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Characterization of the stable, acid-induced, molten globule-like state of staphylococcal nuclease

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Abstract

Titration of a salt-free solution of native staphylococcal nuclease by HCl leads to an unfolding transition in the vicinity of pH 4, as determined by near- and far-UV circular dichroism. At pH 2–3, the protein is substantially unfolded. The addition of further HCl results in a second transition, this one to a more structured species (the A state) with the properties of an expanded molten globule, namely substantial secondary structure, little or no tertiary structure, relatively compact size as determined by hydrodynamic radius, and the ability to bind the hydrophobic dye 1-anilino-8-naphthalene sulfonic acid. The addition of anions, in the form of neutral salts, to the acid-unfolded state at pH 2 also causes a transition leading to the A state. Fourier transform infrared analysis of the amide I band was used to compare the amount and type of secondary structure in the native and A states. A significant decrease in α-helix structure, with a corresponding increase in β or extended structure, was observed in the A state, compared to the native state. A model to account for such compact denatured states is proposed.

Keywords: acid pH; anions; compact intermediate state; folding; FTIR; molten globule; secondary structure; staphylococcal nuclease

It is now generally accepted that protein folding involves a discrete pathway with distinct intermediate stages (Kim & Baldwin, 1990). Intermediate states formed in the early stages of folding have been identified by several techniques including NMR (Roder et al., 1988; Bycroft et al., 1990; Udgaonkar & Baldwin, 1990), CD (Kuwajima et al., 1985, 1987; Ikeguchi et al., 1986; Gilmanshin & Ptitsyn, 1987), and other rapid kinetic methods (Semisotnov et al., 1987; Ptitsyn et al., 1990). These latter investigations provide substantial support for the early involvement of a molten globule-like intermediate (Ohgushi & Wada, 1983). Kuwajima's group has shown that for several proteins the properties of the transient molten globule in folding are essentially identical to those of the stable molten globule formed at low pH (Ikeguchi et al., 1986; Kuwajima et al., 1986; Kuwajima, 1989; Sugawara et al., 1991). According to Ptitsyn (1987), the key characteristics of the molten globule state are substantial secondary structure, little or no tertiary structure (reflecting highly mobile side chains), compact size, and more exposed hydrophobic surface area than in the native state. It should be noted that less compact intermediate states have also been observed (e.g., Goto et al., 1990a).

SNase is well suited for studies of protein folding and as a result has become the focus of much attention in recent times. Of particular interest in the context of the molten globule is the observation by Shortle and coworkers (Shortle & Meeker, 1989; Flanagan et al., 1992) that SNase unfolded in denaturant can adopt a compact structure as well as a more fully unfolded state. We have previously shown that some proteins, on acidification by HCl, initially unfold and then refold to a molten globule state (Goto et al., 1990a). We show here that SNase undergoes a similar phenomenon, although the degree of unfolding and refolding are less marked; in particular the A state is considerably expanded relative to the native state. In addition, characterization of the secondary structure of the SNase A state was carried out using FTIR.

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Abbreviations: ANS, 1-anilino-8-naphthalene sulfonic acid; FPLC, fast performance liquid chromatography; FTIR, Fourier transform infrared spectroscopy; Gdn.HCl, guanidinium chloride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SNase, staphylococcal nuclease.

Results and discussion

Secondary and tertiary structure determined by CD: pH effects

Figure 1 shows selected near- and far-UV CD spectra of SNase as a function of pH. The spectrum of the salt-free solution of SNase at pH 7 is similar to that for the native state in buffer at neutral pH. The far-UV spectrum in 6 M Gdn.HCl, pH 2, indicates substantially, but not completely, unfolded protein (Shortle et al., 1989), whereas the near-UV spectrum is featureless, indicating the absence of tertiary structure. In salt-free HCl, pH 2.1, the far-UV spectrum shows slightly more residual structure than that in 6 M Gdn.HCl, whereas the near-UV spectrum is the same as that in 6 M Gdn.HCl. At pH 0.5, significantly more secondary structure is present than at pH 2, as shown by the far-UV spectrum, whereas the near-UV spectrum is identical to that at pH 2 or 6 M Gdn.HCl.

Trends in the pH-dependent changes in the near- and far-UV spectra are more readily seen in Figure 2, which shows the titration curves for changes in ellipticity at 222 nm and 275 nm for salt-free SNase as a function of HCl concentration. The unfolding transition begins around pH 5 and is complete by pH 3.5; this transition is between the native and acid-unfolded protein, U_A. The acid-unfolded protein appears to be about 80% unfolded in terms of native secondary structure and fully unfolded by near-UV CD. The addition of further HCl leads to a second transition, to the A state manifested only in the far-UV CD. With HCl (pH 0.5) the amount of secondary structure regained (as measured by ellipticity at 222 nm) is only approximately 35% (it is higher with higher chlo-

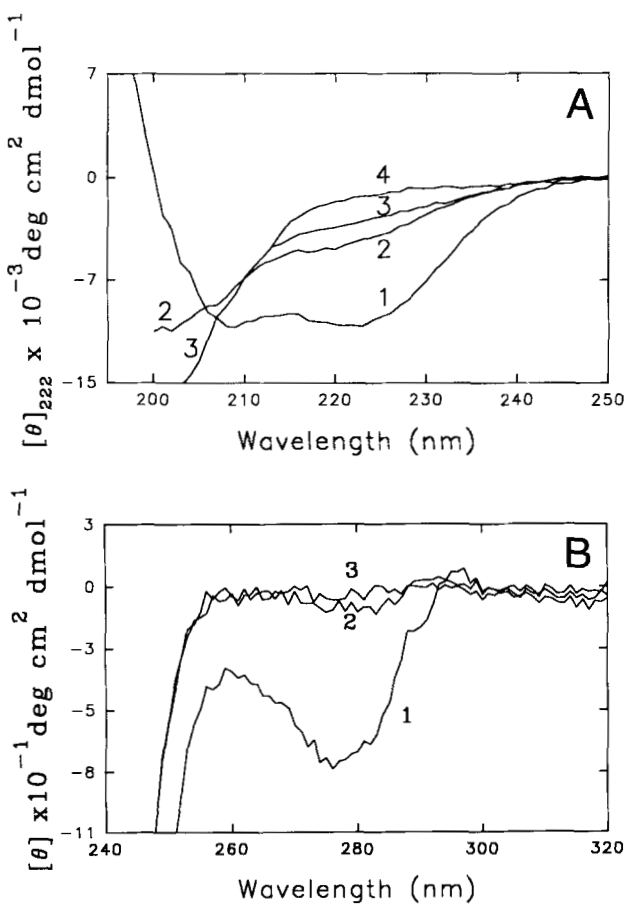

Fig. 1. Circular dichroism spectra for the titration of staphylococcal nuclease by HCl, in the presence and absence of salt. **A:** Spectra from the far-UV region. (1) Native, salt-free, pH 7.2. (2) HCl, pH 0.5. (3) HCl, pH 2.1. (4) Unfolded, 6 M Gdn.HCl, pH 2. Protein concentration 20 μM. **B:** Spectra from the aromatic region (1) Native, salt-free, pH 7.3. (2) HCl, pH 0.5. (3) Unfolded, 6 M Gdn.HCl, pH 2.0. Protein concentration was 50 μM, 20 °C.

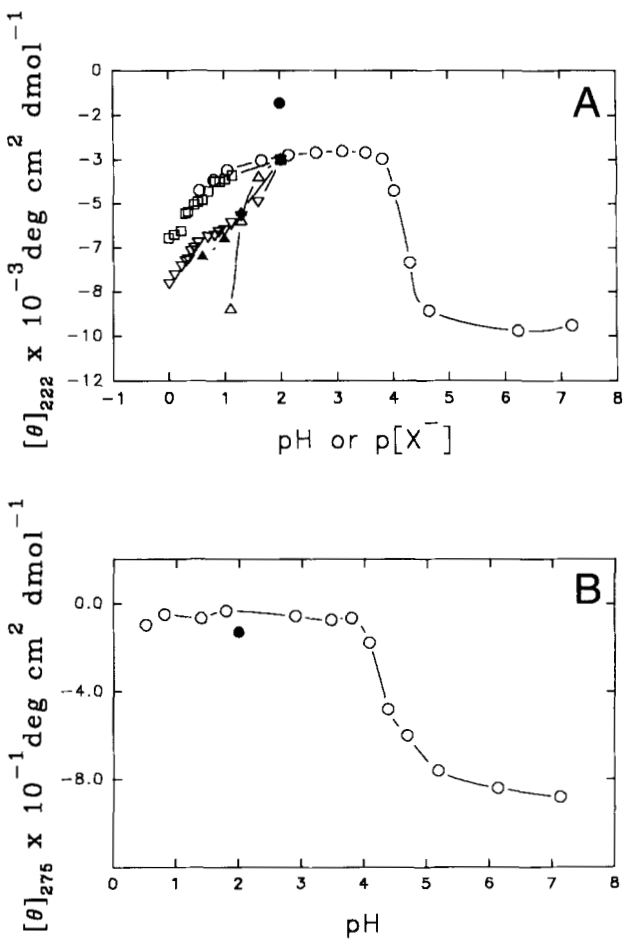

Fig. 2. The HCl titration curves for staphylococcal nuclease as monitored by ellipticity at 222 and 275 nm. **A:** The far-UV data. The value for the protein unfolded in 6 M Gdn.HCl at pH 2 is shown for reference (filled circle). The effect of titrating with neutral salts at pH 2 is shown by squares for KCl, inverted triangles for sodium sulfate, open triangles for trichloroacetate, and filled triangles for perchlorate. pX$^-$ = −log[anion]. **B:** The near-UV data. The value for the protein unfolded in 6 M Gdn.HCl at pH 2 is shown for reference (filled circle).

ride concentration and with other anions; see below). Thus the A state is characterized by significant secondary structure but the absence of native-like tertiary structure. These properties are similar to those of the molten globule, which is a compact species distinguished by substantial secondary structure and a highly mobile, fluctuating tertiary structure, leading to the absence of typical signals from the native tertiary structure.

Anion-induced transition from acid-unfolded state to the A state

We have previously shown that the transition from the acid-unfolded state of apomyoglobin and cytochrome *c* to the corresponding A state is induced by anions, either from acid (as the pH is lowered) or from the addition of neutral salt (Goto et al., 1990b). The addition of anions to the acid-unfolded state of SNase at pH 2, monitored by θ_{222}, showed that the anions induced the transition to the A state. The data have been incorporated in Figure 2A in the form of plots of ellipticity against pX^- ($= -\log[X^-]$), where X^- is the anion. It can be seen that for chloride, the curves for the KCl data essentially superimpose on those for HCl, demonstrating that it is the anion that is responsible for the transition to the A state. These data also show that the anions differ not only in their effectiveness in bringing about the transition, as revealed by the concentration of anion required, but also in the amount of secondary structure present in the induced A state (the maximum anion concentration that could be used was limited by its propensity to induce aggregation of the A state). The fraction of native secondary structure, as measured by the difference in ellipticity at 222 nm between native and guanidinium-unfolded protein, present in the A state, induced by different anions at pH 2 is shown in Table 1. Potassium ferricyanide, a trivalent anion, was even more potent in causing the transition from the U_A state to the A state; however, aggregation occurred at 0.3 mM ferricyanide, at which point some 25% of the secondary structure had returned. It is not clear why different salts (anions) bring about different degrees of return of secondary structure, but one possibility would be specific anion interactions, possibly competing for an internal ligand, i.e., a specific anion binding site formed by ionic side chains, in which either the anion from the salt, or a side-chain ligand compete. It is also possible that it reflects differences in the hydrophobicity of the anions, as well as differences in size and charge density.

Table 1. *Amount of secondary structure in A states induced at pH 2 by different anions*

Anion	Concentration	% Native secondary structure[a]
Chloride	0.5 M	52
Sulfate	1.0 M	67
Trichloroacetate	75 mM	92
Perchlorate	0.25 M	60

[a] As measured by the difference in ellipticity at 222 nm between native and guanidinium-unfolded SNase.

We have previously shown that ANS binding may be a good method for monitoring the presence of molten globule-like states (Goto & Fink, 1989). In the case of SNase we examined the binding of ANS to the various conformational states (Fig. 3). Unbound ANS in aqueous solution has very low fluorescence intensity, and little increase was observed in the presence of the native state, in the absence or presence of KCl (1 M) at pH 7.0. A small increase in ANS fluorescence intensity was observed at pH 2 for the acid-unfolded material and a slightly larger increase for the Gdn.HCl, pH 2 sample, suggesting the possibility that there may be a trace of residual structure in these species, because for most proteins we have examined in 6 M Gdn.HCl at pH 2 there is no increase in fluorescence over the background. As expected, the A state, e.g., pH 2, 1 M KCl, showed a significant fluorescence enhancement, reflecting the binding of ANS to hydrophobic regions of the compact intermediate (Fig. 3).

Measurement of the compactness of the A state

FPLC gel filtration experiments were conducted on the native, guanidine-unfolded and A states to determine the hydrodynamic properties. The Stokes radii of the native and unfolded states were determined to be 18 and

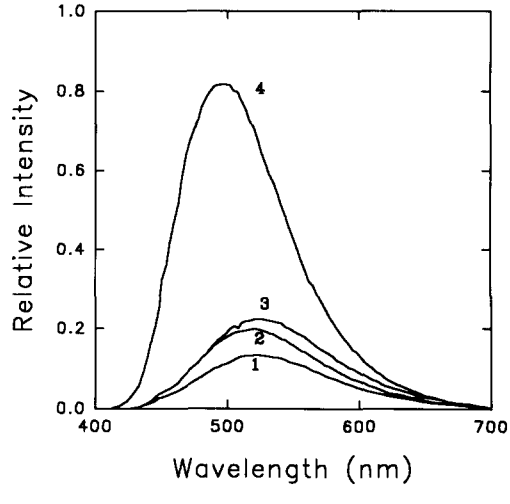

Fig. 3. The interaction of ANS with staphylococcal nuclease. Fluorescence emission spectra of 70 M ANS are shown under various conditions at 20 °C are shown. (1) Native SNase, pH 7.2. (2) Acid-unfolded state, HCl, pH 2. (3) Denatured state, 6 M Gdn.HCl, pH 2. (4) Molten globule state, pH 2, 1.0 M KCl. Excitation was at 390 nm. Protein concentration was 1.0 μM.

40 Å, respectively. For the chloride-induced A state (pH 2, 0.5 M KCl) the Stokes radius was 25 Å. Thus the hydrodynamic radius of the A state is expanded by close to 40% over that of the native state but is still much more compact than the unfolded state (>100% expanded relative to the native state). The volume expansion of the A state would be more than doubled that of the native state, assuming relatively spherical shapes.

Infrared analysis of A state and native structures

FTIR can be used to obtain information about the secondary structure of proteins, especially from the amide I and III bands (Byler & Susi, 1986; Jakobsen & Wasacz, 1987). Examination of the FTIR spectra in the amide I and III regions for the native, fully unfolded, and A states of SNase, as well as other proteins (S.A. Swedberg & A.L. Fink, unpubl.), shows significant differences between the different conformational states (Fig. 4). Fast-Fourier self-deconvolution and second derivative spectra were used to determine the deconvoluted band positions in the amide I region; their positions and assignments are given in Table 2. Note that the data were collected in aqueous, not D_2O solution; there are shifts in band positions, usually small, between the two solvents. Secondary structure content from curve fitting to the amide I band are given in Table 3 for the native and A states. Good agreement for the native state is found between the reported secondary structure content from X-ray structural analysis and the FTIR results. From the deconvolution of the amide I band the major differences in secondary structure in going from the native state to the A state are a 9% decrease in α-helix and a 9% increase in β-sheet. Confirmation for the decrease in helix and increase in β-structure in the A state was obtained from analysis of the amide III band (data not shown). It is likely that some of the increase in β-structure in the A state may correspond to extended chain, rather than β-sheet structure, since at present it is not possible to readily distinguish between the two by IR.

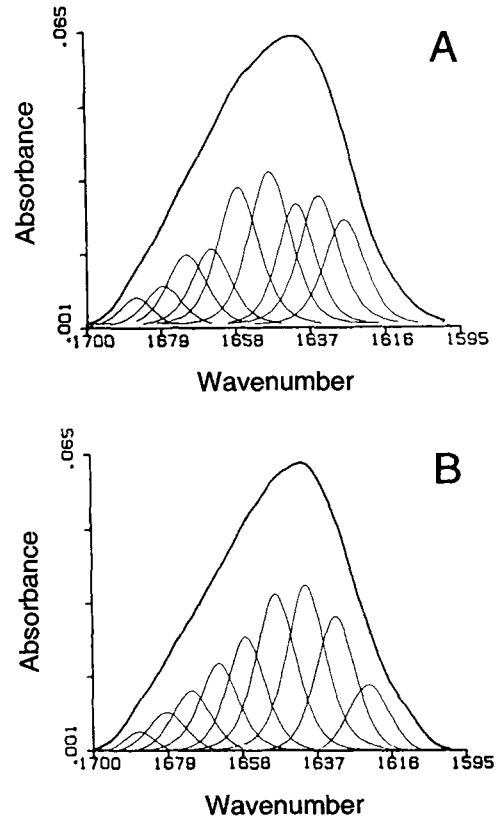

Fig. 4. The FTIR amide I bands for native and A state SNase. The deconvoluted, curve-fitted spectra are shown, the sum of the individual curve areas was indistinguishable from the original spectrum. The assignment of individual bands is given in Table 2.

Table 2. *Assignment of amide I frequencies to secondary structure*[a]

Band position (cm^{-1})		Assigned secondary structure
Native	A state	
1,686 ± 1	1,686 ± 1	Turn
1,678 ± 1	1,679 ± 1	β or extended
1,672 ± 1	1,672 ± 1	Turn
1,665 ± 1	1,664 ± 1	Turn
1,657 ± 1	1,657 ± 1	α-Helix
1,648 ± 1	1,648 ± 1	Unordered
1,641 ± 1	1,641 ± 2	β or extended
1,633 ± 2	1,632 ± 2	β or extended
1,627 ± 1	1,627 ± 1	β or extended

[a] Analysis of the amide I band region of the FTIR spectrum was done using the Nicolet FOCAS procedure. Band assignments were were made using the data of Byler and Susi (1986) and Krimm and Bandekar (1986).

Conclusions

The unfolding of SNase in the vicinity of pH 4 is brought about by the titration of the carboxylic acid side chains, which results in the protein having a net positive charge due to the protonated side chains of lysine, histidine, and

Table 3. *Comparison of secondary structure content of native and A states*[a] *of SNase*

Conformational state	% Helix[b]	% β-Sheet	% Turn	% Unordered
Native	25	44	17	15
A	16	53	14	17
N (X-ray)	27	39	15	19

[a] The A state was formed at pH 2.0, 0.5 M KCl. Protein concentration was 60 and 120 μM.

[b] The estimated overall accuracy of the values is ±5%.

arginine. The consequent electrostatic (Coulombic) repulsion from the ammonium groups causes substantial unfolding of the protein leading to formation of a noncompact denatured state, U_A, in the vicinity of pH 2–3.5. Binding of anions to the ammonium groups leads to shielding of these intramolecular repulsive forces and allows the intrinsic hydrophobic forces to cause collapse to a more compact denatured state (the A state) with many of the properties of a molten globule. The amount of secondary structure present in the A state was found to be a function of the nature and concentration of the anion. This may reflect either of two possibilities: (1) a mixture of U_A and A states, with the equilibrium being shifted in favor of more A state with higher anion concentration, or (2) structurally different A states, where the A state formed by chloride has less secondary structure than that formed by trichloroacetate or perchlorate. Because the anion titration curves level off at higher anion concentrations, the second possibility is more likely to be correct. This implies that a family of intermediate states exists with differing amounts of secondary structure and presumably different degrees of compactness.

The hydrodynamic radius of the A state is substantially expanded compared to that of the native state, suggesting a much more open structure in the A state compared to the native state and consistent with the loss of properties (signals) associated with the tertiary structure. The FTIR data indicate a significant change in the type of secondary structure in the A state compared to the native state; this could imply that secondary structure formed early in the folding process is different than that in the final folded state, if the A state is a good model for transient intermediates on the folding pathway. The FTIR results are also interesting in that they indicate little increase in unordered structure in the A state. It should be noted that the protein concentration in the FTIR experiments is sufficiently high that soluble aggregates of the A state would be present and could conceivably affect the observed secondary structure.

The variation in amount of secondary structure, as reflected by the ellipticity at 222 nm, for the A state induced by different conditions suggests that such compact denatured or intermediate states may adopt a variety of three-dimensional structures, depending on the environmental conditions. One model that would account for such a conformational variation is the following. The native conformation is assumed to consist of a number of structural units, which we will call building blocks, that are tightly packed together via tertiary interactions. These building blocks may consist of regions of secondary structure, or other types of subdomains or autonomous folding units, and will have significant intrinsic stability. Such building blocks would minimally consist of a helix or two-stranded β-sheet. We visualize the initial transition from the native state to the A state or other molten globule-like state, which corresponds experimentally to the loss of tertiary structure but retention of secondary structure, to correspond to the separation of these building block units, presumably with concomitant solvent penetration and with expansion of the molecule as a whole.

In SNase the likely building block units would be the two β-sheets and three helices. A range of species is anticipated, depending on the extent of the loss of side-chain contacts between building blocks. Once the contacts between building blocks are lost, the molecule will become a chain of small structural units (the building blocks) linked by flexible polypeptide chain links. It is likely that the conformations will be quite mobile in the sense that in the species with loss of some of the building block contacts there may still be transient contacts between the structural units. In a small molecule such as SNase, the separation of these building blocks leads to their decreased stability due to loss of the stabilizing influence of the tertiary interactions and thus may also result in significant loss of their secondary structure. The A state is thus pictured as an ensemble of substates ranging from those with substantial contacts between all or most of the building block units, and thus quite compact, to those with relatively few contacts between the building blocks, which are thus quite expanded. Whereas the building block units themselves may retain a rather rigid, native-like structure, there will be substantial mobility between them, as dictated by the freely jointed connecting links. Depending on the destabilizing forces of the external environment the predominant substates may be quite compact and relatively native-like, as seen in the present case with perchlorate or trichloroacetate, or more expanded, as seen with chloride.

Materials and methods

Materials

SNase was grown and purified from a cloned gene kindly supplied by Prof. D. Shortle. The homogeneity of the protein was checked by SDS-PAGE using the Phastsystem (Pharmacia). Protein concentrations were determined from published molar extinction coefficients. The ammonium salt of ANS was purified by recrystallization and gel filtration on Sephadex LH-20 using water as eluant (York et al., 1978).

Methods

CD spectra were recorded on an AVIV model 60DS instrument at 25 °C unless otherwise specified. Either 1.0- or 0.2-mm-pathlength cells were used for far-UV measurements, and a 10.0-mm-pathlength for the near-UV region. Tryptophan fluorescence was measured at 20 °C with a Perkin-Elmer MPF 4 instrument with excitation at 280 nm. The pH measurements were made with a micro combination glass electrode (Microelectrodes Inc., model MI-410).

Hydrodynamic radii were determined using molecular exclusion chromatography on Superose 12 with a Pharmacia FPLC apparatus, using standard MW marker proteins. The Stokes radius was determined using the procedure of Corbett and Roche (1984). Protein sample concentrations were 2 μM. ANS binding was measured using fluorescence emission with excitation at 400 nm. Typically ANS concentrations were in the region of 50 μM, protein concentration in the vicinity of 0.3 mg/mL. FTIR measurements were done in duplicate (with independent analysis) on a Nicolet model 800 instrument. Barium fluoride windows were used with pathlengths of 15 μm for amide I and 25 μm for amide III measurements. Spectral deconvolution was done using the Nicolet FOCAS system.

The pH titrations at low salt conditions used protein samples extensively desalted with deionized water using Centricon membranes. Hydrochloric acid was used to adjust the pH. Salt titrations at low pH were carried out by making a series of solutions of the desired salt concentration and adjusting the pH with HCl. Desalted protein stock solutions were used. Urea and guanidinium concentrations were determined from the refractive index of the solution.

Acknowledgments

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