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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