded lysozyme structures

To compare the locations of cavities and buried water variations within lysozymes from different species, crystal structures were taken from pdb codes 2LZT [42], 1DKJ [43], 2IHL [44], 1JEF [45], 1BB7 [46], 1REX [47], 1JUG [48], and 1QQY [49]; the program PRO_ACT [50] was used to identify buried water molecules.

3. Results and discussion

3.1. Evidence for low-lying excited states

We recorded ^{1}H one-dimensional and ^{15}N/^{1}H two-dimensional HSQC spectra of 1.7 mM ^{15}N-uniformly labeled hen lysozyme (pH 3.8) at every 2.5 °C over the temperature range between 25 °C and −20 °C by taking advantage of the fact that the freezing point of water is decreased to −21 °C at 2000 bar [34]. Representative ^{1}H and ^{15}N/^{1}H HSQC spectra are shown in Fig. 1A and B, respectively. The spectral changes observed were fully reversible with temperature.

Examination of the ^{1}H one-dimensional spectra recorded in this manner reveals (Fig. 1A) that the signals gradually broaden as the temperature is lowered, although the chemical shifts are largely unchanged even at the lowest temperature, −20 °C, reached at 2000 bar. This finding reveals that the overall structure of the native state is retained down to −20 °C at 2000 bar. The features of the ^{15}N/^{1}H HSQC spectra recorded over the corresponding temperature range, however, show very different behavior (Fig. 1B), the cross-peaks being selectively broadened above −10 °C and almost completely disappearing below −15 °C. This phenomenon is not due to the aggregation of protein molecules, as shown by ^{1}H NMR spectra at the corresponding temperatures (Fig. 1A).

A particularly interesting finding in this study is that many HSQC cross-peaks in Fig. 1B disappear at much higher temperatures than do the rest of the cross-peaks. For example, the cross-peaks of residues Ile 55, and Val 92 are broadened beyond detection at 10 °C, as are the cross-peaks of residues Thr 43, Thr 51, Leu 56, Trp 62, Asp 66, Arg 73, and Cys 76 at 7.5 °C, and residues Arg 45, Ser 60, Asn 74, and Gly 104 at 5 °C, and several others become unobservable at 2.5 °C (Fig. 1B). In Fig. 2A, we plot the temperatures at which the individual cross-peak disappears from the spectra as a function of the residue number. We note that the residues whose signals disappear above 2.5 °C are largely confined into the β-domain of the protein.

The disappearance of some cross-peaks is often accompanied by the appearance of new peaks, indicating that a segment of the protein is denatured. This is not the case, however, for the data shown in Fig. 1B, revealing that the β-domain is not yet fully denatured but is likely to be partially disordered and heterogeneous in conformation around the original natively folded structure N. One possible mechanism for the disappearance of all the cross-peaks below −10 °C is the greater viscosity of water; the value increases by a factor of 4 between 20 °C and −20 °C [51], giving rise to a corresponding increase in ^{1}H and ^{15}N line widths by the same factor of 4. A question arises, however, as to whether the selective disappearance of cross-peaks above −10 °C could be due to intrinsic differences in the line width (or the transverse relaxation rate R_2) between individual residues. To test this possibility, the temperatures at which signals disappear have been plotted against the reported values of ^{15}N R_2 at 25 °C [52] in Fig. 2B. Although there is a variation in ^{15}N R_2, it is apparent that this variation is not correlated with the temperature at which a given signal disappears, indicating that the selective disappearance of HSQC cross-peaks must be caused by an additional mechanism that is specific to residues in the β-domain, apart from the viscosity effect on R_2.

The high degree of broadening can be attributed to exchange effect that simply requires an increased dispersion of ^{1}H and ^{15}N chemical shifts, which is not averaged out by fluctuations. An increase in chemical shift dispersion in the β-domain will result, for example, from an increase in the heterogeneity of the backbone and side-chain conformations at low temperature and high pressure. To cause the broadening of the NMR signals, the rate of exchange among these heterogeneous conformations must be rather slow on the NMR time

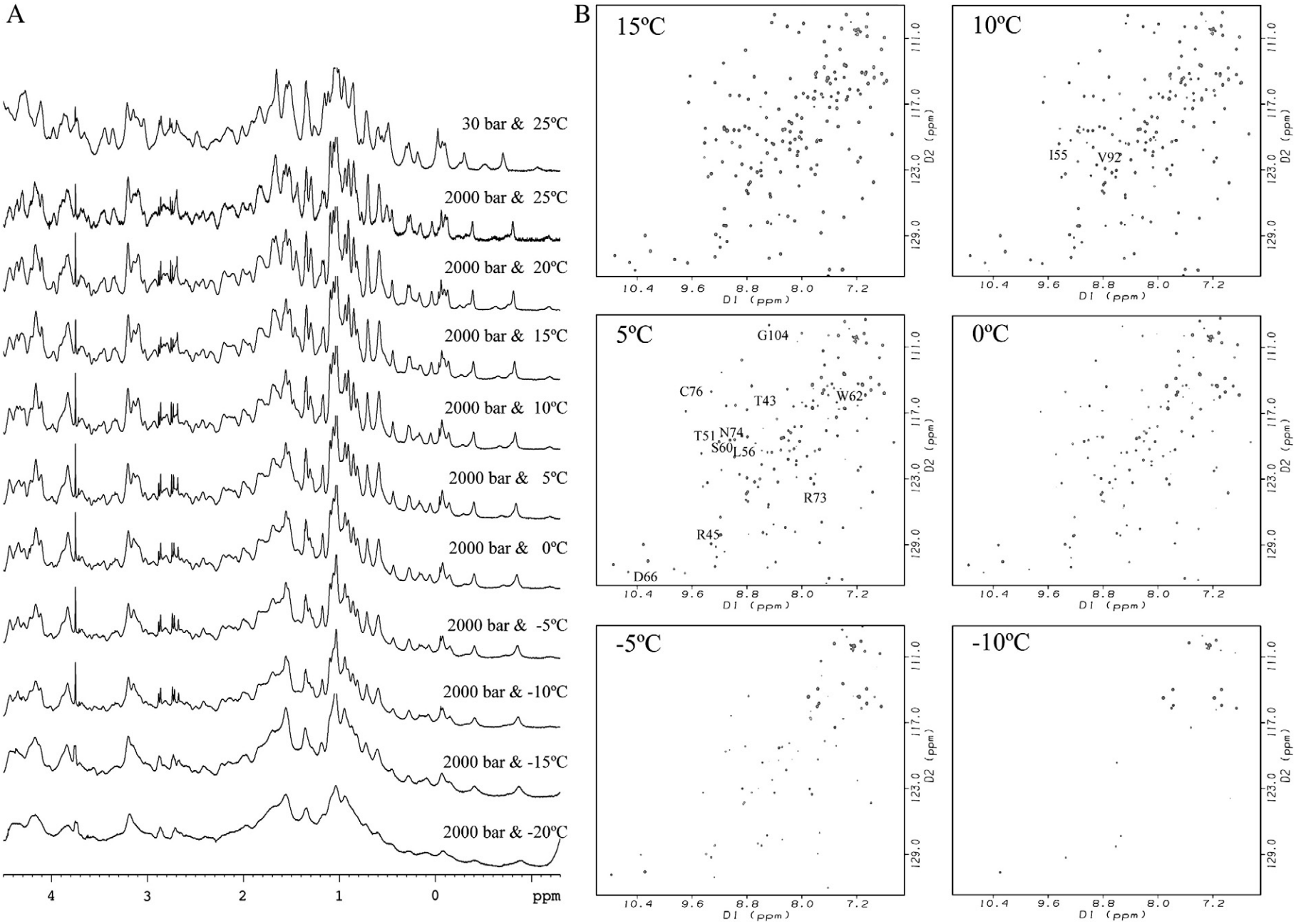

Fig. 1. Changes in NMR spectra of hen lysozyme at high pressure and low temperature. (A) ^{1}H one-dimensional NMR spectra of ^{15}N-uniformly labeled hen lysozyme (1.7 mM, pH 3.8) at 2000 bar and various temperatures indicated. (B) ^{15}N/^{1}H two-dimensional HSQC spectra at 2000 bar and various temperatures. Cross-peaks that disappear between 15 °C and 10 °C are labeled on the spectrum recorded at 10 °C and that disappear between 10 °C and 5 °C are labeled on the spectrum at 5 °C.

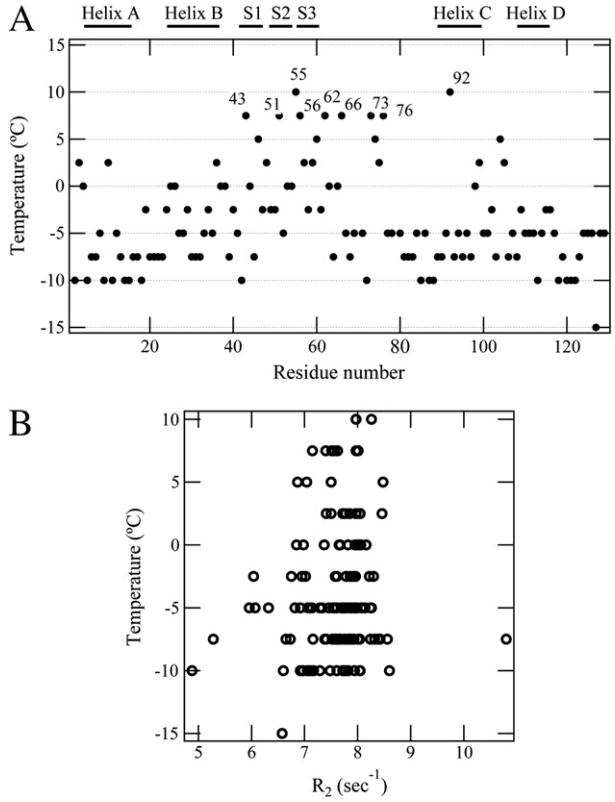

Fig. 2. Disappearance of $^{15}N/^{1}H$ cross-peaks at lower temperatures. (A) Plot of the temperature below which the cross-peak disappears in the two-dimensional HSQC spectrum against the residue number in ^{15}N-labeled hen lysozyme at pH 3.8. (B) Plot of the temperature below which the cross-peak disappears in the $^{15}N/^{1}H$ two-dimensional HSQC spectrum against the transverse relation rate R_2 at 25 °C [52].

scale (~μs~ms). The ensemble of conformers with substantial heterogeneity in the β-domain can be attributed to an ensemble of low-lying excited state conformers (N′, N″, N‴, etc.) stabilized under the condition of low temperature and high pressure relative to the conformer N. The fact that their populations are increased at high pressure and low temperature suggests that they could have a slightly smaller partial molar volume than the conformer N and, therefore, that they are probably more hydrated than the N conformer [53,54].

3.2. Structural characterization of the low-lying excited states

The residues whose main-chain amide resonances effectively disappear from the HSQC spectrum at temperatures above 0 °C are indicated in color on the crystal structure of hen lysozyme in Fig. 3A. In this figure, we note that the signals that disappear are those of residues close to the large water-containing cavity in the cleft between the α- and β-domains and to the smaller cavity in the β-domain, and extend to the whole region of the β-domain. The disappearance of the NMR signals of residues in the vicinity of water-containing cavities suggests that more water penetrates into the cavities at lower temperature, producing an ensemble of partially solvated heterogeneous conformations (low-lying excited states N′, N″, N‴, etc.).

In our previous $^{15}N/^{1}H$ HSQC NMR study of hen lysozyme carried out at pressures of 30–2000 bar at pH 2 and 25 °C [30], conditions under which the folded conformer is quite stable, we showed from pressure-induced chemical shifts that the fluctuations of the folded structure are most evident in the vicinity of the water-containing cavities (Fig. 3B). If we compare Fig. 3A and B, residues in the vicinity of the water-containing cavities are commonly perturbed. This finding suggests that the two dynamic processes, i.e., conformational fluctuations within the ensemble of folded conformations and conformational transitions to low-lying excited states, are correlated in such a way that the conformational fluctuations within the folded manifold are localized to the vicinity of the cavities (Fig. 3B) but give rise to significant heterogeneity and disorder in the conformational ensemble involving a large number of residues in the β-domain (Figs. 2A and 3A).

3.3. Implications for protein folding and the nature of the low-lying excited states

Detection of intermediates is a key aspect of understanding the process of protein folding and its associated energy landscape. Equilibrium folding and unfolding experiments using calorimetry and fluorescence [18,19], however, suggest that the folding of hen lysozyme is fully cooperative and proceeds in a two-state manner. An earlier high pressure ^{1}H one-dimensional NMR spectroscopy experiment with multiple chromophores [31], however, showed a degree of heterogeneity in the denaturation process. On the other hand, kinetic folding experiments carried out using site-specific hydrogen exchange methods [21,22] clearly show that an intermediate is formed in the process of folding, in which the α-domain is formed, while

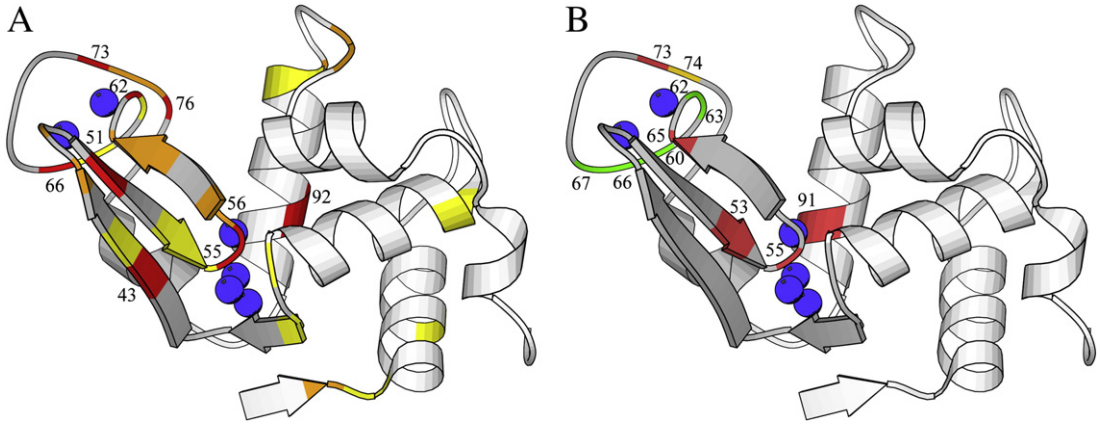

Fig. 3. NMR images of native hen lysozyme. (A) From $^{15}N/^{1}H$ HSQC cross-peak intensities at low temperature and 2000 bar. The residues whose main-chain amide resonances disappear from the HSQC spectrum at temperatures above 7.5 °C, 2.5 °C, and 0.0 °C are shown in red, orange, and yellow respectively. (B) From $^{15}N/^{1}H$ chemical shifts at 2000 bar and 25 °C (cited from Ref. 21). Residues with large main-chain ^{15}N pressure shifts (>1.3 ppm or ≤−0.20 ppm) are shown in red (Tyr 53, Ile 55, Ser 60, Arg 73, and Ser 91), those with large ^{1}H pressure shifts (>0.25 or <−0.08 ppm) in green (Trp 62, Trp 63, Asn 65, Asp 66, and Gly 67) and that with large ^{15}N and ^{1}H pressure shifts in yellow (Asn 74) [30]. The main-chain trace of native hen lysozyme (structure 2lzt) showing the positions of the six buried water molecules (indicated by blue spheres) in cavities within the structure. The α-domain of the protein (residues 1–35, 85–129) is shown in light gray and the β-domain (residues 84–129) in dark gray. The images were created by MolScript [58].

the β-domain is largely unstructured (designated the "α-domain intermediate").

The existence of multiple conformations, including low-lying excited states, is a general characteristic of proteins in solution [11–13,29]. In view of the general coincidence between the equilibrium intermediate stabilized by pressure and the kinetic intermediate in folding [28,55,56], high pressure NMR can provide a unique opportunity for studying structural details of kinetic intermediates involved in protein folding. Indeed, our specific focus is the direct observation of the "α-domain intermediate" of hen lysozyme in equilibrium studies. In order to try to detect this species, therefore, we set out to explore the structure of hen lysozyme just prior to denaturation. We reduced the temperature down to the lowest permissible value (−20 °C) by exploiting the fact that at a pressure of 2000 bar the freezing point of water is −21 °C. We utilized $^{15}N/^{1}H$ two-dimensional HSQC spectroscopy and gradually lowered the temperature at 2000 bar, examining any changes in conformation prior to denaturation for residue-specific information. As the temperature is decreased, the signals from residues in the β-domain disappeared gradually and selectively while the signals from the α-domain remain relatively unchanged (Figs. 2A and 3A). The disappearance of the β-domain signals can be attributed to an increased conformational heterogeneity resulting from preferential hydration at high pressure and low temperature.

Water penetration into protein matrices is thought to be the primary step leading to denaturation at high pressure. A selective increase in the number of water molecules located in preexisting cavities has been demonstrated by direct X-ray analysis of a cavity mutant of T4 lysozyme at high pressure [57]. Thus, the preferential disappearance of signals observed for the β-domain of hen lysozyme may represent the events just prior to denaturation resulting from the increased hydration of cavities prior to the complete solvation of the β-domain, which could lead to the formation of the "α-domain intermediate", observed in kinetic experiments [21,22]. The preferentially hydrated pre-denaturation intermediates N′, N″, N‴ etc. represented by Fig. 3A may be illustrated as shown in Fig. 4A, with the low-lying excited states and the α-domain intermediate indicated in the schematic free energy landscape of hen lysozyme in Fig. 4B.

3.4. Cavities are universally present in lysozymes

Our present study suggests the view that cavities are a source of water penetration that leads to the creation of low-lying excited states of proteins. The conformational heterogeneity observed in the vicinity of the cavities located at the α–β domain interface, which includes the active site of the enzyme, suggests that hydration of this cavity could be coupled with the mechanism of action of the enzyme. To examine the possible generality of this view, we have compared the locations of water-containing cavities in lysozymes from a variety of species by analysis of their crystal structures (Fig. 5). The results show that the location of the water-containing cavities is remarkably similar in each lysozyme structure, despite the fact that the sequences are not always highly conserved (for example, the sequence identity of hen and dog lysozyme is less than 50%). This observation suggests that the cavities could be an important feature of the lysozyme structures, and is consistent with the view that they might play a crucial role in the catalytic cycle of lysozymes by giving a degree of mobility in the active site, always with a well-defined population of water molecules that could contribute to the hydrolysis of the substrate molecules.

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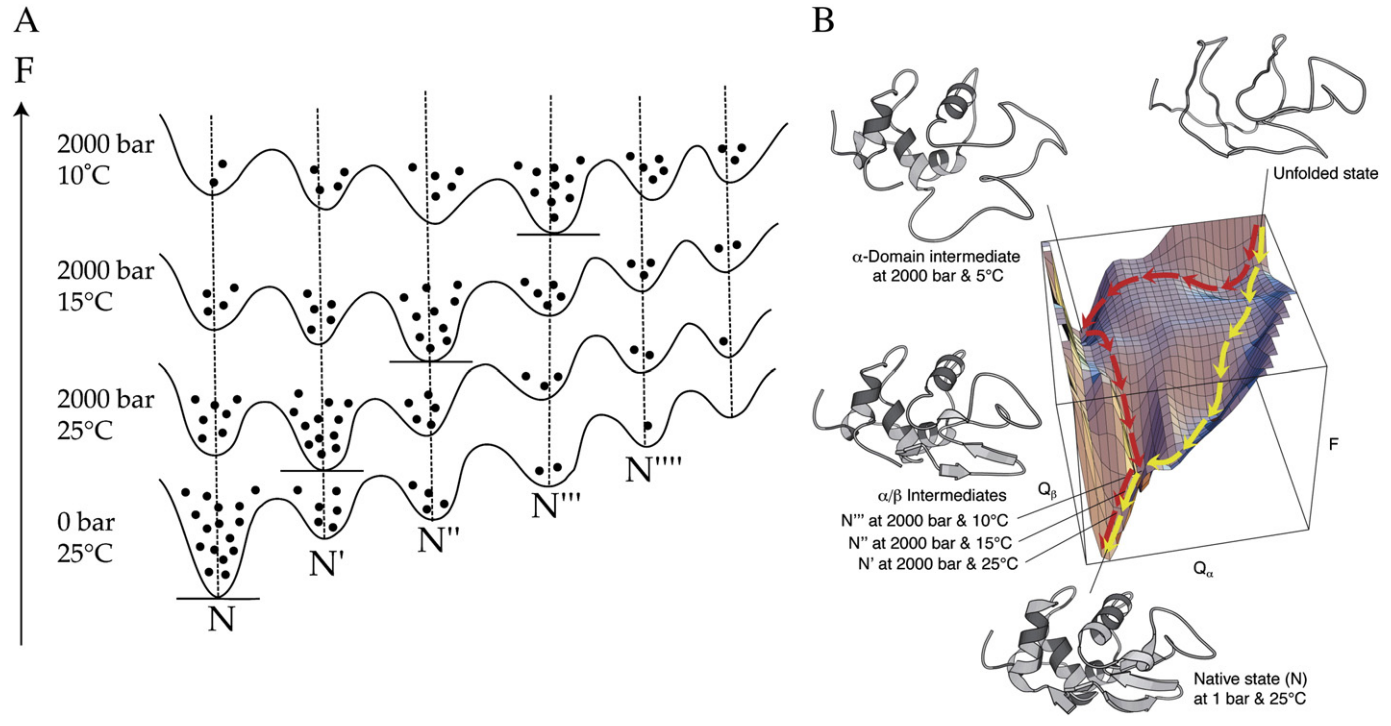

Fig. 4. Low-lying excited states of hen lysozyme. (A). Schematic free-energy (F) surface indicating how the equilibrium between the native and low-lying excited states is shifted by high pressure and low temperature. (B). Probable locations of low-lying excited states (N′, N″, N‴, …) and α-domain intermediate depicted in this study on the schematic free-energy (F) surface representing features of the folding of hen lysozyme (adapted from [2]). Q_α and Q_β are the numbers of contacts in the α- and β-domains. The yellow trajectory represents a "fast track" in which α/β intermediates are populated only transiently. The red trajectory represents a "slow track" in which both α/β and α intermediates are populated transiently, depending on the time-scale.

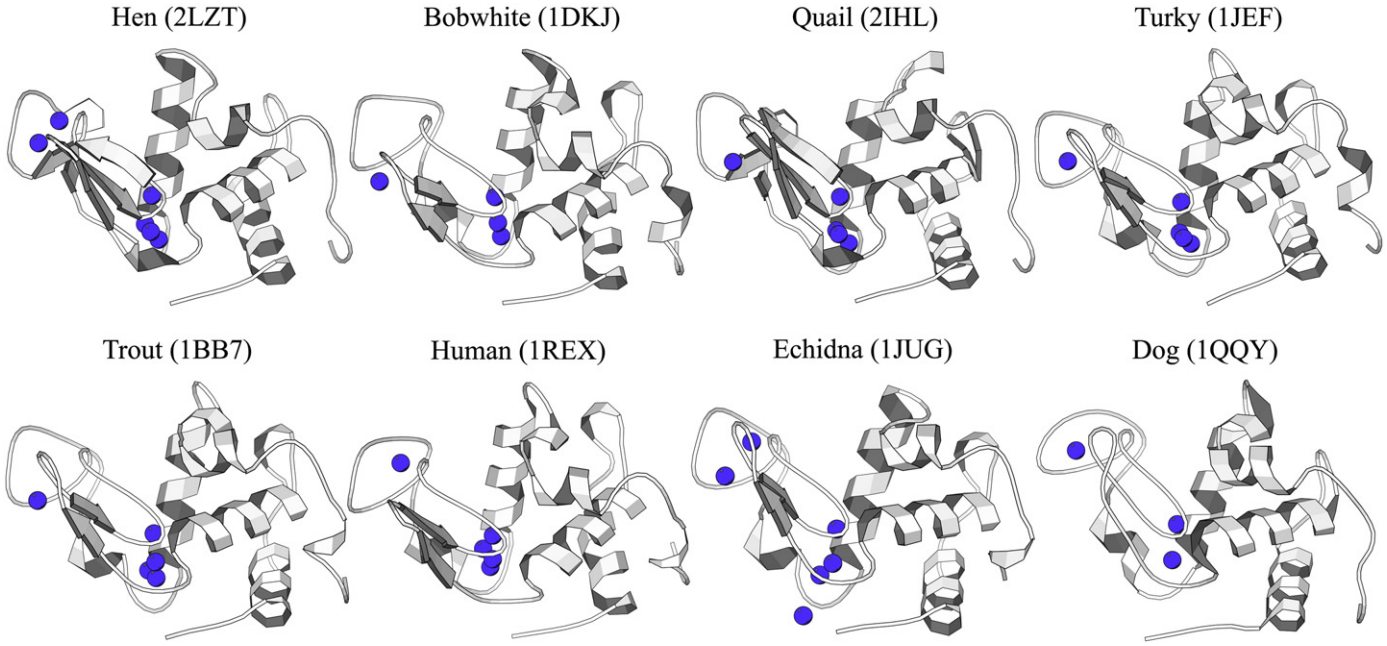

Fig. 5. Locations of cavities and buried water molecules (indicated by blue spheres) in a variety of structures of lysozyme from different biological species: hen, bobwhite, quail, turkey, trout, human, echidna, and dog lysozyme.

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