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Double Point Mutant F34W/W140F of Staphylococcal Nuclease Is in a Molten Globule State but Highly Competent to Fold into a Functional Conformation[1]

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The double point mutant F34W/W140F of staphylococcal nuclease was created and then characterized by far and near-UV CD, size-exclusion chromatography, ANS-binding fluorescence. The results show that this mutant has properties consistent with the classical definition of a molten globule, *i.e.*, substantial secondary structure but no unique tight packing of tertiary structure, a relatively compact size and a larger exposed hydrophobic surface area as compared with the wild type enzyme, indicating that a molten globule can occur under physiological conditions. However, the activity assay showed that the mutant still maintains wild-type levels of activity. To further clarify the mechanism of the substrate-induced reactivation, enzymatic parameters such as K_M^{DNA}, K_S^{DNA}, K_M^{Ca}, K_A^{Ca}, K_d^{pdTp}, and V_{max} were determined, showing that all the parameters of this mutant are similar to those of the wild type enzyme. The results indicate that the F34W/W140F mutant has a similar substrate affinity to the wild type enzyme, and the functional conformation can be restored by substrate binding, which corresponds to the conformational adjustment capability of the mutant upon binding to ligands pdTp and Ca^{2+}. The severely disrupted tertiary structure and high activity of the mutant indicate that it is highly competent to fold to its functional conformation. The results suggest that the primary structure can only guide the mutant to a molten globule state and that ligand-binding causes the mutant to fold further into its functionally active conformation, indicating that ligand-binding plays an important role in protein folding and catalysis.

Key words: folding, ligands-binding, molten globule, mutant, staphylococcal nuclease.

Molten globules have been extensively studied as general intermediates in protein folding, and their presence is widely accepted (*1–4*). However, the stable molten globule states of globular proteins were usually obtained at acid pH, high temperature, and intermediate concentrations of strong denaturants (*1, 5*), at which most proteins lose their activities. A question of interest is whether they can gain their functional conformations, and, if they do, what is the driving force. Fortunately, some proteins, such as α-lactalbumin (*6, 7*) and $p21^{H\text{-}ras}$ protein (*8*), have been found to exhibit some properties of molten globules after the removal of their ligands at neutral pH, *i.e.*, the removal of the ligands completely releases side chain packing but leaves substantial secondary structure. Furthermore, the restoration of the native conformation occurs upon binding to ligands. However, these proteins can not easily be used to trace the function expression following conformational restoration. A model protein that is in a molten globule state and able to fold into a functional conformation upon ligand binding is needed for studying the folding and binding mechanisms and to show the generality of the molten globule formation.

Staphylococcal nuclease (SNase) is a well-known protein used for studies of protein folding (*9–14*). Ca^{2+} is necessary for its DNA or RNA hydrolyzing activity, and thymidine 3′,5′-bisphosphate (pdTp) is a competitive inhibitor and substrate analogue of SNase (*15*). The ternary complex of SNase-Ca^{2+}-pdTp is usually used as a model of SNase in the catalytic state (*13, 14*). In this paper, the F34W/W140F mutant of SNase was created and its structural properties were characterized by far and near-UV CD, ANS-binding fluorescence, and size-exclusion chromatography experiments. The results show that the mutant has all the properties of a molten globule, however, the enzymatic parameter measurements show that the mutant has similar or only slightly smaller values for K_M^{DNA}, K_S^{DNA}, K_M^{Ca}, K_A^{Ca}, K_d^{pdTp} and V_{max}, indicating that the mutant has similar functional properties to the wild type SNase. The results clearly suggest that a molten globule can occur under physiological conditions and the functional expression of the mutant is mediated through ligand (substrate) binding.

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Abbreviations: SNase, staphylococcal nuclease; CD, circular dichroism; UV, ultraviolet; pdTp, thymidine-3′,5′-bisphosphate; ANS, 1-anilinonaphthalene-8-sulfate.

EXPERIMENTAL PROCEDURES

Materials—Plasmid pBVS-1 for the expression of SNase was constructed by Jing *et al.* (*16*). The mutants were created using a PCR technique and confirmed by DNA sequencing. All the proteins used here contained six extra amino acid residues at the N-terminus, which have no effect on the SNase properties (*17*). The proteins were expressed and purified according to Jing *et al.* (*16*). The purity of the proteins was confirmed by SDS-PAGE to be greater than 95%. Protein concentration was determined according to Gill and Hippel (*18*). 1-Anilinonaphthalene-8-sulfonate (ANS) and salmon sperm DNA were purchased from Sigma and pdTp was purchased from Pharmacia. All other reagents were of analytical grade.

Methods—Circular dichroism spectra were obtained on a Jasco-700 spectropolarimeter at 25°C using a 0.1 mm pathlength for the peptide region with a protein concentration of 0.3 mg/ml, and a 10 mm pathlength for the aromatic region with protein concentration of 1 mg/ml in 30 mM Tris-HCl, pH 7.4. Eight scans, taken at scan speeds of 50 nm/min and bandwidths of 1 nm, were averaged and corrected for the buffer baseline. The apparent dissociation constant of pdTp (K_d^{pdTp}) was determined by titration of the binary complex of the mutant and Ca^{2+} with pdTp according to the methods described by Zhou and Jing (*19*).

Size-exclusion chromatography was carried out on a Bio-Rad Biologic chromatography workstation with a Superose-12 column. Each sample containing a specified concentration of protein in 30 mM Tris-HCl, pH 7.4, was applied to the column and eluted with the same buffer at a flow-rate of 0.4 ml/min at 25°C. The effluent was monitored at 280 nm. Stokes radii were determined after calibration with four proteins whose radii are known from the literature (bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease A) according to Uversky's method (*20*).

ANS-binding fluorescence emission spectra were measured with a Hitachi F4010 spectrofluorometer at 25°C. Each sample contained 60 μM ANS and 5 μM protein in 30 mM Tris-HCl, pH 7.4. The excitation wavelength was 345 nm and the slit width was 10 nm.

Nuclease activity was measured with a UV1601 spectrophotometer at 25°C by observing the increase in absorbance at 260 nm upon the addition of enzyme to 1 ml of a solution containing 50 μg/ml denatured salmon sperm DNA/30 mM Tris-HCl, pH 7.4/10 mM $CaCl_2$. One unit is defined as an increase of 1 OD/min (*21*). The kinetic parameters of the mutant and the wild type SNase were obtained by analyzing the initial velocity of the reactions according to the methods described by Serpersu *et al.* (*22*).

RESULTS

Near and Far-UV CD Spectra of SNase and Its F34W/W140F Mutant—The mean residue ellipticity at 222 nm ($[\theta]_{222\ nm}$) in far-UV CD usually represents the secondary structure content, especially the characteristics of the α-helical conformation. The ellipticity in the near-UV CD region reflects the asymmetry of the environment of the aromatic groups and is usually considered as an index of the uniqueness of protein tertiary structure. Figure 1A shows the far-UV CD spectra of the F34W/W140F mutant in the presence and absence of ligands. It can be seen that the secondary structure of the F34W/W140F mutant is less than that of the wild type SNase but much greater than that of the wild type SNase in 6 M GuHCl, indicating the partially folded secondary structure of the mutant. However, the relative value of $[\theta]_{222\ nm}$ for the mutant is 68% that of wild type SNase. The α-helix content in a stable acid–induced molten globule-like state of wild type SNase is 64% that of the native state (*5*). Thus, the mutant still retains substantial secondary structure. Upon increasing the pdTp concentration in the presence of 10 mM $CaCl_2$, the far-UV spectrum of the F34W/W140F mutant changed greatly and became almost the same as that of the wild type SNase at 60 μM pdTp and 10 mM Ca^{2+}. This suggests that ligand binding induces the partially folded mutant to fold further into a native-like structure showing that the mutant is in a competent state.

The apparent dissociation constant of pdTp (K_d^{pdTp}) was determined by titration of the binary complex of the mutant and Ca^{2+} with pdTp according the methods described by Zhou and Jing (*19*). The value of K_d^{pdTp} for the mutant is 10.9 ± 2.4 μM, which is very similar to that of wild type SNase (13.1 ± 5.8 μM), suggesting that this mutant is sim-

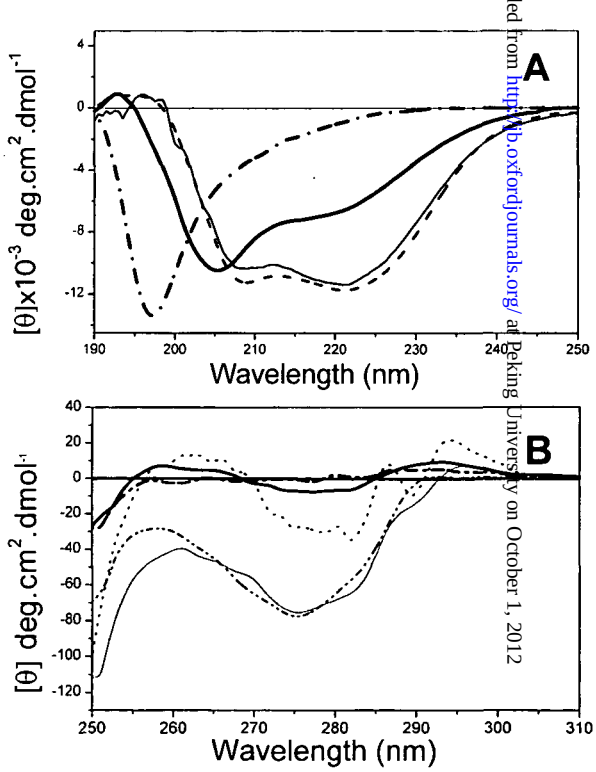

Fig. 1. **(A) Far-UV CD spectra of the F34W/W14F mutant and wild type SNase under different conditions.** Protein concentration was 0.3 mg/ml in 30 mM Tris-HCl, pH 7.4. The spectra were measured at 25°C, and the sample cell pathlength was 0.1 mm. (——) F34W/W140F; (——) F34W/W140F in the presence of 60 μM pdTp and 10 mM Ca^{2+}; (------) wild type SNase; (—.—) wild type SNase in 6 M GuHCl. **(B) Near-UV CD spectra of the F34W/W140F, F34W, and W140F mutants and wild type SNase under different conditions.** Protein concentration is 1.0 mg/ml in 30 mM Tris-HCl, pH 7.4. The spectra were measured at 25°C, and the sample cell pathlength was 10 mm. (——) F34W/W140F; (——) wild type SNase; (------) F34W; (—··) W140F; (—·) wild type SNase in 6 M GuHCl.

ilar to the wild type SNase in terms of pdTp binding.

It can be seen from Fig. 1B that the near-UV CD spectrum of the F34W/W140F mutant is similar to that of SNase in 6 M GuHCl, suggesting a disruption of the tight packing around the aromatic chains and a lack of rigid tertiary structure in the mutant. The wild type SNase has a single Trp at position 140, which would make a significant contribution to the near-UV CD spectra. Therefore, we examined the near-UV spectra of the two single mutants, F34W and W140F, as controls. Mutant W140F shows a very similar near-UV CD spectrum to wild type SNase, while mutant F34W shows a much shallower near-UV CD spectrum than either wild type SNase or mutant W140F, Fig. 1B, even though it has two tryptophan residues at positions 34 and 140. These results clearly show that the loss in the near-UV CD is not due to the residue substitution, but reflects a loss of tertiary structure in the case of the double point mutant F34W/W140F. Because of the strong absorption of pdTp in the near-UV region, we did not measure the near-UV CD spectra of SNase and the mutant in the presence of ligands. However, the high activity of the mutant suggests that it must adopt a specific tertiary structure in the presence of substrate since the specific functional expression requires a specific tertiary structure.

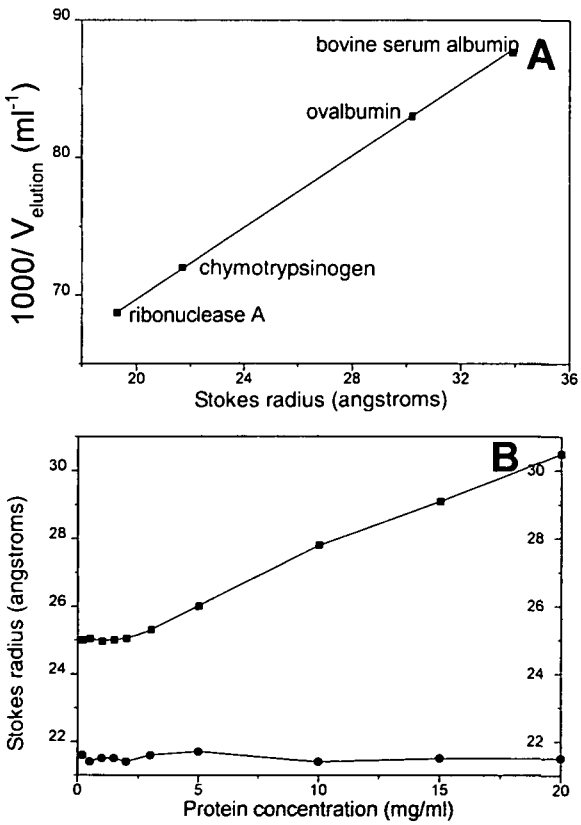

Fig. 2. **(A) The calibration curve for Superose-12 column with bovine serum albumin, ovalbumin, chymotrypsinogen, ribonuclease A.** The curve was obtained as described in the text. Protein concentration is 0.2 mg/ml. **(B) The Stokes radii of the F34W/W140F mutant and the wild type SNase at different protein concentrations.** (■) the F34W/W140F mutant; (●) the wild type SNase. All chromatography was carried out in 30 mM Tris-HCl, pH 7.4, at a flow-rate of 0.4 ml/min at 25°C. The effluent was monitored at 280 nm.

Size-Exclusion Chromatography Results—The Stokes radius can be used as an indicator of compactness and molecular size of globular proteins. After calibration according to Uversky's method (*20*), the equation below was used to calculate the Stokes radii of the proteins:

$$1000/V_{el} = (1.2957 \pm 0.0091) * R_s + (43.7997 \pm 0.2465)$$

where R_s is the Stokes radius and V_{el} is the elution volume. The value of V_{el} is the product of Retention time and flow-rate. The chromatogram for the calibration is shown in Fig. 2A.

The Stokes radii of the wild type SNase and the F34W/W140F mutant are 21.5 ± 0.1 and 25.0 ± 0.1 Å, respectively, at protein concentrations from 0.2 to 2 mg/ml. These values indicate that the mutant is loose to some extent. However, the Stokes radius of the mutant is only 16% larger than that of the wild type SNase showing that it still has a relatively compact molecular size as compared with the Stokes radius of 37.2 Å for the unfolded wild type SNase (*4, 23*). It is noteworthy that the Stokes radius of the mutant seems to show a protein concentration dependence at concentrations greater than 2 mg/ml, giving a Stokes radius of 30.5 Å at 20 mg/ml, while the wild type SNase shows no detectable increase in the Stokes radius over a concentration range of 0.2 to 20 mg/ml, as shown in Fig. 2B. This phenomenon was also observed by Kataoka *et al.* when they characterized apo-α-lactalbumin, which is a typical molten globule (*24*). They found that the increase in the gyration radius (R_g) of apo-α-lactalbumin with increasing protein concentration is not due to aggregation and proposed that it should come from different surface properties that lead to differences in the interparticle interference effect (*24*).

Because of the high absorbance background of pdTp, the Stokes radius of the mutant in the presence of pdTp can not be measured in this way. However, a comparison of the chromatographic behaviors of the mutant and wild type SNase indicates that the surface properties of the mutant are quite different from those of the wild type SNase.

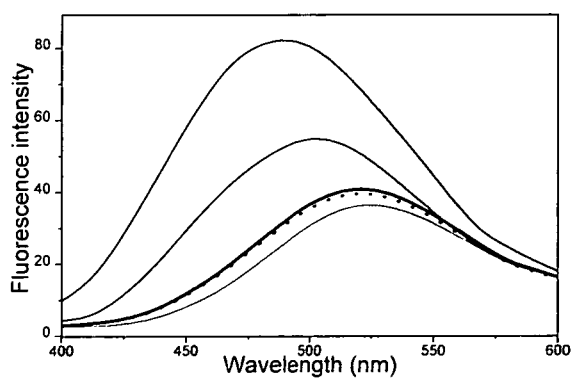

Fig. 3. **ANS fluorescence spectra in the presence of the F34W/W140F mutant and wild type SNase under different conditions.** Each sample contained 60 μM ANS and 5 μM protein in 30 mM Tris-HCl, pH 7.4. The spectra were measured at 25°C. The excitation wavelength was 345 nm and the slit width was 10 nm. From top to bottom: F34W/W140F mutant alone; F34W/W140F mutant with 60 μM pdTp and 10 mM Ca^{2+}; wild type SNase alone; wild type SNase with 60 μM pdTp and 10 mM Ca^{2+} (dotted line) and 60 μM ANS itself.

TABLE I. **Enzymatic parameters of the wild type SNase and F34W/W140F mutant.**

	K_M^{DNA} [a] (μg/ml)	K_S^{DNA} [b] (μg/ml)	K_M^{Ca} [c] (μM)	K_A^{Ca} [d] (μM)	K_d^{pdTp} [e] (μM)	V_{max} (ΔAbs·mg^{-1}·min^{-1})
Wild type SNase	4.31 ± 0.2	18.7 ± 2.4	129 ± 21	447 ± 15	13.1 ± 5.8[f]	683 ± 41
F34W/W140F mutant	4.09 ± 0.1	11.5 ± 1.1	111 ± 31	250 ± 11	10.9 ± 2.4	595 ± 20

[a] K_m^{DNA} is the Michaelis constant for DNA at saturating [DNA]. [b] K_S^{DNA} (the dissociation constant) is the K_m for DNA extrapolated to zero [Ca^{2+}]. [c] K_M^{Ca} is the Michaelis constant for Ca^{2+} at saturating [DNA]. [d] K_A^{Ca} is the K_m of Ca^{2+} extrapolated to zero [DNA]. [e] K_d^{pdTp} is the dissociation constant of the binary complex of the mutant and Ca^{2+} with pdTp. [f] K_d^{pdTp} of the wild type SNase is from Serpersu *et al.* (*22*).

ANS-Binding Fluorescence Spectra of SNase and Its F34W/W140F Mutant—ANS is used as a hydrophobic fluorescence probe to measure changes in the surface hydrophobicity of proteins (*25*). The increase in ANS-binding fluorescence intensity reflects the exposure of the hydrophobic side chains of proteins. The F34W/W140F mutant shows a considerable increase in ANS fluorescence intensity and a significant blue-shift compared with the wild type SNase, Fig. 3, indicating that the mutant has a unique hydrophobic surface area that usually appears in molten globules. The results correspond to those of the size-exclusion chromatography. It is interesting that the mutant has a lower fluorescence intensity with a red-shift when bound by pdTp and Ca^{2+}, Fig. 3, indicating that ligand binding induces changes in the surface hydrophobicity of the mutant. This means that ligand binding at the local binding site can cause the exposed hydrophobic side chains of the mutant to be reburied in the interior of the molecule, which further shows that the mutant is in a competent state.

Enzymatic Parameters of the Wild Type SNase and Its F34W/W140F Mutant—To clarify further the mechanism of the substrate-induced reactivation, the enzymatic parameters were measured and are listed in Table I. Surprisingly, the mutant has similar or only slightly lower values for K_M^{DNA}, K_S^{DNA}, K_M^{Ca}, K_A^{Ca}, K_d^{pdTp}, and V_{max} compared with the wild type SNase, although the tertiary structure of the mutant is disrupted as shown in Fig. 1B. The similarity of the kinetic parameters between the mutant and wild type SNase shows that they have similar substrate or ligand affinities, and that the functional conformation of the mutant can be restored by substrate (ligand) binding. This also explains why the mutant has almost the same activity as the wild type enzyme.

DISCUSSION

As described above, the partially folded F34W/W140F mutant has a substantial secondary structure but no unique tight packing of the tertiary structure, a relatively compact size and a larger exposed hydrophobic surface area compared with the wild type SNase. All the conformational properties characterize the mutant as being in a molten globule state. It is known that molten globules usually occur when proteins are denatured by acid pH, high temperature, or intermediate concentrations of strong denaturants (*1*). However, the present experiments show that a molten globule can also occur under physiological conditions due to amino acid substitutions in a protein molecule. Of particular interest is the finding that a mutant enzyme in a molten globule state shows a specific activity very similar to that of the wild type protein. It is generally accepted that the structural uniqueness of proteins determines their biological function. However, the fact that the F34W/W140F mutant does not have a unique tertiary structure but does have high activity raises the question: what is the structural basis for the functional activity of such a protein? A reasonable explanation is that the F34W/W140F mutant as a molten globule is highly competent to fold into a functional conformation in the presence of substrate as shown by the conformational changes in the presence of its ligands, pdTp and Ca^{2+}, as monitored by the CD spectra and ANS-binding fluorescence, which indicates that ligand-binding induces the partially folded enzyme to fold further into a native-like structure. This competent property of the mutant enables it to regain high activity upon binding the substrate.

Flanagan *et al.* characterized a truncated Staphylococcal nuclease that lacks 13 amino acids from the carboxyl terminus of the original 149–amino acid sequence by nuclear magnetic resonance, circular dichroism, and small-angle X-ray scattering measurements (*26*). The results showed that the truncated nuclease is a denatured, partly unfolded molecule that lacks a persistent secondary structure but is compact under physiological conditions. The truncated nuclease shows wild-type levels of activity in the presence of calcium and is found to fold into a native-like conformation in the presence of pdTp, a similar result to that seen for the F34W/W140F mutant described above. Ermacora *et al.* also reported another truncated nuclease that lacks 14 amino acids from the carboxyl terminus of the nuclease and has the same characteristics as the truncated nuclease described by Flanagan *et al.* (*26*). However, since the large truncated nucleases are not in neither a native nor a completely unfolded state, there is some doubt as to whether the truncated nucleases are in the molten globule state (*26–32*). Recently we studied the structural features of a family of C-terminally truncated nucleases with different peptide chain lengths by comparing the changes in conformation during peptide chain extension of SNase. The results showed that a truncated SNase that lacks 28 amino acids from the carboxyl terminus of the nuclease has the typical properties of a molten globule state, and the truncated nucleases that lack 13 or 14 amino acids from the carboxyl terminus of the nuclease do not represent the molten globule state but lie between the molten globule and native states. This can be characterized as a post-molten globule state, although the large truncated nucleases have some of the properties of a molten globule, such as a relatively pronounced secondary structure and compact size, as well as a relatively higher ANS-binding fluorescence (*28*).

Recently Kataoka *et al.* further examined the structural properties of two types of molten globule forms of α-lactalbumin, the apo-α-lactalbumin at neutral pH and the acid molten globule, using small-angle X-ray scattering measurements (*24*). They revealed that the radius of gyration for the native holo-protein was 15.7 Å, but the two molten globules both had radii of gyration of 17.2 Å. The maximum

dimension of the molecule was also increased from 50 Å in the native state to 60 Å for the molten globules, indicating that the molten globule is not as compact as the native state, a different finding from that reported prevously by Dolgikh *et al.* (*33, 34*). As shown above, the Stokes radius (R_s) for the native SNase is 21.5 ± 0.1 Å, but the F34W/W140F mutant has a Stokes radius of 25.0 ± 0.1 Å. The increase in R_s also indicates that the molten globule state of the F34W/W140F mutant is not as compact as the native state of SNase as the increase in R_s is comparable to the increase in R_g described by Kataoka *et al.* (*24*).

Finally, the substrate- (or ligand-) induced restoration of the structure and function of the F34W/W140F mutant also suggests that the amino acid sequence of the mutant may only contain information to direct the formation of a molten globule state. Further folding of the mutant is driven by ligand or substrate binding to form the functionally active conformation as described by Zhang and Matthews (*8*). As an in-creasing number of biologically important proteins and protein domains have been found to be only partially structured or unstructured (unfolded) under physiological conditions, and ligand or substrate binding plays an important role in their structural formation and functional expression (*35*), the concept that ligand binding is required to drive the formation of a specific tertiary structure or functionally active conformation, as explained by Tsai *et al.* (*36, 37*) using a different hypotheses, will become widely accepted.

REFERENCES

1. Ptitsyn, O.B., Pain, R.H., Semisotnov, G.V., Zerovnik, E., and Razagulyaev, O.I. (1990) Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* **262**, 20–24
2. Jennings, P.A. and Wright, P.E. (1993) Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science* **262**, 892–896
3. Ptitsyn, O.B. (1995) Structures of folding intermediates. *Curr. Opin. Struct. Biol.* **5**, 74–78
4. Ptitsyn, O.B. (1995) Molten globule and protein folding. *Adv. Protein Chem.* **47**, 83–229
5. Fink, A.L., Calciano, L.J., Goto, Y., Nishimura, M., and Swedberg, S.A. (1993) Characterization of the stable, acid-induced, molten globule-like state of staphylococcal nuclease. *Protein Sci.* **2**, 1155–1160
6. Kuwajima, K., Mitani, M., and Sugai, S. (1989) Characterization of the critical state in protein folding. Effects of guanidine hydrochloride and specific Ca^{2+} binding on the folding kinetics of α-lactalbumin. *J. Mol. Biol.* **206**, 547–561
7. Yutani, K., Ogasahara, K., and Kuwajima, K. (1992) Absence of the thermal transition in apo-α-lactalbumin in the molten globule state. A study by differential scanning microcalorimetry. *J. Mol. Biol.* **228**, 347–350
8. Zhang, J. and Matthews, C.R. (1998) Ligand binding is the principal determinant of stability for the p21(H)-ras protein. *Biochemistry* **37**, 14881–14890
9. Shortle, D. (1983) A genetic system for analysis of staphylococcal nuclease. *Gene* **22**, 181–189
10. Shortle, D. (1989) Probing the determinants of protein folding and stability with amino acid substitutions. *J. Biol. Chem.* **264**, 5313–5318
11. Shortle, D. and Meeker, A.K. (1989) Residual structure in large fragments of staphylococcal nuclease: effects of amino acid substitutions. *Biochemistry* **28**, 936–944
12. Hynes, T.R. and Fox, R.O. (1991) The crystal structure of staphylococcal nuclease refined at 1.7 Å resolution. *Proteins Struct. Funct. Genet.* **10**, 92–105
13. Loll, P.J. and Lattman, E.E. (1989) The crystal structure of the ternary complex of staphylococcal nuclease, Ca^{2+}, and the inhibitor pdTp, refined at 1.65 Å. *Proteins Struct. Funct. Genet.* **5**, 183–201
14. Sugawara, T., Kuwajima, K., and Sugai, S. (1991) Folding of staphylococcal nuclease A studied by equilibrium and kinetic circular dichroism spectra. *Biochemistry* **30**, 2698–2706
15. Anfinsen, C.B., Cuatrecasas, P., and Taniuchi, H. (1971) Staphylococcal nuclease, chemical properties and catalysis in *The Enzymes* (Boyer, P.D., ed.) Vol. 4, 3rd ed., pp. 177–204, Academic Press, New York
16. Jing, G.Z., Liu, L.J., Jiang, M.Y., Zou, Q., and He, R.Q. (1992) High-level expression of staphylococcal nuclease R gene in *Escherichia coli*. *J. Biotechnol.* **22**, 271–282
17. Griko, Y.V., Privolav, P.L., Sturtevant, J.M., and Venyaminov, S.Y. (1988) Cold denaturation of staphylococcal nuclease. *Proc. Natl. Acad. Sci. USA* **85**, 3343–3347
18. Gill, S.C. and Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326
19. Zhou, B. and Jing, G.Z. (1998) Conformational features of a truncated staphylococcal nuclease R (SNR135) and their implications for catalysis. *Arch. Biochem. Biophys.* **360**, 33–40
20. Uversky, V.N. (1993) Use of fast protein size-exclusion liquid chromatography to study the unfolding of proteins which denature through the molten globule. *Biochemistry* **32**, 13288–13298
21. Cuatrecasas, P., Fuchs, S., and Anfinsen C.B. (1967) Catalytic properties and specificity of the extracellular nuclease of *Staphylococcus aureus*. *J. Biol. Chem.* **242**, 1541–1547
22. Serpersu, E.H., Shortle, D., and Mildvan, A.S. (1986) Kinetic and magnetic resonance studies of effects of genetic substitution of a Ca^{2+}-liganding amino acid in staphylococcal nuclease. *Biochemistry* **25**, 68–77
23. Uversky, V.N., Karnoup, A.S., Segel, D.J., Seshadri, S., Doniach, S., and Fink, A.L.(1998) Anion-induced folding of *Staphylococcal* nuclease: characterization of multiple equilibrium partially folded intermediates. *J. Mol. Biol.* **278**, 879–894
24. Kataoka, M., Kuwajima, K., Tokunaga, F., and Goto, Y. (1997) Structural characterization of the molten globule of α-lactalbumin by solution X-ray scattering. *Protein Sci.* **6**, 422–430
25. Semisotnov, G.V., Rodionova, N.A., Razgulyaev, O.I., and Uversky, V.N. (1991) Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers* **31**, 119–128
26. Flanagan, J.M., Kataoka, M., Shortle, D., and Engelman, D.M. (1992) Truncated staphylococcal nuclease is compact but disordered. *Proc. Natl. Acad. Sci. USA* **89**, 748–752
27. Ermacora, J.J., Ledman, D.W., and Fox, R.O. (1996) Mapping the structure of a non-native state of staphylococcal nuclease. *Nat. Struct. Biol.* **3**, 59–66
28. Zhou, B., Tian, K.G., and Jing, G.Z. (2000) An in vitro peptide folding model suggests the presence of the molten globule state during nascent peptide folding. *Protein Eng.* **13**, 35–39
29. Griko, Y.V., Gittis, A., Lattman, E.E., and Privalov, P.L. (1994) Residual structure in a staphylococcal nuclease fragment. Is it a molten globule and is its unfolding a first-order phase transition? *J. Mol. Biol.* **243**, 93–99
30. Shortle, D. and Abeygunawardana, C. (1993) NMR analysis of the residual structure in the denatured state of an unusual mutant of staphylococcal nuclease. *Structure* **1**, 121–134
31. Gittis, A.G., Stites, W.E., and Lattman, E.E. (1993) The phase transition between a compact denatured state and a random coil state in staphylococcal nuclease is first-order. *J. Mol. Biol.* **232**, 718–724
32. Carra, J.H., Anderson, E.A., and Privalov, P.L. (1994) Three-state thermodynamic analysis of the denaturation of staphylococcal nuclease mutants. *Biochemistry* **33**, 10842–10850
33. Dolgikh, D.A., Abaturov, L.V., Bolotina, I.A., Brazhnikov, E.V., Bychkova, V.E., Gilmanshin, R.I., Lebedev, Y.O., Semisotnov, G.V., Tiktopulo, E.I., and Ptitsyn, O.B. (1985) Compact state of a protein molecule with pronounced small-scale mobility:

bovine alpha-lactalbumin. *Eur. Biophys. J.* **13**, 109–121
34. Dolgikh, D.A., Gilmanshin, R.I., Brazhnikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venyaminov, S.Y., and Ptitsyn, O.B. (1981) α-Lactalbumin: compact state with fluctuating tertiary structure? *FEBS Lett.* **136**, 311–315
35. Baskakov, I.V. and Bolen, D.W. (1998) Forcing thermodynamically unfolded proteins to fold. *J. Biol. Chem.* **273**, 4831–4834
36. Tsai, C.J., Ma, B., and Nussinov, R. (1999) Folding and binding cascades: shifts in energy landscapes. *Proc. Natl. Acad. Sci. USA* **96**, 9970–9972
37. Tsai, C.J., Kumar, S., Ma, B., and Nussinov, R. (1999) Folding funnels, binding funnels, and protein function. *Protein Sci.* **8**, 1181–1190