Communication

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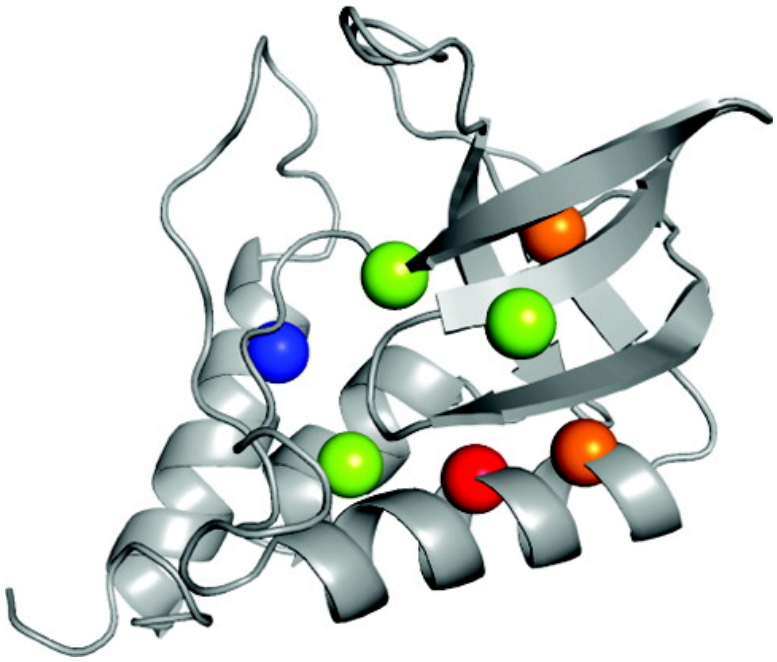

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V_i-Value Analysis: A Pressure-Based Method for Mapping the Folding Transition State Ensemble of Proteins

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We introduce an approach to mapping the structure of the folding transition state ensemble (TSE) of proteins, based on the pressure dependence of protein stability. Pressure leads to the unfolding of most proteins at ambient temperatures because the specific volume of the unfolded state is smaller than that of the folded state. Although a fully quantitative parametrization of volume changes accompanying protein conformational changes remains one of our main objectives, recent advances based on pressure perturbation calorimetric studies[1] have confirmed our earlier conclusions[2] that the relatively small, negative ΔV of unfolding measured near ambient temperatures is the result of positive contributions from increased hydrophobic hydration and negative contributions from increased hydration of polar moieties and the hydration of cavities that are opened to solvent upon unfolding. Thus, overall, the volume changes reflect the change in hydration of the protein upon unfolding. The effect of pressure on the folding and unfolding rate constants depends upon the activation volumes and hence on the relative hydration of the TSE with respect to the folded and unfolded states. Here we use Staphylococcal nuclease (SNase) to illustrate how pressure can be used evaluate the state of hydration of the folding TSE and hence to map its structure.

Since it was first introduced in the late 1980s, ϕ-value analysis has provided considerable insight into the structure of the TSE in the protein folding reaction and into the overall energetics and landscape of protein folding.[3−6] The ϕ-value analysis requires that the mutations destabilize the TSE enough for the effect to be measurable, but not sufficiently to alter the structure of the TSE significantly. In contrast, our approach based on activation volumes relies on significant disruption of the TSE structure by mutations to identify structured regions in the WT TSE.

Previously,[2,7] we have shown that the activation volume for folding of WT SNase is large and positive, and hence that the rate-limiting step, as for a number of other proteins,[8−14] in folding involves significant dehydration. We refer to this measure of the fractional dehydration of the TSE with respect to the unfolded state as the V value. It is calculated as the ratio of the folding activation volume, ΔV^*_f, over the total equilibrium volume change upon folding, $\Delta V°_f$.

$$V = \Delta V^*_f / \Delta V°_f \quad (1)$$

Unlike m values, which rely on denaturant binding to estimate solvent-exposed surface area, this V value is directly related to the fractional change in hydration. A V value near 1 corresponds to a TSE that is as dehydrated as the folded state.

Recent pressure perturbation studies on a series of variants of a stabilized form of SNase,[15−17] in which valine 66, which is buried in the hydrophobic core of the protein, was substituted by ionizable residues, revealed that their TSE, unlike that of WT SNase, was highly hydrated. For example, the variant with the V66K substitution has a V value ~0.25 compared to 0.88 for WT SNase.[18] To compare these variants with the WT, we introduce the V_i value, defined as the ratio of the V values for the variant with the internal ionizable group and for the WT protein:

$$V_i = V^v / V^{WT} \quad (2)$$

We have now performed a systematic study of variants in which several internal positions were substituted with Lys. Under the conditions of pH that were used for these studies, the Lys residues are neutral when they are internal and charged when they are in water (see pK_a values in Table 1, Supporting Information). Substitution of internal positions that are ordered in the WT TSE with Lys is expected to disrupt the TSE structure because the Lys residues near neutral pH are charged, thus they promote structural states where they can remain charged by virtue of being exposed to bulk water. This should lead to completely different pressure-dependent kinetics. The sites that were studied were selected to sample different interfaces between elements of secondary structure (Figure 1A). V23 and V74 are both in the β-barrel; their side chains are in the most hydrophobic region of SNase. T62 and V66 are on the same α-helix, and they are also members of the hydrophobic core. L36 and L103 are in the region between the α- and β-subdomains, and L125 is on the C-terminal α-helix. By far UV−CD and/or X-ray crystallography, these variants have been shown to have structures that are indistinguishable from the WT.[16,17,19−21] They undergo cooperative unfolding transitions.[22] In the variants studied here, the internal Lys residues titrate with highly perturbed pK_a values, consistent with the neutral side chain of the Lys residues being buried inside the protein.

The pressure-jump fluorescence relaxation profiles for the variants were analyzed with single exponential decays. The plots of ln τ versus pressure (τ, relaxation time) were fitted for the folding (or unfolding) rate constant and the folding (or unfolding) activation volume, using as constraints the equilibrium constant and volume change obtained from the equilibrium unfolding profile, as described previously[17] (e.g., Figure 1C and Table 1, Supporting Information). Because the variant proteins have high stability, GuHCl was added to some of them at low concentration to allow unfolding in the range of P that is experimentally accessible. We have shown previously that the equilibrium and activation volume changes for SNase and many of its variants are independent of pH and GuHCl up to 1 M.[23,24] The equilibrium $\Delta V°_f$ values for all the variants are within the range of 65 ± 6 mL/mol except for those at positions 62 and 36. Relaxation at position 62 was extremely slow (several hours), and hence the results were somewhat less accurate, but clearly all of the effect of pressure was on the unfolding rate constant, which increased with pressure. The response of the V74K

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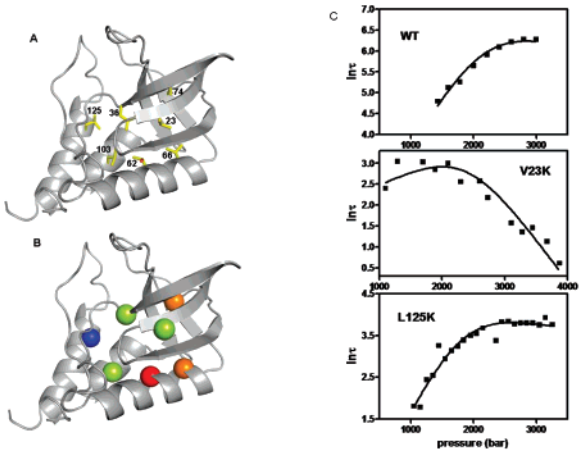

Figure 1. (A) Positions in SNase that were substituted with Lys residues. (B) High (blue, 1), low (red, orange, 0−0.3), and intermediate V_i values (green, 0.5−0.7). (C) Plots of the ln τ versus pressure for WT and two of the Lys-containing variants. Lines through points correspond to fits of the data for the activation volumes and rate constants as described in ref 17. All experiments except WT were performed with a hyperstable variant of SNase known as Δ+PHS. Structures were rendered using PyMol 0.99c6.

variant to pressure perturbation was comparable to that of V66K.[18] Like the T62K variant, the large negative activation volume for unfolding for both V74K and V66K, as opposed to the large positive activation volume for folding observed for WT SNase, implies that, in terms of hydration, the folding TSEs of these variants are much closer to the unfolded than to the native state in terms of their fractional hydration. The L36K, V23K, and L103K variants exhibited an intermediate behavior, with pressure leading to both an increase in the unfolding rate constant and a decrease in the folding rate constant. At position 36, even if there is some ground state (unfolded state) perturbation by the substitution, as suggested by the much larger negative equilibrium volume change upon unfolding, this would only lead to a small decrease in the calculated V_i value and not alter the intermediate nature of the effect of this substitution. Finally, the L125K variant exhibited behavior similar to that of WT SNase, indicating that its TSE is nearly identical to that of the wild type protein, that is, largely dehydrated relative to the unfolded state.

The V_i values are superimposed on the structure of SNase in Figure 1B. It can be seen that the sites with the lowest V_i values (red, red-orange), corresponding to the highest degree of perturbation of the TSE by the internal Lys residue, are in the β-barrel and in the α-helix that packs against it. The burial of unpaired ionizable residues by substitution of one member of a buried ion pair with a neutral side chain likewise destabilized the TSE of barnase.[25] Substitution of residues 23, 36, and 103 with Lys disrupts the TSE to a lesser extent than substitutions in the core (green in Figure 1B). Lys125 (in subdomain 2) has no effect on the structure of the TSE (blue), suggesting that the C-terminal helix of SNase does not participate in the transition state, and that it folds against the more stable β-subdomain after passage of the barrier. These studies suggest that the structure of the TSE of WT SNase includes part of the β-barrel and part of the first α-helix (residues 55−67). In the WT TSE, these elements of secondary structure are sufficiently well ordered to exclude solvent from the interior of the hydrophobic core. The region linking this hydrophobic core to subdomain 2 appears to adopt an ensemble of conformations with an intermediate probability to be folded in the TSE, while the C-terminal α-helix remains disordered.

Our approach to mapping the TSE of SNase differs from the much more widely used ϕ-value analysis in that the goal of ϕ-value analysis is to perturb the TSE energetically without perturbing its structure significantly. More often than not, this is actually possible, and for this reason, ϕ-value analysis has proven very useful. An alternative to ϕ-value analysis is ψ-value analysis[26] in which the TSE structure is stabilized by cross-linking, which is considered to occur only when the structure pre-exists. In the V_i-value analysis introduced in this paper, the goal is to make substitutions that are sufficiently destabilizing to disrupt the TSE in the mutant, especially at sites that would be ordered in the TSE of the wild type protein. Use of ionizable residues was essential for this purpose. This study was possible owing to the availability of the highly stable Δ+PHS variant of SNase, which tolerates substitutions of internal positions with ionizable groups.[22] In other protein systems, a V_i-value analysis will likewise require enhancement of the stability of the protein by mutation or solution conditions. The unique advantage of V_i-value analysis is that it conveys direct information about the state of hydration of the TSE, which has been recognized as a key factor in the protein folding transition.[27]

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Supporting Information Available: Data acquisition protocol, data analysis, complete results table including the rate constants, examples of the fits of the relaxation profiles to single exponential decays, and ln τ versus pressure plots for all of the tested variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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