Extensively Hydrated but Folded: A Novel State of Globular Proteins Stabilized at High Pressure and Low Temperature

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ABSTRACT We studied conformational fluctuations of the transcription factor c-Myb R2 subdomain (52 residues with three Trp) at high pressure and low temperature (5°C) using two different spectroscopic methods, Trp fluorescence and ^{1}H NMR, on its chemically stable mutant C130I (pseudo-wild-type (WT^S)), which has a large internal cavity. As pressure was increased from 3 to 300 MPa, the Trp fluorescence λ_{max} of WT^S shifted from 342 to ~355 nm, clearly showing that the three Trp rings become fully exposed to the polar environment, which usually is taken to indicate that the protein underwent unfolding. In contrast, as pressure was increased from 3 to 300 MPa, the high-field-shifted ^{1}H NMR signals characteristic of the folded state showed a still higher-field shift, but no significant changes in their intensity. The last result unequivocally shows that the protein remains largely folded at 300 MPa. The apparent discrepancy between the two predictions would only be solved if one were to postulate the existence of an extensively hydrated but folded state in WT^S. Intriguingly, such a state was not found in a cavity-filling mutant of WT^S, C130I/V103L, suggesting that this state is mediated by cavity hydration. The generality and significance of this state in proteins are discussed.

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Trp fluorescence is commonly used for monitoring the unfolding of globular proteins (1,2). In most globular proteins, tryptophan side chains occupy the hydrophobic core. The fluorescence-emission maximum wavelength (λ_{max}) of fully buried Trp is ~335 nm, and that of a fully exposed one is ~355 nm. When the λ_{max} value becomes ~355 nm, we consider that the protein is in a fully unfolded state. NMR spectroscopy is also a good tool for monitoring the unfolding of globular proteins (2). Resonance peaks corresponding to the folded structure of the protein will be absent in the NMR spectrum of the unfolded protein (2). Variable-pressure techniques are particularly useful for increasing the population of minor conformational species of a protein that could be crucial for function, without increasing their thermal energy under closely physiological conditions (3–7). In this study, we applied both variable-pressure Trp fluorescence and variable-pressure ^{1}H NMR spectroscopy on a globular protein c-Myb R2 subdomain. The results of the two experiments, taken together, provide evidence for the presence of an extensively hydrated but folded state in c-Myb R2.

c-Myb is a transcription factor involved in proliferation and differentiation of hematopoietic cells (8–11). The DNA-binding domain of c-Myb consists of three structural repeats, R1, R2, and R3, of 51–52 amino acids, each forming a helix-turn-helix-related motif containing three helices (12,13). Three conservative tryptophans in each repeat participate in forming a hydrophobic core, which definitely characterizes the structure of the repeat. Comparison of the three repeat structures indicates that there exists a large cavity in the hydrophobic core of the R2 subdomain (residues 90–141). This internal cavity appears to be crucially important for the function of the c-Myb protein, because when the protein binds to DNA, its Trp^{95} side chain slides into this cavity (14). The cavity-filling mutation of V103L, whose side chain is directly oriented to the internal cavity, reduces the flexibility and the DNA-binding activity of the c-Myb protein (14,15).

We have used a chemically stable pseudo-wild-type of the c-Myb R2 subdomain with a C130I mutation (WT^S), retaining the large internal cavity and its cavity-filled V103L mutant for the experiment presented here. The amino acid sequences of these proteins are given in Table S1 in the Supporting Material. These two proteins were obtained by solid-phase synthesis using F-moc chemistry strategy. The synthesis protocol used is described in the Supporting Material. Variable-pressure Trp fluorescence in the range 3–600 MPa and ^{1}H-NMR in the range 3–300 MPa were carried out on the WT^S and V103L proteins at pH 7.5 and temperature 5°C. Details of these experiments are described in the Supporting Material.

A reversible change in the combined fluorescence emission maximum (λ_{max}) of all the three Trp residues in WT^S with respect to pressure from 3 to 600 MPa is shown in Fig. 1 *b*. The λ_{max} value shows a significant red shift from 342 nm

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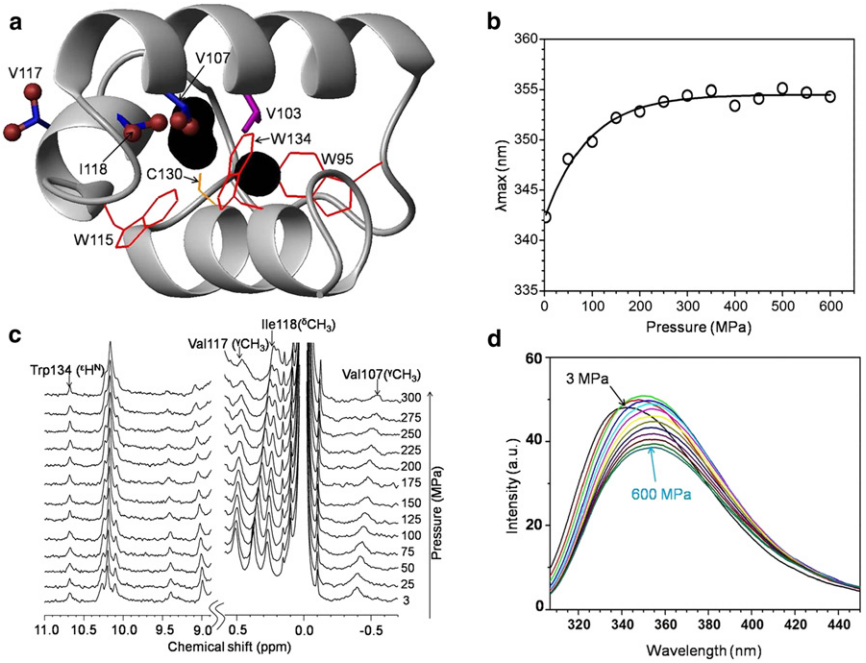

FIGURE 1 (*a*) Three-dimensional structure of the c-Myb R2 subdomain (PDB ID 1GV5). Cavity positions are black. Three Trp side chains are red. The side-chain methyl groups, which were assigned in the NMR spectrum, are shown as brown balls. The C130 side chain is yellow. (*b*) Change in the Trp fluorescence emission maximum (λ_{max}) of the WT^S protein as a function of pressure. Here, the solid line is drawn simply to follow the data points visually. (*c*) ^1H NMR spectra of WT^S protein at various pressures from 3 to 300 MPa at 5°C and pH 7.5. A 0.8 mM protein solution in 25 mM Tris buffer is used. Only the isolated signals are shown. The signals corresponding to Val^{107} ($^{\gamma}CH_3$), Val^{117} ($^{\gamma}CH_3$), Ile^{118} ($^{\delta}CH_3$), and Trp^{134} ($^{\varepsilon}H^N$) groups are labeled. (*d*) Trp fluorescence emission spectra of the WT^S protein at various pressures from 3 to 600 MPa at 5°C and pH 7.5. A 30 μM protein solution in 25 mM Tris buffer is used. The cavity-filling mutation site V103 is magenta (cf. Fig. S2 *a*).

to ~355 nm as pressure is increased from 3 to 300 MPa and it remains constant up to 600 MPa. The shift of λ_{max} to ~355 nm indicates that the immediate environments surrounding all the three Trp side chains occupying the core of R2 (cf. Fig. 1 *a*; PDB ID 1GV5) become fully polar at 300 MPa. This often happens when the protein is in a fully unfolded state and the Trp side chains are fully exposed to the aqueous environment. An array of 1D ^1H NMR spectra of the WT^S at different pressures ranging from 3 to 300 MPa is shown in Fig. 1 *c* It demonstrates continuous shifts of the high-field-shifted methyl signals, characteristic of the hydrophobic core, toward a still higher field without much loss of intensity, indicating that the protein remains largely folded with increasing compaction of the core with increasing pressure.

The apparent discrepancy between Trp fluorescence and NMR results would arise from the naïve assumption that when the fluorescence λ_{max} is shifted to ~355 nm, the Trp residues are exposed to the solvent water, causing the protein to unfold. The discrepancy would be resolved only if we assume a state of the c-Myb R2 domain in which water penetrates into the core and nearly fully hydrates the Trp side chains, but the Trp side chains remain in the core, basically keeping the native fold (N*), in closer vicinity with nearby methyl groups, e.g., of Val^{107} and Ile^{118}.

The presence of the peculiar conformer (N*) is further supported by the nonlinear nature of the pressure-induced chemical-shift changes (5). The pressure-induced changes in the well isolated signals of the side-chain groups, Val^{107} ($^{\gamma}CH_3$), Ile^{118} ($^{\delta}CH_3$), and Trp^{134} ($^{\varepsilon}H^N$), occupying the core of the protein and that of Val^{117} ($^{\gamma}CH_3$) occupying the periphery of the protein (cf. Fig. 1 *a*) but close to the Tyr^{110} ring, are marked in Fig. 1 *c*. The pressure-dependent chemical-shift changes of the side-chain signals occupying the structural core are clearly nonlinear and are found at different parts of the protein molecule. This suggests that the WT^S protein undergoes a cooperative conformational transition to a second conformer (N*) that coincides with the compaction of the core. The pressure-dependent changes in chemical shifts of the V107 ($^{\gamma}CH_3$) and I118 ($^{\delta}CH_3$) groups, which are very close to the internal cavity and also to the side-chain indole rings of W115 and W134, are shown in Fig. 2, *a* and *b*. These results suggest that in R2, the basic folded conformer N, as that found in crystal, is in equilibrium with another folded conformer N* in solution, $N \rightleftarrows N^*$. Furthermore, many of the NMR signals of N* exhibit considerable line broadening, suggesting that N* is more heterogeneous and flexible than N (cf. Fig. 1 *c*). Now, the data points for the emission maximum (λ_{max}) of the combined three-Trp fluorescence of Fig. 1 *b* can be fitted reasonably well with a two-state transition (Eq. 8 of Maeno et al. (2), giving $\Delta G^0 = 3.5 \pm 0.8$ kJ/mol (0.83 kcal/mol) at 0.1 MPa and $\Delta V = -42 \pm 8$ mL/mol (Fig. S1). The small ΔG^0 and relatively large ΔV values appear to be consistent with the notion that N* is an extensively hydrated but folded state.

In the case of the cavity-filled mutant V103L (cf. Fig. S2 *a*), the Trp fluorescence λ_{max} showed only a small red shift from 341 to ~346 nm at 300 MPa and even up to 600 MPa (cf. Fig. S2 *b*). The result indicates that internal hydration is taking place, but in a much limited manner, and no extensively hydrated and folded state N* is produced even at 600 MPa in the cavity-filled mutant. When we estimate the solvent-accessible surface area of Trp side chains in R2 (cf. Fig. S3), it is observed that the indole rings are partially exposed to the solvent through the external surface,

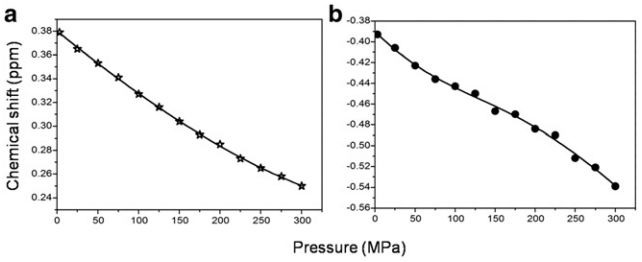

FIGURE 2 (*a*) Change in the chemical shift of the Ile^{118} ($^{\delta}CH_3$) group ^{1}H NMR signal in the WT^S protein as a function of pressure from 3 to 300 MPa. (*b*) Change in the chemical shift of Val^{107} ($^{\gamma}CH_3$) group ^{1}H NMR signal in the WT^S protein as a function of pressure from 3 to 300 MPa. Solid lines show the fitting of the two-state equation (Eq. 3 in Kitahara et al. (3)) to the experimental data points.

and the small red shift from 341 only to ~346 nm may be caused by the water penetration into the interior from the surface. In ^{1}H NMR, the nonlinear nature of the pressure-induced chemical shift changes of Val^{107} ($^{\gamma}CH_3$) and Ile^{118} ($^{\delta}CH_3$) signals (cf. Fig. S2 *c* and Fig. S4) indicates that a conformational change is taking place in addition to the general compaction of the protein molecule. The splitting of Val^{107} ($^{\gamma}CH_3$) group signal indicates that the pressure-stabilized conformer is heterogeneous. However, the pressure-stabilized conformer of the cavity-filled mutant is not as extensively hydrated as that in WT^S, even at 600 MPa. We conclude that the extensively hydrated folded state N* of the R2 subdomain is likely to be mediated by the preferential hydration of its large internal cavity. The conformational flexibility gained by the hydration of the large cavity would facilitate the binding of the R2 subdomain to DNA.

The existence of an extensively hydrated folded state in the c-Myb R2 subdomain becomes unambiguously clear from the discrepancy between the high-pressure Trp fluorescence and NMR results. Recently, a similar discrepancy was also found in staphylococcal nuclease, in which the ^{1}H NMR signals showed complete folding, whereas the fluorescence showed a significant fraction of unfolding at high pressure (C. A. Royer, Centre de Biochimie Structurale, personal communication, 2011). Furthermore, an extensively hydrated folded state mediated by cavity hydration has been detected in hen lysozyme by ^{1}H NMR spectroscopy at high pressure and low temperature (4). The extensively hydrated and folded state (N*) is likely to be present in globular proteins with large internal cavities, as was inferred earlier from pressure-induced nonlinear shift of NMR signals (5). This becomes clearer by pressure studies at low temperature, because of the lower stability of the folded conformer N (approaching cold denaturation) and the larger volume decrease with hydration at lower temperature (16). The notion that the cavity-hydration-mediated N* state is present in many globular proteins under physiological conditions in equilibrium with N to regulate their dynamics and function appears to be supported by the observation that internal cavities are evolutionarily preserved (4).

Finally, this report gives a caution against a naive interpretation of the conformational state of a protein based on Trp fluorescence, at the same time pointing to the critical role of high-pressure NMR spectroscopy in delineating protein conformations with varying degrees of hydration.

SUPPORTING MATERIAL

Supporting methods, figures, a table, and reference (17) are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)05416-6.

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