Effect of Salts on the Stability and Folding of Staphylococcal Nuclease†

Chiaki Nishimura,‡ Vladimir N. Uversky, and Anthony L. Fink*

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064

Received April 17, 2000; Revised Manuscript Received December 14, 2000

ABSTRACT: The stability and folding kinetics of wild-type and a mutant staphylococcal nuclease (SNase) at neutral pH are significantly perturbed by the presence of moderate to high concentrations of salts. Very substantial increases in stability toward thermal and urea denaturation were observed; for example, 0.4 M sodium sulfate increased the free energy of wild-type SNase by more than 2 kcal/mol. For the NCA SNase mutant, the presence of the salts abolished the cold denaturation observed at neutral pH with this variant, and substantially increased its stability. Significant effects of salts on the kinetics of refolding were also observed. For NCA SNase, the presence of the salts markedly increased the folding rates (up to 5-fold). On the other hand, chloride, in particular, substantially decreased the rate of folding of the wild-type protein. Since the rates of the slow phases due to proline isomerization were increased by salt, these steps must be coupled to conformational processes. Fluorescence energy transfer between the lone tryptophan (Trp140) and an engineered fluorescent acceptor at residue 64 revealed that the addition of a high concentration of KCl led to the formation of a transient folding intermediate not observed at lower salt concentrations, and in which residues 140 and 64 were much closer than in the native state. The salt-induced effects on the kinetics of folding are attributed to the enhanced stability of the transient folding intermediates. It is likely that the combination of the high net charge, due to the high isoelectric point, and the relatively low intrinsic hydrophobicity, leads to staphylococcal nuclease having only marginal stability at neutral pH. The salt-induced effects on the structure, stability, and kinetics of staphylococcal nuclease are attributed to the binding of counterions, namely, anions, resulting in minimization of intramolecular electrostatic repulsion. This leads to increased stability, more structure, and greater compactness, as observed. Consequently, localized electrostatic repulsion is present at neutral pH in SNase, probably contributing to its marginal stability. The results suggest that, in general, marginally stable globular proteins will be significantly stabilized by salts under conditions where they have a substantial net charge.

The relative role of the contribution of electrostatic interactions to protein stability, compared to that of hydrophobic interactions, has been the subject of long-standing controversy (*1*). Part of the difficulty in discriminating the contributions from electrostatic effects stems from the fact that there are several different ways in which they contribute to the net stability of the native conformation. In addition, both attractive and repulsive electrostatic interactions are possible. In general, models to account for cosolute effects on protein stability may be classified in a number of ways; two major classifications are those in which the cosolute directly interacts with the protein, i.e., binding, or those involving effects on the solvent, e.g., excluded volume effects (*2*).

Previously, we have shown that many proteins are significantly unfolded at low pH due to charge−charge repulsion emanating from multiple positively charged ammonium groups, and that the addition of low concentrations of anions leads to substantial refolding (*3*−*7*). The effect of the anions is attributed to their minimizing the charge repulsion by binding to the positively charged groups and hence minimizing the repulsive interactions (*4*). Similar effects, in this case involving the presence of cations, appear to result in the refolding of natively unfolded proteins that have a net negative charge at neutral pH (V. N. Uversky and A. L. Fink, unpublished observations).

Staphylococcal nuclease is a widely used model protein for folding studies due to its many favorable structural aspects: it is a small monomeric protein with representative secondary structure (three helices and five β-strands), a single tryptophan residue, and no cysteine residues. Due to its high isoelectric point, it is strongly positively charged at neutral pH. It is likely that the combination of the high net charge and the relatively low intrinsic hydrophobicity leads to staphylococcal nuclease having only marginal stability at neutral pH. In terms of the overall spectrum of protein stabilities, however, ranging from natively unfolded proteins at one extreme to very stable, tightly packed proteins at the other, SNase[1] is in the middle. In other words, there are many other proteins that have marginal or even lower stability

† This research was supported by a grant from the National Science Foundation. Small-angle X-ray scattering data were collected at beamline 4-2 at the Stanford Synchrotron Radiation Laboratory (SSRL). SSRL is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, and in part by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and by the Department of Energy, Office of Biological and Environmental Research. C.N. was supported in part by a fellowship from the Uehara Memorial Foundation.

* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064. Telephone: (831) 459-2744. Fax: (831) 459-2935. E-mail: enzyme@cats.ucsc.edu.

‡ Present address: Department of Molecular Biology (MB2), The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037.

10.1021/bi000861k CCC: $20.00
Published on Web 01/25/2001

under physiological conditions. Thus, the effects of salts on SNase are likely to be applicable to a large number of proteins, as well as to further illuminating the broad range of ionic interactions with proteins.

The refolding of acid-unfolded SNase induced by anions was previously investigated, and revealed the existence of three different partially folded intermediates, A states (*6*). These three intermediates lacked the rigid tertiary structure characteristic of native states, and differed in their degree of folding as measured by probes of secondary structure, size, stability, and globularity. The least structured conformation, A_1, is stabilized by chloride or sulfate. It is ~50% folded [based on circular dichroism and small-angle X-ray scattering (SAXS) data]. The more structured intermediate, A_2, is induced by trifluoroacetate and has ~70% native-like secondary structure. The most structured intermediate, A_3, is stabilized by trichloroacetate, has native-like secondary structure content, and is almost as compact as the native state. The stability toward urea denaturation increased with an increasing level of intermediate structure. Kratky plots, based on the SAXS data, indicated that the two more structured intermediates have significant globularity (i.e., a tightly packed core), whereas the less structured intermediate has very little globularity. It is likely that these intermediates represent the equilibrium counterparts of transient kinetic intermediates.

In the study presented here, the effects of salts on the structure, stability, and kinetics of staphylococcal nuclease at neutral pH were examined by using wild-type SNase and NCA SNase, a relatively unstable mutant (*8*−*10*). The latter contains a five-amino acid type I β-turn from concanavalin A in place of residues 27−30 of SNase (*8*). The crystal structure of this mutant superimposes very well with that of the wild type, and the hydrogen bonding of the guest turn has been preserved (*8*, *11*). NCA S28G SNase, however, is 3.6 kcal/mol less stable at 2 °C than the wild type at pH 7 (*10*).

MATERIALS AND METHODS

Wild-type SNase and a mutant (NCA S28G), in which residues 27−30 of SNase (Tyr-Lys-Gly-Gln), found in a type I′ β-turn conformation, were replaced with a type I + G_1 β-bulge structure from concanavalin A (residues 160−164, Ser-Gly-Asn-Gly-Ser), were expressed and isolated from a culture of *Escherichia coli* strain AR120 having the inducible plasmid pL9. The wild-type expression system was kindly provided by D. Shortle, that of NCA SNase by R. Fox. The expression system for K64C SNase was generously provided by W. Stites. The proteins were purified by ethanol precipitation followed by SP-Sepharose column chromatography (Pharmacia). The homogeneity of the protein samples was checked electrophoretically using the Phastsystem (Pharmacia). The labeling of the single cysteine in SNase K64C was performed by reaction with the thiol-specific probe 5-({2-[(iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid (IAEDANS, Molecular Probes) in 100 mM Tris-HCl buffer at pH 8.0 after reducing the intermolecular disulfide bond of SNase K64C with 10 mM DTT. The labeled protein was purified by using a Sephadex G-25 column (Pharmacia). The protein solution was concentrated and buffer-exchanged to water by using a Centriprep 10 cartridge (Amicon). Protein concentrations were determined using a molar extinction coefficient ϵ_{280} of 15 630 M^{-1} cm^{-1} for wild-type SNase, 14 140 M^{-1} cm^{-1} for NCA S28G SNase, and 17 080 M^{-1} cm^{-1} for IAEDANS-labeled K64C SNase.

Steady-State Fluorescence Measurements. Fluorescence measurements were made with a SPEX FluoroMax-2 fluorescence spectrometer with an excitation wavelength of 280 nm for tryptophan and 350 nm for ANS and bis-ANS. The cell path length was 1 cm, and excitation and emission bandwidths were 2.5 nm. All sample concentrations were 2 μM. Acrylamide quenching studies were performed by adding aliquots from a stock solution of the quencher into a cuvette containing protein. Fluorescence intensities were corrected for any dilution effects and for acrylamide absorbance at the excitation wavelength. Fluorescence quenching data were analyzed according to the general form of the Stern−Volmer equation, taking into account not only dynamic but also static quenching (*12*):

$$I_o/I = (1 + K_{SV}[Q]) \exp(V[Q]) \quad (1)$$

where I_o and I are the fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} is the dynamic quenching constant, V is a static quenching constant, and [Q] is the total quencher concentration.

Stopped-Flow Fluorescence Measurements. Rapid kinetic events were studied using a Biologic SFM2 stopped-flow module with Bio-Kine software, version 3, an ALX-220 lamp power supply (Molecular Kinetics), and a monochromator H10 (Jobin Yvon), with an excitation wavelength of 280 nm and a bandwidth of 4 nm. Glass filters were used to pass only light with a wavelength exceeding 325 nm for wild-type and NCA SNase, and 420 nm for IAEDANS-labeled K64C SNase. The refolding experiments from the acid-unfolded (U_A) state were carried out at 25 °C by mixing 1 volume of the unfolded protein at pH 2.0 with 9 volumes of 100 mM Tris-HCl buffer (pH 7.5) in the presence of various salts. The final protein concentration in the flow cell, except for the experiments measuring the dependency of protein concentration, was 5 μM for wild-type SNase and NCA S28G and 3 μM for IAEDANS-labeled K64C SNase.

Manual mixing experiments were also performed for the studies of slow kinetic events using a FluoroMax-2 fluorescence spectrometer with an excitation wavelength of 280 nm. The emission wavelength was 338 nm for wild-type and NCA SNase and 488 nm for IAEDANS-labeled K64C SNase. The refolding experiments from the U_A state were performed at 2 and 25 °C by mixing the solutions at the same ratios as in the stopped-flow experiments. In the cases of the refolding experiments from states A_1−A_3 to the native state, 5 volumes of the partially folded protein solution in the presence of various anions (chloride, sulfate for A_1, trifluoroacetate for A_2, and trichloroacetate for A_3) at pH 2.0 (except 3.3 volumes for 0.4 M Na_2SO_4) was mixed with 1 volume of 1.0 M Tris-HCl buffer containing the salts at pH 7.5 and 2 °C. Salt-induced folding experiments were

[1] Abbreviations: SNase, staphylococcal nuclease; WT, wild-type; IAEDANS, 5-({2-[(iodoacetyl)amino]ethyl}amino)naphthalene-1-sulfonic acid; NCA SNase, S28G mutant of staphylococcal nuclease in which a type I β-turn from concanavalin A replaced residues 27−30 of SNase; TFA, sodium trifluoroacetate; SAXS, small-angle X-ray scattering; R_g, radius of gyration.

performed at 2 °C, at both pH 7.5 and 2.0, by mixing 1 volume of the protein solution in 100 mM Tris-HCl buffer (pH 7.5) or 10 mM HC1 (pH 2.0) with 9 volumes of the same buffers in the presence of various salts. The final protein concentration in the cell, except for the experiments measuring the dependency of protein concentration, was 2 μM. The dead time for manual mixing was ~10 s.

Circular Dichroism Measurements. Circular dichroism spectra were measured with an AVIV 60DS spectropolarimeter equipped with a RTE-110 temperature controller (Neslab). For the thermal denaturation experiments, data were collected at 222 nm every 20 s at a heating rate of 0.33 °C/min. The cell path length was 1.0 mm. Manual mixing experiments were also performed at 2 °C at the same mixing ratios as the fluorescence experiments that were carried out at 222 nm. The dead time for manual mixing was ~20 s. The final protein concentration in the cell, except for the experiments measuring the dependency of protein concentration, was 10 μM.

Small-Angle X-ray Scattering. SAXS measurements were carried out at Stanford Synchrotron Radiation Laboratory (SSRL) on beamline 4-2. X-ray energy was selected at 9 keV by a pair of Mo/B4C multilayer monochromator crystals. Scattering patterns were recorded by a linear position-sensitive proportional counter, which was filled with an 80% Xe/20% CO_2 gas mixture. Scattering patterns were normalized by incident X-ray flux measured with a short-length ion chamber before the sample. The sample-to-detector length was 230 cm. Radii of gyration (R_g) were calculated according to the Guinier approximation (*13*), $\ln I(\mathbf{Q}) = \ln I(0) - R_g^2\mathbf{Q}^{2/3}$, where $\mathbf{Q}$ is the scattering vector given by the equation $\mathbf{Q} = (4\pi \sin \theta)/\lambda$ (where 2θ is the scattering angle and X is the wavelength of X-ray) and $I(0)$ is the forward scattering amplitude.

RESULTS

Effects of Salts on the Structure of NCA and Wild-Type SNase. At low pH (≤3), chloride and sulfate ions transform the acid-unfolded state of SNase into the A_1 partially folded intermediate, and trifluoroacetate causes the conversion to the A_2 state (*6*). The effects of these salts were investigated at neutral pH. We first examined the far-UV circular dichroism spectra of wild-type and NCA SNase in the presence of various concentrations of potassium chloride, sodium sulfate, and sodium trifluoroacetate at pH 7.5 and 25 °C. In the case of NCA SNase, the addition of KCl, Na_2SO_4, and TFA increased the content of α-helical structure in a concentration-dependent manner (Figure 1) (for clarity, intervening concentrations of chloride and sulfate are not shown). In the absence of salts, NCA SNase has about 60% of the α-helical structure of the wild type; however, in the presence of 3.2 M KCl or 0.4 M Na_2SO_4, the α-helix content in SNase NCA is almost the same as that of SNase WT (Figure 1). Thus, the presence of moderate to high concentrations of salts leads to a substantial increase in the secondary structure content of NCA SNase at neutral pH. There appears to be a similar, but much smaller, effect with the wild-type protein upon addition of the same concentrations of these salts (Figure 1).

To determine if there were corresponding effects on the tertiary structure of SNase, we examined both the intrinsic tryptophan fluorescence and the near-UV circular dichroism as probes. The data in Figure 2 show that the presence of added salts had a very significant effect on the Trp fluorescence of NCA SNase, and only minimal effects for the wild-type protein. The addition of KCl, Na_2SO_4, and TFA to NCA SNase increased the intensity of the intrinsic Trp fluorescence in a concentration-dependent manner (data not shown). The intensity of the Trp fluorescence of NCA SNase in the presence of 3.2 M KCl or 0.4 M Na_2SO_4 was almost same as that of WT SNase. Control experiments for measuring the effect of the salts on the fluorescence of *N*-acetyl-L-tryptophan ethyl ester revealed that the salts caused small decreases in the fluorescence emission, i.e., in the direction that was opposite of that observed with the proteins. For simplicity, we will mostly just show the effects of the highest salt concentrations that were examined, 3.2 M KCl or 0.4 M Na_2SO_4. Lower concentrations yielded either the same effects or smaller-amplitude effects. For example, with KCl the effects of increasing concentration were essentially linear to 1 M, then leveled off between 1 and 2 M, and showed a small increase between 2 and 3.2 M.

The near-UV CD spectra of wild-type and NCA SNase at pH 7.5 in the absence of added salts were significantly different; in particular, the major trough in ellipticity at 280 nm was greatly attenuated in the NCA SNase mutant (Figure 3). The addition of salts increases the aromatic ellipticity for both proteins, but had a much larger effect on the mutant; for example, with 3.2 M KCl, the negative ellipticity in the vicinity of 280 nm was more than doubled, although the signal was still significantly smaller than that for the wild-type protein. Thus, elevated salt concentrations significantly increase the amounts of both secondary and tertiary structure in NCA SNase.

As a further probe of the structure, we investigated the effect of acrylamide quenching on tryptophan fluorescence. The Stern−Volmer plots exhibited upward curvature, indicating the presence of both dynamic (or collision) and static quenching. The data were analyzed according to the general form of the Stern−Volmer equation (see Materials and Methods). There was a significantly larger effect of the acrylamide quenching in the presence of salts for NCA SNase than for the wild type (Figure 2 insets). Analysis of the data suggests that an increased level of burial of Trp occurs with increasing anion concentrations, and that this effect is much more pronounced with the mutant protein.

The effect of ANS binding to SNase was investigated to determine whether there was a significant amount of exposed hydrophobic surface in the native states. As shown in Figure 4, there was very little increase in the ANS fluorescent signal in the presence of the wild-type protein. Furthermore, the addition of salts had only a small effect. In contrast, the presence of NCA SNase led to a substantial increase in the ANS fluorescence signal, which decreased significantly when salts were added (Figure 4). These data indicate that NCA SNase has a substantial amount of solvent-exposed nonpolar surface that is significantly decreased in the presence of salts.

Small-angle X-ray scattering was used to assess the effect of the salts on the compactness of SNase. For Gdn-HCI-unfolded NCA SNase, the radius of gyration (R_g) was 38.8 Å; for the native state at pH 8.0 and 20 °C, R_g = 21.6 Å. The latter was reduced by 2−3 Å in the presence of 1.6 M KCl or 0.4 M Na_2SO_4. For WT SNase, the native state had

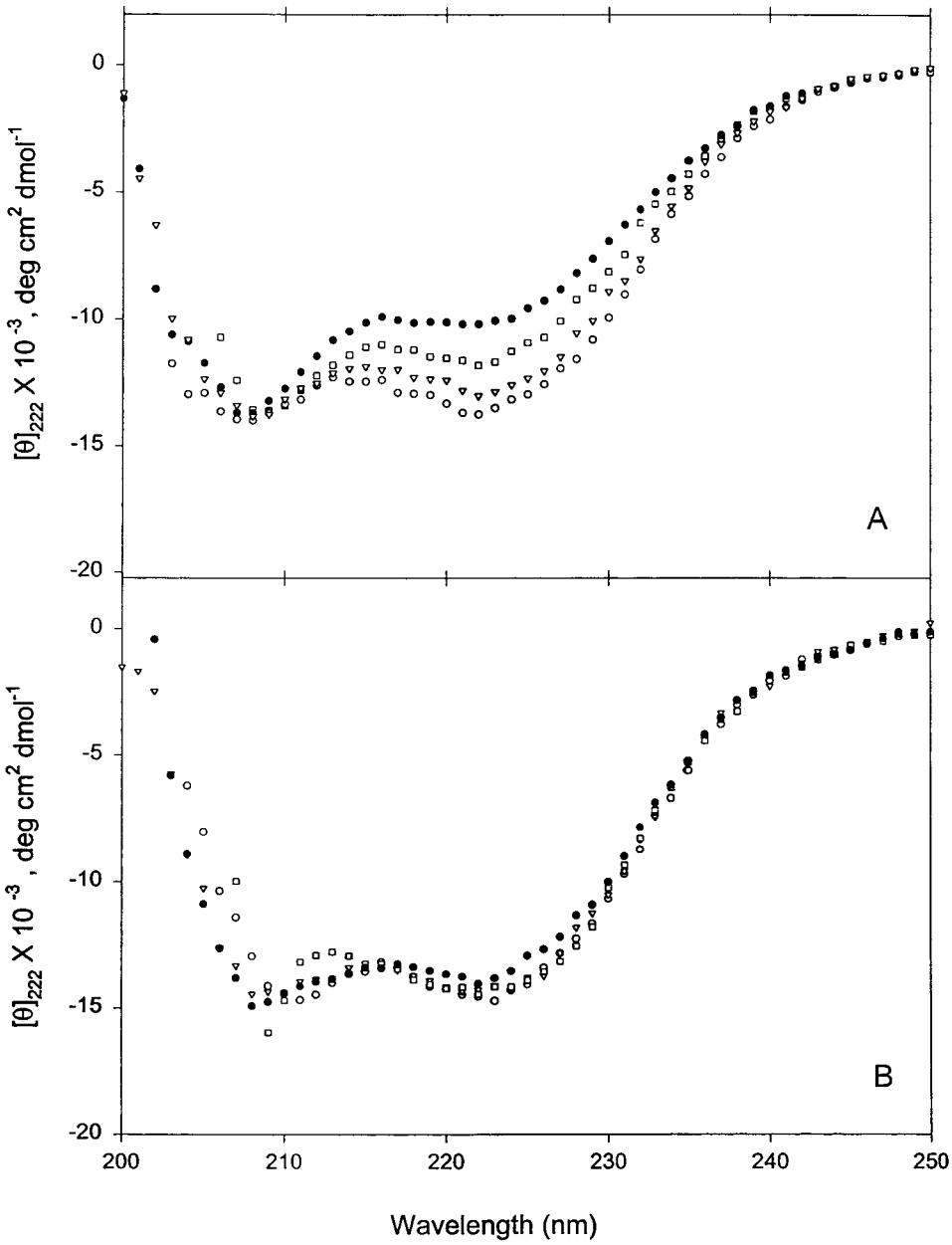


FIGURE 1: Circular dichroism spectra of NCA (A) and wild-type (B) SNase in the presence of salts at pH 7.5 and 25 °C. CD spectra were measured in the presence or absence of 3.2 M KCl (○), 0.4 M Na_2SO_4 (▽), or 0.3 M TFA (□) in 100 mM Tris-HC1 at pH 7.5 (●).

an R_g of 19.7 Å in the absence of added salt, and the presence of 1.6 M KCl or 0.4 M Na_2SO_4 reduced this to 18.3 Å. Thus, high salt concentrations cause a small contraction of wild-type SNase, and decreased the size of NCA SNase to that of the wild type.

Effects of Salts on the Stability of NCA and Wild-Type SNase. NCA SNase has previously been shown to be subject to cold denaturation at neutral pH (*10*), as well as to be rather unstable (*8*−*10*). Far-UV circular dichroism was used to monitor the thermal unfolding. In the absence of salts, NCA SNase undergoes cold denaturation at pH 7.5 and is most stable at 15 °C (Figure 5). The T_m values were 28 ± 1 °C for NCA SNase and 52 ± 1 °C for WT SNase in 100 mM Tris buffer (pH 7.5) in the absence of added salts. Both the cold denaturation and much of the decreased stability of NCA SNase relative to the WT protein were abolished in the presence of high chloride and moderate sulfate concentrations (3.2 M KCl or 0.4 M Na_2SO_4) (Figure 5). More surprisingly, these salts also had a significant stabilizing effect on the wild-type protein (Figure 5). However, the stability of NCA SNase in the presence of these salts was still substantially lower than that of the WT. The increase in T_m in the presence of the salts was 14 °C for NCA SNase and 8 °C for WT SNase. For NCA SNase, the stability is very dependent on pH, being maximal in the vicinity of pH 7.5−8.0, and decreasing as the pH is lowered (data not shown).

The stability of SNase in the presence of salts was also determined by measuring their effect on urea-induced unfolding. Figure 6 shows that the addition of 2.6 M KCl or 0.4 M Na_2SO_4 to the protein solution substantially increased the stability against urea denaturation for both NCA and WT SNase. In the absence of salts, the C_m values were 0.21 and 2.31 M, respectively (Table 1). In the presence of salts, both C_m and $\Delta G_u(H_2O)$ values increased, whereas the *m* values decreased, compared with those in the absence of salts. In particular, the free energies of stabilization for both proteins

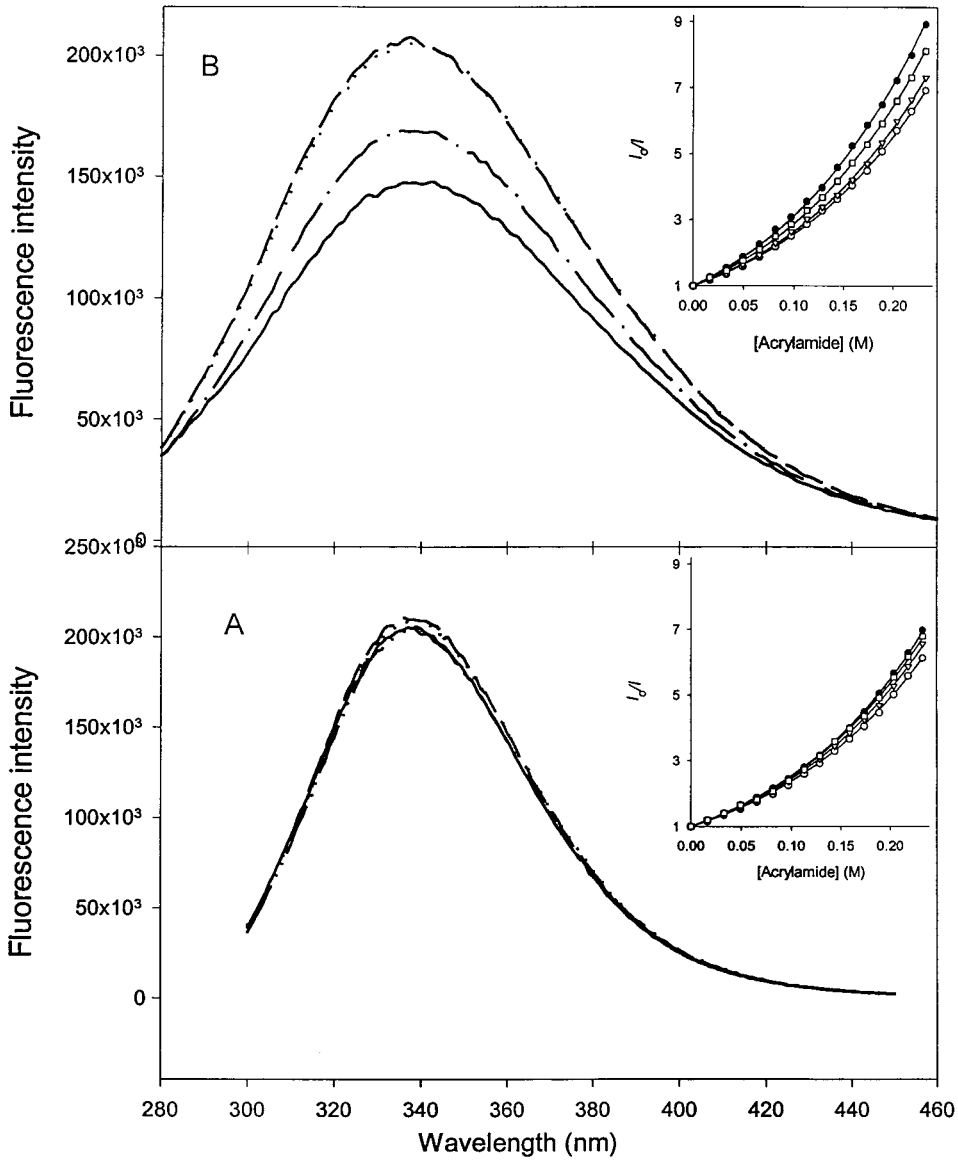


FIGURE 2: Fluorescence spectra of NCA (B) and wild-type (A) SNase in the presence of salts at pH 7.5 and 25 °C. Trp fluorescence spectra were measured in the presence or absence of 3.2 M KCl (···), 0.4 M Na_2SO_4 (− − −), or 0.3 M TFA (−·−) in 100 mM Tris-HCl (pH 7.5) (—). Insets represent Stem−Volmer plots of acrylamide quenching of SNase tryptophan fluorescence in the presence of salts at pH 7.5 and 25 °C. Measurements were performed in the presence or absence of 3.4 M KCl (○), 0.4 M Na_2SO_4 (▽), or 0.3 M TFA (□) in 100 mM Tris-HCl (pH 7.5) (●).

increased substantially in the presence of the salts: 1.7 kcal mol^{-1} for NCA SNase and 1−2.3 kcal mol^{-1} for the wild type.

Salt-Induced Refolding of NCA SNase at Low pH. The addition of anions to acid-unfolded WT SNase has been shown to lead to the formation of three different partially folded intermediates (A states), depending on the anion (*6*). Further, at a high salt or protein concentration, soluble associated states were observed in which the association led to increased secondary structure (*7*, *14*). When NCA SNase at pH 2.0 and 2 °C was titrated with chloride, sulfate, or trifluoroacetate, the concentration of anion required to complete the transition to the corresponding A state was approximately twice that required for the WT protein (Figure 7). The ellipticity values for the A states were similar to those for the WT. As with the WT protein, the addition of higher concentrations of anions led to association, as revealed by a second transition (*14*). In all cases with NCA SNase, the transition to the A state during the salt titration was less cooperative than with WT SNase. Furthermore, the transition for NCA SNase to the A_1 state (ellipticity ∼ −8000 deg cm^2 $dmol^{-1}$) was biphasic, indicating an intermediate conformation with an θ_{222} of approximately −5500 deg cm^2 $dmol^{-1}$. The transition with Na_2SO_4 (to the A_1 state) was shown to be independent of the concentration of protein from 2 to 20 μM. The titrations for the wild-type SNase were also carried out at 2 °C and were essentially the same as those at 25 °C. Thus, the transformation of the acid-unfolded state into the partially folded intermediate A states is significantly less favorable in the NCA SNase variant, compared with the wild type, suggesting that the intrinsic driving force for folding is significantly reduced.

Effects of Salts on the Kinetics of Refolding of Staphylococcal Nuclease. The kinetics of refolding of WT SNase are well-known to be complex due to the presence of multiple proline isomerizations, which results in slow kinetic phases (*15*−*20*). Tryptophan fluorescence was used to monitor the effect of salt on the refolding kinetics of WT and NCA SNase

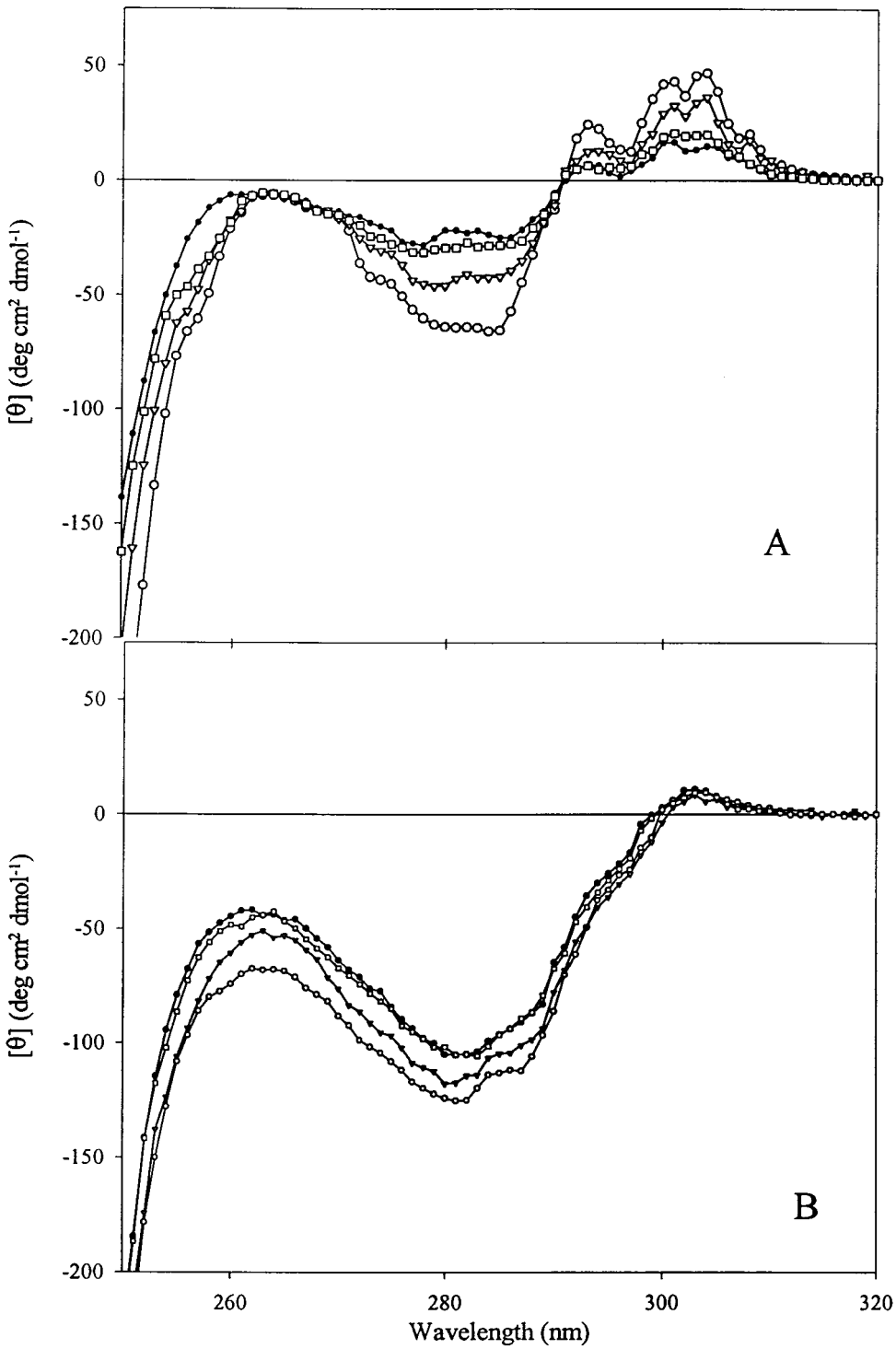


FIGURE 3: Near-UV CD spectra of NCA (A) and wild-type (B) SNase in the presence of salts at pH 7.5 and 25 °C. Spectra were measured in the presence or absence of 3.4 M KCl (○), 0.4 M Na_2SO_4 (▽), or 0.3 M TFA (□) in 100 mM Tris-HCl (pH 7.5) (●). The protein concentration was kept below 0.5 mg/mL. The cell path length was 4 mm.

at pH 7.5 and 25 °C by diluting the protein unfolded at pH 2 into pH 7.5 buffer (the observed kinetics for the WT protein were 10% faster using acid unfolding than when unfolding was accomplished with Gdn-HCI and the final denaturant concentration was 0.3 M Gdn-HCI; the amplitudes were the same).

The results of stopped-flow refolding experiments of NCA and WT SNase in the presence of anions are shown in Figure 8, and the rate constants and amplitudes are summarized in Table 2. The addition of high concentrations of salt (either 3.2 M KCl or 0.4 M Na_2SO_4) significantly affected the folding kinetics of both NCA and WT SNase. For wild-type SNase, chloride decreased k_1, whereas sulfate and trifluoroacetate increased k_1. Higher concentrations of all the anions decreased k_2 and significantly increased k_3 for the wild-type protein. The latter observation is interesting since k_3 corresponds to a slow phase due to non-native proline conformation. Thus, the 4-fold increase in k_3 with 0.4 M sulfate suggests that the proline isomerization step is tightly coupled to a folding conformation change. Only the burst phase and

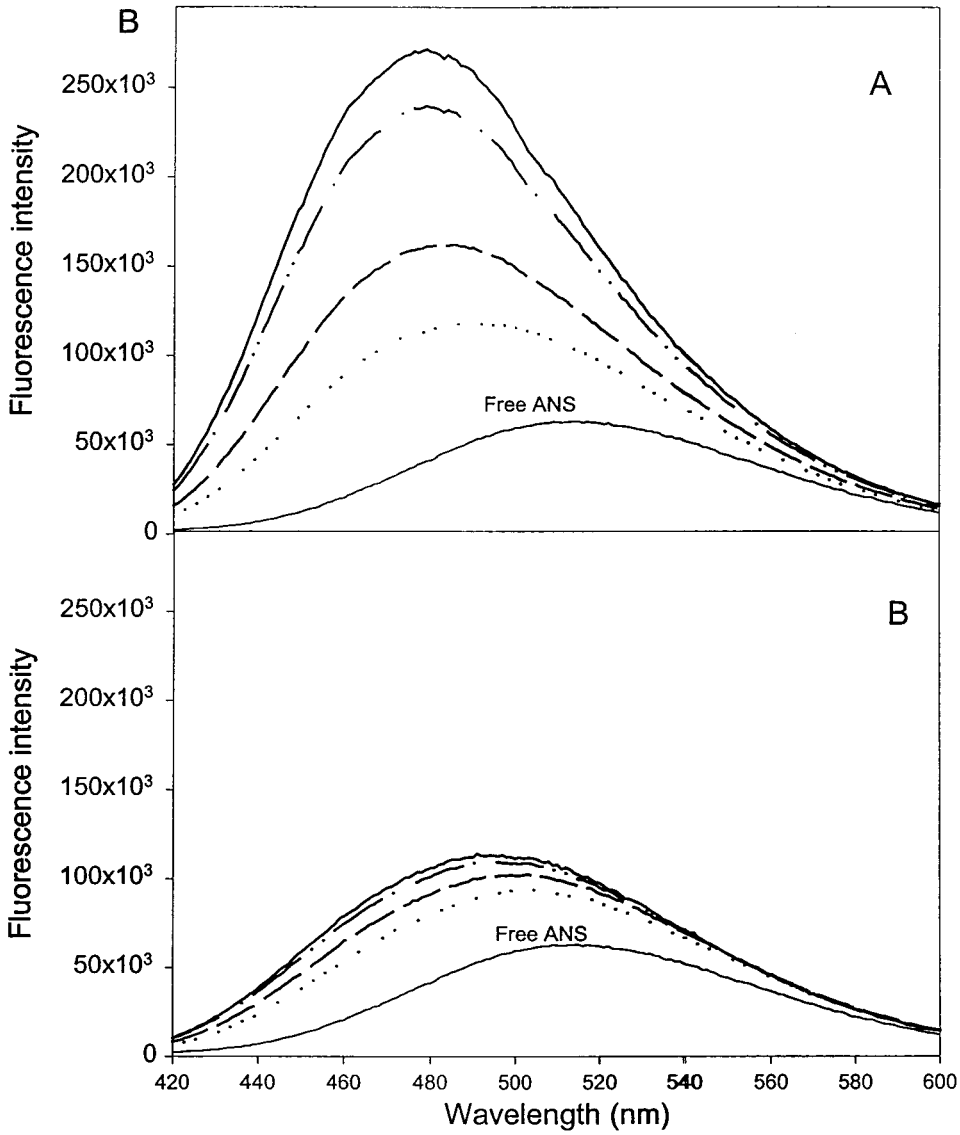


FIGURE 4: ANS fluorescence spectra in the presence of NCA (A) and wild-type (B) SNase in the presence of salts at pH 7.5 and 25 °C. Spectra were measured in the presence of 3.4 M KCl (•••), 0.4 M Na_2SO_4 (− − −), or 0.3 M TFA (−•−) in 100 mM Tris-HCl (pH 7.5) (—). The protein concentration was 0.01 mg/mL, and the ANS concentration was 10 μM. Fluorescence was excited at 350 nm.

k_1 and k_2 are observed with proline-free staphylococcal nuclease (*20*). The effects on k_3 were comparable with the NCA variant. With NCA SNase, the values of k_1 and k_2 were quite significantly increased by the presence of sulfate and TFA [as much as 8-fold for k_2 with 0.4 M sodium sulfate (Table 2)]. In general, there were relatively small effects on the amplitudes of the kinetic phases, the major exception being 3.2 M KCl in the refolding of the WT, for which the magnitude of the burst was decreased by ~30%, and sulfate, which increased the burst phase by a corresponding amount.

The very slow refolding kinetics of NCA SNase at 2 °C permitted manual mixing experiments on a long time scale. Some of the results are shown in Figure 8. In these experiments, the same concentrations of salt were used as in the stopped-flow experiments. Due to the relatively long effective dead time of mixing, only the two slowest kinetic phases were observed in these experiments. The data are summarized in Table 3. In all cases, the presence of the salts increased the rate of refolding for NCA SNase. The k_3 was also monitored by ellipticity at 222 nm; the rate constants were essentially identical to those measured by Trp fluorescence, within experimental error (Table 3). In contrast, with the wild-type protein, the general effect of the presence of the salts was to decrease the folding rate, although the responses were more varied (Figure 8 and Table 3). Again, the major effect was observed with high concentrations of chloride, which caused substantial (5-fold) decreases in k_2.

In addition to examining the effects of salts on the refolding of acid-unfolded NCA SNase, we also examined the effects of starting with one of the three previously identified A states (*6*), and jumping to the native state. No significant differences in the rates and amplitudes of refolding were observed whether we started with the acid-unfolded state and jumping to pH 7.5 with the salt present in the native buffer or with one of the A states with the salt already present and jumping to pH 7.5 (data not shown). This indicates that the transition from the U_A state to the A states is fast for NCA SNase. The kinetics of the slowest phase were also monitored by circular dichroism, and the rates were effectively the same as those determined by Trp fluorescence.

To ensure that transient association was not responsible for any of the observed kinetic phases, the experiments were

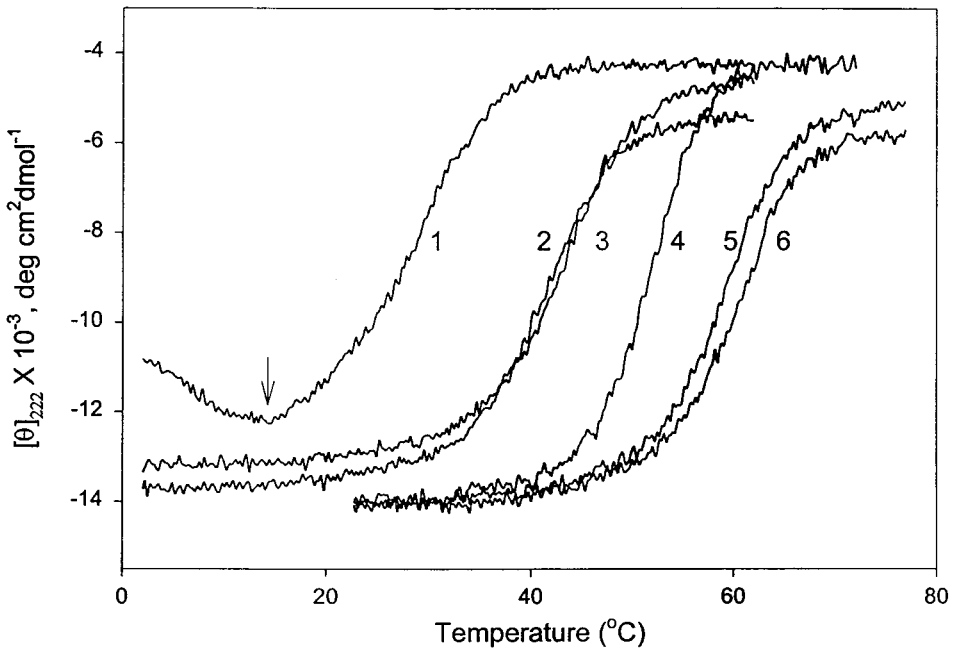


FIGURE 5: Effect of anions on the thermal denaturation of NCA (traces 1−3) and WT (traces 4−6) SNase at pH 7.5. Denaturation was monitored by the change in ellipticity in the far-UV CD at 222 nm in the absence (traces 1 and 4) or presence of 3.2 M KCl (traces 2 and 6) or 0.4 M Na_2SO_4 (traces 3 and 5) in 100 mM Tris-HC1 (pH 7.5). The arrow shows the temperature of maximum stability for NCA SNase; at lower temperatures, the protein unfolds due to cold denaturation.

repeated at three different protein concentrations, 2−10 μM, for both NCA and WT SNase. The rates and amplitudes were independent of the protein concentration that was used, within experimental error, indicating that the observed changes in folding kinetics in the presence of salt are not due to intermolecular interactions such as transient aggregation.

Fluorescence resonance energy transfer between suitably positioned donor and acceptor groups can be used to monitor the rate at which a protein collapses during refolding. To determine the effect of a high salt concentration on the initial rate of collapse, we used a modified staphylococcal nuclease in which Lys46 was converted to Cys and then labeled with the fluorescence acceptor IAEDANS. By exciting the single Trp residue and monitoring the IAEDANS emission, we were able to measure the rate of change in the distance between these two residues. In the absence of a high salt concentration, a burst, complete within 1 ms, followed by four transient phases is observed (Figure 9). However, and very surprisingly, in the presence of 3.2 M KCl a major overshoot in signal is observed in the burst reaction, which is then followed by a *decrease* in fluorescence emission (Figure 9 and Table 4). The magnitude of the burst in the presence of the salt is more than twice the value in its absence (Table 4). Varying the protein concentration over the range of 0.33−3.0 μM had no effect on the kinetics or amplitudes of the observed reaction, indicating that transient association of the protein is not responsible for the increased burst size. Two possible explanations are that the high concentration of salt leads to SNase adopting a more compact collapsed structure than in the native state, or that the high salt leads to an alternate conformation in the collapsed state in which Trp140 is closer to residue 64.

As seen in Figure 2, the presence of a high salt concentration leads to a significant increase in the fluorescence emission of Trp140 in NCA SNase. The kinetics of the transition between these two "states" were monitored using Trp fluorescence and salt concentration jumps with 3.2 M KCl or 0.4 M Na_2SO_4 at 2 °C and pH 7.5 (Table 5). The kinetics were very slow; under both conditions, a biphasic increase in Trp emission was observed, with identical kinetics and amplitudes. The magnitude of the amplitude is of note, namely, a total of 75% for both transient phases. The rate constants are of the same magnitude as those of the two slowest for refolding from the acid-unfolded state to the native state in the presence of the salts and which are believed to reflect proline isomerization.

Monitoring the rates of anion-induced refolding at pH 2, i.e., from the U_A state to an A state, revealed that at lower salt concentrations (e.g., <2.4 M KCl, 0.4 M Na_2SO_4, or 0.9 M TFA) the rate of refolding was fast ($\leq$1 s). However, at high salt concentrations, e.g., 3.2 M KCl or 1.2 M TFA, the kinetics were much slower (Figure 10). This was true for both NCA and WT SNase (data not shown). These observations suggest that the effects of the ions are qualitatively different at lower and higher concentrations.

DISCUSSION

Although the major effects of salts (especially the anions) on protein structure at low pH are reasonably well understood (*3*, *4*, *21*−*26*), in general the effects at neutral pH are much more subtle, and hence more difficult to elucidate (*27*). At low pH, the major effect is due to binding of the anions to the positively charged protonated amine groups, leading to decreased Coulombic repulsion (*28*). At neutral pH, for a folded globular protein, such as staphylococcal nuclease, the effects are likely to be more complex, since there is probably no one aspect that dominates (*29*). For example, the consequences could range from effects on water structure to binding of ions to specific sites on the protein, resulting in local attractive or repulsive interactions. In addition, the effects may also vary depending on the salt concentration. There have been many studies on the role of electrostatics

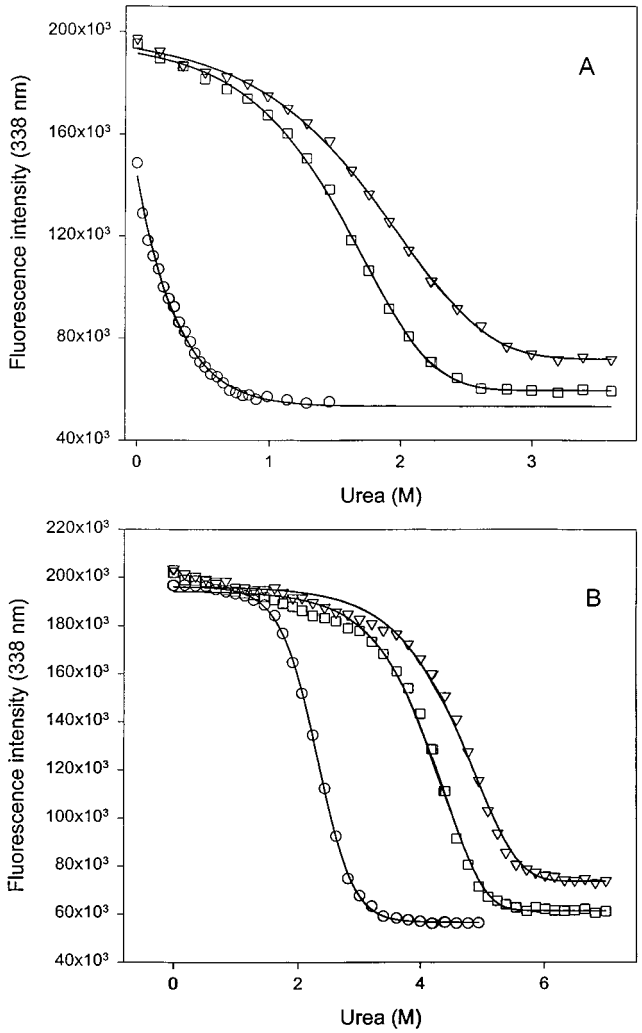


FIGURE 6: Urea titrations of NCA (A) and wild-type (B) SNase in the presence of salts. Urea denaturation was monitored at 25 °C by fluorescence intensity at 338 nm in the presence or absence (○) of 2.6 M KCl (▽) or 0.4 M Na_2SO_4 (□) in 100 mM Tris-HCl (pH 7.5). The solid lines are sigmoidal fits as guides.

Table 1: Urea Titration of NCA and WT SNase in the Presence of Salts at pH 7.5 and 25 °C[a]

additive	$\Delta G_u(H_2O)$ (kcal/mol)	m (kcal mol^{-1} M^{-1})	C_m (M)
NCA			
none	0.70	3.4	0.2
2.6 M KCl	2.3	1.3	1.7
0.4 M Na_2SO_4	2.4	1.6	1.5
WT			
none	4.6	2.0	2.3
2.6 M KCl	5.6	1.2	4.5
0.4 M Na_2SO_4	6.9	1.7	4.2

[a] The errors were all within ±12%.

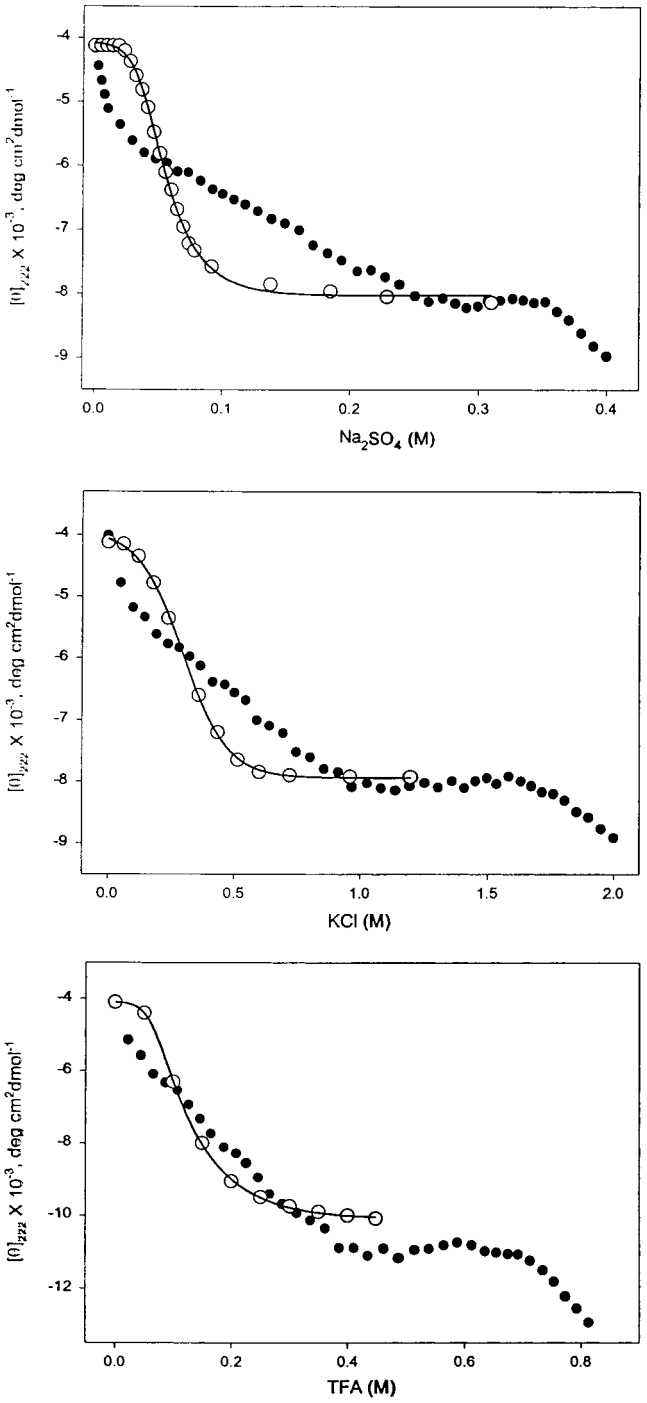


FIGURE 7: Salt-induced transitions from the acid-unfolded state of NCA SNase to a partially folded intermediate A state (●). The titrations were monitored by CD at 222 nm at pH 2.0 and 2 °C. Titration with KCl and Na_2SO_4 leads to the A_1 state, with a θ_{222} of -8×10^3 cm^2 dmol^{-1}, and titration with TFA leads to the A_2 state, with a θ_{222} of -11×10^3 cm^2 dmol^{-1} (*6*). The beginning of a second transition is seen at a high salt concentration; this corresponds to association-induced secondary structure (*7*). The open circles are the data for the wild-type protein (data from ref *6*).

in the stability of proteins, both experimental and theoretical, going back to the pioneering work of Linderstrom-Lang and Kirkwood. In contrast, the number of investigations specifically on the effects of salts on protein stability and folding kinetics is much more limited. Ribonuclease T1, a very acidic protein, of relatively low stability in the absence of salt, was shown to be significantly stabilized by the presence of salts (*30*). The effects were predominantly due to relatively weak specific ion binding. No such effects were observed with ribonuclease A or lysozyme (which is more stable). Carboxylate salts have been shown to increase the thermal stability of several proteins (*31*). An investigation of the effect of replacing charged side chains of SNase by Ala or Gly concluded that ionizable amino acids do not significantly contribute to stability through electrostatic interactions (*32*). There are several reports that increasing ionic strength eliminates shifts in calculated and measured pK_a values, probably

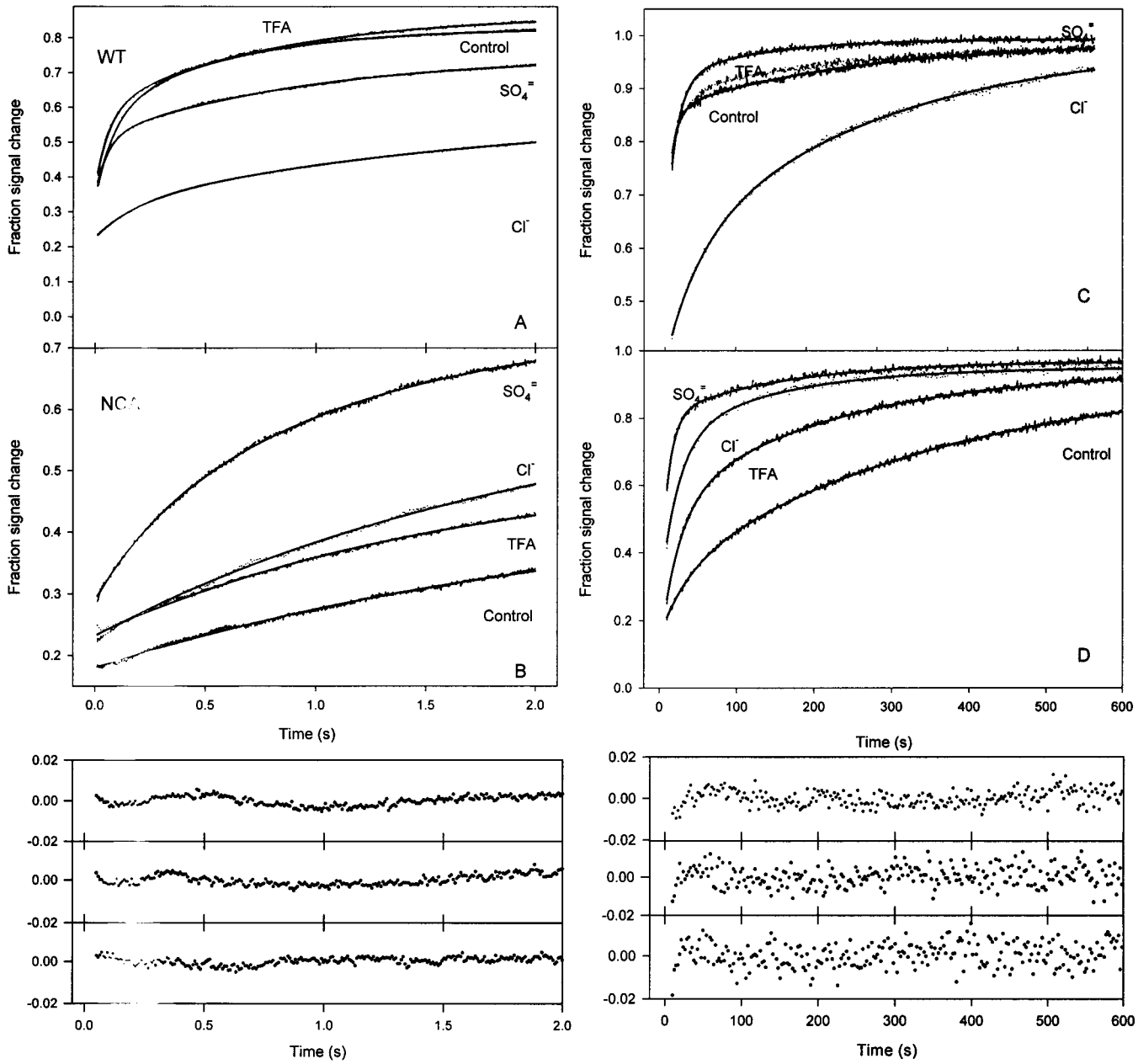


FIGURE 8: Effect of anions on the kinetics of refolding of wild-type (A and C) and NCA (B and D) SNase. The ordinate corresponds to the observed signal as a fraction of that expected, based on the signals for the unfolded and native states. (A and B) Stopped-flow traces of the first 2 s of reaction monitored by changes in Trp fluorescence intensity. Refolding was initiated by a 10-fold dilution of protein unfolded at pH 2.0 into 100 mM Tris-HC1 buffer (pH 7.5) in the presence or absence of 3.2 M KCl, 0.4 M Na_2SO_4, or 0.3 M TFA at 25 °C. (C and D) Refolding at 2 °C. The theoretical curves for the data in Tables 1 and 2 are shown as solid lines under the data. Representative plots of the residuals are shown below. The ordinate scale shows the fraction folded, based on the observed signal compared to that for the unfolded and folded states for the particular conditions. Refolding was initiated by a 10-fold dilution of protein unfolded at pH 2.0 into 100 mM Tris-HCl buffer (pH 7.5) in the presence or absence of 3.2 M KCl, 0.4 M Na_2SO_4, or 0.3 M TFA. The refolding was monitored by changes in fluorescence intensity at 338 nm.

due to effects in the denatured state (*33*, *34*). Favorable interactions between charged surface residues have been shown to make major contributions to protein stability (*35*). Such favorable electrostatic interactions are expected to be weakened by increasing ionic strength, through Debye−Hückel screening. The fact that surface-charged residues can make a significant negative contribution to overall stability was nicely demonstrated using theoretical and mutagenesis approaches (*36*).

Our results show that for both wild-type and NCA SNase the presence of moderate to high concentrations of salt at neutral pH significantly increases the thermodynamic stability of the proteins. In the case of the relatively unstable NCA mutant, this is also accompanied by the induction of significant secondary and tertiary structure, and increased compactness of the molecule. Similarly, in refolding experiments the higher salt concentrations have significant effects on the rates of some of the folding steps. These effects are more marked at lower pH, where the net charge on the molecule will be more positive (the pI of SNase is >9). Given the net positive charge on the protein, it is most likely that it is the anions that are the key components in the action of the salts. One indication that the effects involve a significant direct interaction with the protein is that both

Table 2: Stopped-Flow Fluorescence Kinetic Data (observed rate constants and amplitudes) for the Refolding of NCA and WT SNase in the Absence and Presence of Salts at pH 7.5 and 25 °C[a]

additive	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	burst	Φ_1 (%)	Φ_2 (%)	Φ_3 (%)
NCA							
none	0.75	0.11	0.028	16	6	25	53
0.8 M KCl	1.21	0.17	0.036	18	15	28	39
3.2 M KCl	0.54	0.12	**0.052**	17	13	30	33
0.1 M Na_2SO_4	0.81	0.13	0.031	18	13	25	44
0.4 M Na_2SO_4	**3.66**	**0.93**	**0.200**	22	7	31	25
0.3 M TFA	**2.14**	**0.23**	0.035	20	7	27	46
WT							
none	11.47	1.70	0.016	31	26	28	15
0.8 M KCl	10.11	1.40	**0.041**	33	24	30	13
3.2 M KCl	**5.31**	**0.56**	**0.029**	22	8	25	45
0.1 M Na_2SO_4	17.64	1.68	0.027	39	24	26	11
0.4 M Na_2SO_4	17.58	**1.04**	**0.062**	38	21	29	12
0.3 M TFA	15.59.	1.29	**0.039**	33	24	30	13

[a] Rate constants that are significantly different from the controls are in bold. The amplitudes (Φ) are shown as % folded. Experimental errors were less than or equal to ±15%.

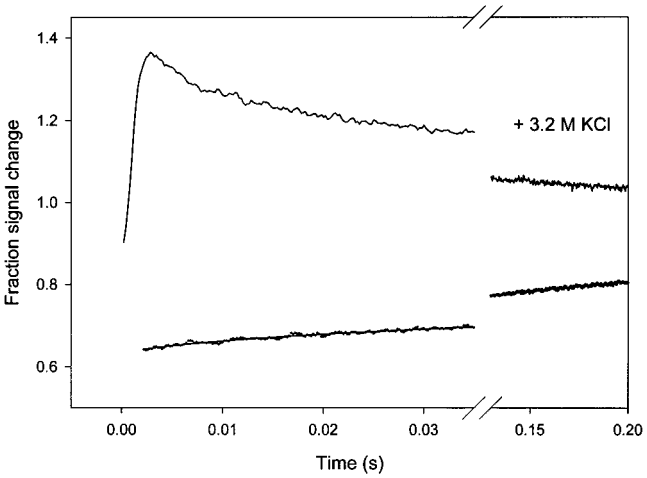


FIGURE 9: Effect of 3.2 M KCl on the early kinetics of refolding of SNase, monitored by fluorescence energy transfer at 25 °C. The first 200 ms of the refolding reaction is shown, with excitation of Trp140 at 280 nm and emission from Cys64 labeled with IAEDANS at ≥420 nm. The ordinate corresponds to the observed signal as a fraction of that expected, based on the signals for the unfolded and native states. Refolding was initiated by a 10-fold dilution of the unfolded protein (pH 2.0) into 100 mM Tris-HCl buffer (pH 7.5) in the presence (top trace) or absence (bottom trace) of 3.2 M KCl.

sulfate and chloride have similar effects, whereas sulfate is a known stabilizing (kosmotrope) agent for proteins; however, chloride is relatively neutral in this regard (*37*). Thus, it is more likely that the effects are due to specific interactions of the ions with the protein, rather than effects on water structure. It is important to note that the crystallographic structure of NCA SNase is essentially identical to that of the wild-type protein except in the immediate vicinity of the changed residues (*8*, *11*). There are two main differences between the wild-type and NCA SNase, namely, the replacement of a positively charged lysine residue with a neutral serine and the insertion of an extra residue, both of which likely contribute to the decreased stability of NCA SNase, along with the local tertiary structure disruptions. The original reason for the mutation was to exchange one β-turn with another, in which both ends of the turns were in corresponding spatial positions. The loss of the lysine disrupts a surface salt bridge with Glu10, and may thus contribute to the decreased stability relative to the wild type. Examination of the structure of SNase indicates that the loss of this lysine residue is likely to lead to increased electrostatic repulsion among other surface carboxylates in the vicinity. It is thus possible that one factor contributing to the enhanced effects of salts on the stability of NCA SNase is due to the abolition of this unfavorable electrostatic interaction by the added cation.

The fact that the folding kinetics and stability of NCA and WT SNase were unaffected when different concentrations of protein solutions were used indicates that the effects of anions shown in this study do not reflect intermolecular interactions of protein molecules such as transient association or protein aggregation.

Salt-Induced Structure in NCA Staphylococcal Nuclease. NCA SNase is significantly less stable than the wild-type protein, as manifested by significant cold denaturation at

Table 3: Manual Mixing Fluorescence Kinetic Data (observed rate constants and amplitudes) for the Refolding of NCA and WT SNase in the Absence and Presence of Salts at pH 7.5 and 2 °C[a]

additive	k_3 (s^{-1})	k_4 (s^{-1})	burst	Φ_3 (%)	Φ_4 (%)	k_4 from CD (s^{-1})
NCA						
none	0.0278	0.0029	15	19	66	0.0023
0.9 M KCl	0.0352	0.0039	13	42	45	0.0024
3.2 M KCl	**0.0427**	**0.0069**	26	51	23	**0.0070**
0.2 M Na_2SO_4	0.0362	0.0037	12	44	44	0.0025
0.4 M Na_2SO_4	**0.0920**	**0.0064**	25	59	16	**0.0064**
0.3 M TFA	**0.0403**	**0.0047**	10	46	44	**0.0036**
50 mM TCA	0.0383	0.0036	17	31	52	
WT						
none	0.0932	0.0039	65	22	13	
0.9 M KCl	0.1083	**0.0064**	68	21	11	
3.2 M KCl	**0.0206**	0.0034	39	20	41	
0.2 M Na_2SO_4	0.0788	0.0042	68	22	10	
0.4 M Na_2SO_4	0.0662	**0.0079**	60	32	8	
0.3 M TFA	0.0580	0.0042	68	22	10	

[a] Rate constants that are significantly different from the controls are in bold. Experimental errors were less than or equal to ±15%.

Table 4: Kinetic Data (observed rate constants and amplitudes) for the Refolding of IAEDANS-Labeled K64C SNase Monitored by Fluorescence Energy Transfer[a]

additive	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})	burst	Φ_1 (%)	Φ_2 (%)	Φ_3 (%)	Φ_4 (%)
none	108.66	17.73	2.43	0.019	63	2	6	24	5
3.2 M KCl	147.72	15.43		0.032	140	−13	−19		−8

[a] Refolding was initiated by a 10-fold dilution of the unfolded protein (pH 2.0) into 100 mM Tris-HCl buffer (pH 7.5) using both stopped-flow and manual mixing experiments to cover the complete time range for folding. Trp was excited at 280 nm, and IAEDANS emission was measured at ≥420 nm. The amplitudes (Φ) are shown as a fraction of the total signal for folding, i.e., fraction folded. Note that the observed rate constants in Tables 2 and 3, k_1-k_3, correspond to the observed rate constants k_2-k_4 in this table. Experimental errors were ±10%.

Table 5: Rate Constants and Amplitudes for the Transitions Induced by the Addition of Salt to NCA SNase at pH 7.5 and 2 °C Monitored by Trp Fluorescence[a]

additive	k_3 (s^{-1})	k_4 (s^{-1})	burst	Φ_3 (%)	Φ_4 (%)
3.2 M KCl	0.0688	0.0047	27	46	27
0.4 M Na_2SO_4	0.0726	0.0049	23	48	29

[a] Experimental errors were ±10%.

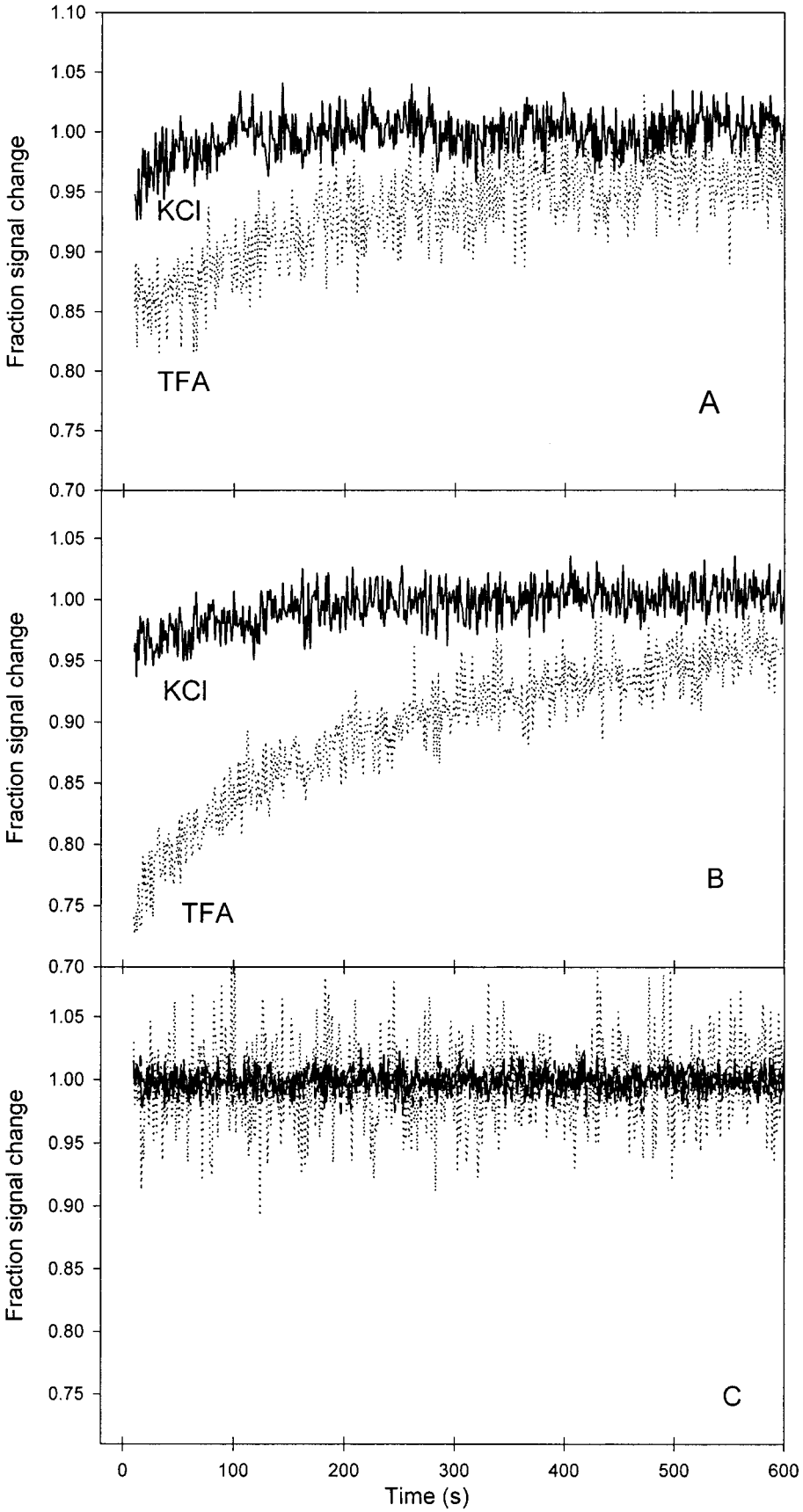


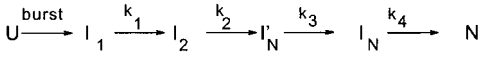
FIGURE 10: Kinetics of anion-induced folding from the acid-unfolded state at 2 °C. Panels A (wild-type SNase) and B (NCA SNase) show the effect of 3.2 M KCl and 1.2 M TFA. Panel C (NCA SNase) shows the effects of 2.4 M KCl, 0.4 M Na_2SO_4, and 0.9 M TFA. The ordinate corresponds to the observed signal as a fraction of that expected, based on the signals for the unfolded and native states. The data for the wild type (not shown) were very similar to those for the NCA variant. Refolding was initiated by a 10-fold dilution of protein in 10 mM HCl (pH 2.0) into the same solution in the presence of 2.4 or 3.2 M KCl, 0.4 M Na_2SO_4, or 0.9 or 1.2 M TFA. The reaction was monitored by fluorescence emission at 338 nm.

neutral pH and a much reduced free energy of stabilization and C_m. The cold denaturation means that the amounts of secondary and tertiary structure in the mutant protein are smaller than in the wild type, at 25 °C. The presence of a moderate to high concentration of salts induced structural changes in NCA SNase that led to circular dichroism and fluorescence spectra similar to those of the wild-type protein. In other words, the presence of the salt shifted the equilibrium from the cold-denatured form to the native state. This is also confirmed by the small-angle X-ray scattering data, and loss of exposed hydrophobic sites for ANS binding. However, the observed spectral changes are smaller than anticipated for the ellipticity at 222 nm and for Trp fluorescence emission, and larger than expected for the changes in the near-UV CD signal and ANS binding. At 25 °C and pH 7, where most of these studies were performed, NCA SNase is only 60% native (Figure 5), and thus, an increase of 167% in signal is anticipated. However, the increase in the near-UV CD signal was 275%, and the decrease in the ANS fluorescence signal was 220%. Thus, it appears that more than simple shifting of the equilibrium between cold-denatured and native SNase is occurring.

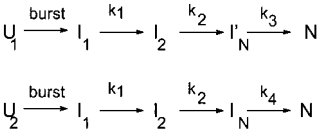
The question arises as to the underlying mechanism and physical basis for this stabilizing effect. The changes are reminiscent of the effects of added anions on acid-unfolded staphylococcal nuclease (*6*, *38*), which have been attributed to binding of the anions to the positively charged ammonium groups of the basic amino acids, resulting in a decrease in the level of Coulombic repulsion (*4*). Given the high pI of SNase, the molecule will have a substantial positive charge at neutral pH (+11). Since the balance of attractive and repulsive forces must be very close in the NCA SNase variant, since the folded state is just marginally stable, it is not unreasonable to assume that a small decrease in the level of repulsive interactions (resulting from proximal positive side chain charges) will lead to increased levels of folding and structure. In fact, the relatively high net charge and low hydrophobicity of SNase suggests that it should be a rather weakly folded protein (*39*). Although the effects are much more marked with NCA SNase, there appear to be similar, albeit attenuated, effects of salts on the structure of the wild-type protein. For both proteins, the effects of salts are most marked on the near-UV circular dichroism spectra, confirming that the major effects are on the tertiary structure. This suggests that even in wild-type SNase there are significant electrostatic repulsive interactions, which are overcome by binding of anions.

Salt-Induced Stability of Staphylococcal Nuclease. The increased stability in the presence of the salts in both wild-type and NCA SNase is seen in the greater resistance to urea and thermal denaturation. For example, in the presence of 0.4 M sodium sulfate, the free energy of stabilization of wild-type SNase is increased by more than 2 kcal/mol. The simplest interpretation is that even at neutral pH electrostatic repulsion destabilizes the structures, and the effect is much more pronounced with NCA SNase. Thus, although a high salt concentration had relatively little effect on the secondary and tertiary structure in WT SNase, both proteins were substantially stabilized by the addition of ions against urea and heat. This indicates that localized electrostatic repulsion occurs even in the wild-type molecule, and that the binding of the counterions leads to a reduced level of repulsion. This observation is one of very few examples demonstrating that the net stability of a folded protein may be determined by a combination of both attractive and repulsive electrostatic effects.

Scheme 1



Scheme 2



Previously, it was recognized that one of the key factors in the cold denaturation of NCA SNase at neutral pH was its relative instability (*10*). The loss of sensitivity to cold denaturation of NCA SNase in the presence of high concentrations of salt demonstrates that the ions dramatically increase the stability. Again, this is most likely a reflection of the decreased level of electrostatic repulsion due to binding of anions to the positively charged basic residues. The fact that at low pH significantly higher concentrations of anions (2−4-fold greater than for the wild type) are required to bring about the transition from the acid-unfolded state to the A state in the NCA SNase variant is a reflection of the weaker intrinsic folding forces in this molecule, compared with the wild type.

Salt-Induced Effects on the Kinetics of Staphylococcal Nuclease Folding. Previous investigations of the refolding kinetics of staphylococcal nuclease have demonstrated that the multiphasic kinetics are in part due to cis−trans prolyl isomerization. Two general classes of kinetic schemes have been proposed: those involving parallel refolding pathways from different populations of unfolded molecules (*19*, *40*) and the presence of partially folded intermediates on the folding pathway, probably with a parallel pathway as well (*20*, *41*). Given that the proline-free variant shows multiple transients with rate constants in the millisecond range or faster (*20*), proline isomerization cannot be responsible for these transients. Consequently, the slowest two observed transients can be assigned to proline isomerization, and the faster ones to transient intermediates. The simplest scheme is one in which there are two transient partially folded intermediates leading to two very slow steps due to proline isomerization, as shown in Scheme 1, where I_N and I'_N differ from N due to proline isomerization. The rate constants correspond to those in Tables 2 and 3.

Alternatively, there could be two parallel pathways, each with two transient partially folded intermediates, but starting with different states of proline isomerization in the unfolded state, and leading to two different slow steps for the proline isomerization (Scheme 2).

The presence of added salt had significant effects on the refolding kinetics for both proteins. In the case of NCA SNase, all of the salts that were tested led to a substantial increase in folding kinetics, especially sulfate (the only exception being 3.2 M KCl which resulted in a decrease in k_1). In the case of wild-type SNase, high concentrations of KCl had a strongly retarding effect on the rate of folding, whereas sulfate had a small accelerating effect on some of the folding transients.

The simplest explanation for salt-enhanced refolding rates is that the presence of the ions, especially the anions, screens the potential repulsive interaction between positively charged residues, thus effectively stabilizing transient intermediates, and allowing a more compact initially collapsed state. It is likely that this repulsion is weaker and occurs in a more localized region of the protein at neutral pH, compared with the situation at low pH. In the case of high concentrations of KCl (>2 M) where the rate of refolding of the WT is significantly reduced, it is clear that an additional factor is at work.

Since the slowest kinetic phases (k_3 and k_4) are associated with proline isomerization (*17*, *19*, *20*), it is interesting to note that these rates are significantly increased by the presence of salt in both WT and NCA SNase. Since previous investigations have shown that proline isomerization is acid-catalyzed, but not affected by ions at neutral pH, this observation means that these proline isomerization steps must be tightly coupled to conformational steps in the folding pathway. Further evidence that the k_3 and k_4 steps correspond to proline isomerization is found in the observation that the k_1 and k_2 values of NCA SNase are about 10 times smaller than those of the WT, whereas the k_3 and k_4 values are almost the same. Since the rate enhancements on k_3 and k_4 are different, this is most consistent with two parallel pathways (Scheme 2).

The two faster transients observed, k_1 and k_2, generally yielded effects that were the opposite of those of the slower transients, in that high concentrations of chloride decreased the rates whereas higher concentrations of the other anions increased k_1 and decreased k_2. This indicates that the net electrostatic interactions (positive or negative) are different in I_1 and I_2. The burst amplitude, which was twice as large in the wild type, was little affected by the presence of the salts. The general trend was for a small increase in the magnitude of the burst in the presence of the salts. This may reflect either a faster initial collapse or a more compact collapsed state in the presence of the salts. In general, the amplitudes of the transients are not significantly affected by the presence of the salts, indicating that the effects of the ions are mainly on the kinetics and not conformation on the transient species.

If the A state intermediates of SNase are reasonable models of the transient intermediates present during folding, as seems to be the case (*41*), then comparison of the kinetics when starting with unfolded protein or A state in refolding in the presence of salt should provide information regarding the relative position of the A states in the folding process. Since no significant difference in refolding rates for NCA SNase was observed when starting from either the U_A state or the A_1 or A_2 state in refolding, the rate-limiting step in folding must be late in the pathway, between the A_2 and native states. That is, the I_2 species in Schemes 1 and 2 probably correspond to the A_2 intermediate. This is also consistent with the observation that folding from the U_A to A_1 or A_2 state was much faster than that from the A_1 or A_2 state to the native state.

Stopped-flow experiments with IAEDANS-labeled K64C SNase in the presence of 3.2 M KCl showed an unusual burst phase with a signal that was 40% greater than that of the native conformation, followed by a gradual decrease in fluorescence intensity. This observation means that the high concentration of salt revealed a transient intermediate during the folding process, in which the distance between Trp40 and AEDANS at position 64 is much closer than in the native structure. Since the enhanced fluorescence from this intermediate is not observed in the absence of the high salt concentration, there are three likely explanations: (1) the presence of high salt concentration affects the folding kinetics such that a transient intermediate in which residues 64 and 140 are closer than in the native conformation which is normally not observed becomes apparent, (2) the added salt leads to a new intermediate not present in folding in the absence of the salt in which the residues of the fluorescence energy transfer pair are closer than in the native conformation, or (3) the presence of the salt leads to a more compact intermediate in which the residues of the fluorescence energy transfer pair were closer together. Since the kinetic transients in the presence and absence of salt are of comparable magnitude, it is likely that the folding pathway is the same, indicating that the enhanced energy transfer in the initial collapsed state in the presence of high salt is due to a more compact conformation, rather than to an alternative pathway. Interestingly, an increased salt concentration has been observed to stabilize a normally meta-stable high-energy intermediate in the folding of lysozyme (*42*).

Notably, the enhancement of structure observed in NCA SNase at neutral pH upon addition of salt was accompanied by very slow kinetics at 2 °C, consisting of a fast phase followed by two slow phases with rates similar to those observed for the slowest rates in refolding at pH 7.5 and 2 °C, k_3 and k_4 (Table 3). Such kinetics are more consistent with the idea that NCA SNase at neutral pH consists of a mixture of unfolded and relatively folded molecules, rather than a single population of a relatively folded protein. The folding kinetics at pH 2 for the acid-unfolded state to the A states were quite fast except in the presence of 1.2 M TFA or 3.2 M KCl. Thus, it takes longer to form the more ordered tertiary structure in the native state at pH *7.5* (induced by the addition of salt) compared to formation of the less ordered A states at pH 2.0.

Underlying Basis for the Effects of Salts on the Stability and Structure of Staphylococcal Nuclease. By most criteria, wild-type staphylococcal nuclease is a representative globular protein, with typical tight packing and tertiary structure. The only indication that it might not be a representative globular protein is that the free energy of stabilization is marginal (4.6 kcal/mol under the experimental conditions used here). On the basis of our hypothesis that the reason for the enhanced stability in the presence of added salts is due to a decreased level of intramolecular electrostatic repulsion, we predict that other marginally stable globular proteins will also be significantly stabilized by salts under conditions where they have a substantial net charge. In fact, this is observed with natively unfolded proteins such as α-synuclein (V. N. Uversky, J. Lie, and A. L. Fink, in press) and prothymosin α (*43*).

The effectiveness of different ions (in the present case anions, but in general cations or anions) in decreasing the level of intramolecular electrostatic repulsion and leading to enhanced protein structure and stability will vary, depending on the properties of the hydrated ions. On the basis of studies with anion binding to acid-unfolded proteins, the main factor appears to be the effective charge density of the ion.

However, especially under conditions where the protein is in a native-like conformation at the start, the possibility of specific ion binding sites could lead to different specificities. Precedence for specific anion (chloride) binding to native states, leading to increased stability, has been previously noted for ubiquitin at pH 2 (where the protein is native) (*44*) and RNase T1 (*30*).

The interactions between salts and proteins are known to involve both electrostatic and hydrophobic effects. The presence of ions in water, at least at higher ion concentrations, leads to effects on the structure of water that are equivalent to those at very high pressure (*45*). It is this solvent effect that is, at least in part, responsible for the effect of salts on the hydrophobic effect (*46*). It has been suggested that the effects of salts on electrostatic interactions largely affect α-helices, whereas salt effects on hydrophobic interactions mostly affect β-sheets (*47*). If this is correct, then the effects of salts on SNase emanate from electrostatic effects, as proposed, since they result in an increase in helicity. At low to moderate concentrations of sodium and potassium salts, the expected effects on interaction with proteins will also involve preferential hydration, i.e., preferential exclusion of the salt from the vicinity of the surface of the protein (*48*−*50*).

Kosmotropic (stabilizing) salts (*37*) such as sulfate may affect protein structure either through direct interaction with the protein or through effects on water structure, depending mostly on the salt concentration. For low concentrations of salts, both specific and nonspecific electrostatic interactions can result in protein stabilization, whereas for high concentrations of salts, the major effects result from effects on water structure and preferential exclusion of the ions from the vicinity of the protein (*50*). The ion-induced effects on water structure at high salt concentrations could in turn affect the hydrophobic interactions within the protein (*51*).

Although we propose that the major effects of moderate to high salt concentrations on SNase structure and stability result from anion binding to ammonium groups, it is likely that there will also be additional effects on the hydrophobic interactions, especially at the highest salt concentrations. These may be responsible for the differing effects of the salts on the refolding kinetics of the wild-type and mutant SNase. It has been shown that the degree of preferential exclusion and increase in chemical potential of a cosolute is directly proportional to the protein surface area. Thus, the system will favor the protein state with the smallest area (*52*); hence, the preferential exclusion of the ions could also lead to increased compactness of the native states, especially in view of the unfavorable interactions between water and the polypeptide backbone (*53*).

ACKNOWLEDGMENT

We thank Dan Segel for assistance with the small-angle X-ray scattering experiments.

REFERENCES

1. Pace, C. N. (1995) *Methods Enzymol. 259*, 538.
2. Saunders, A. J., Davis-Searles, P. R., Allen, D. L., Pielak, G. J., and Erie, D. A. (2000) *Biopolymers 53*, 293−307.
3. Goto, Y., Calciano, L. J., and Fink, A. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573−577.
4. Goto, Y., Takahashi, N., and Fink, A. L. (1990) *Biochemistry 29*, 3480−3488.
5. Fink, A. L., Calciano, L. J., Goto, Y., Kurotsu, T., and Palleros, D. R. (1994) *Biochemistry 33*, 12504−12511.
6. Uversky, V. N., Karnoup, A. S., Segel, D. J., Seshadri, S., Doniach, S., and Fink, A. L. (1998) *J. Mol. Biol. 278*, 879−894.
7. Uversky, V. N., Segel, D. J., Doniach, S., and Fink, A. L. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 5480−5483.
8. Hynes, T. R., Kautz, R. A., Goodman, M. A., Gill, J. F., and Fox, R. O. (1989) *Nature 339*, 73−76.
9. Eftink, M. R., Ghiron, C. A., Kautz, R. A., and Fox, R. O. (1991) *Biochemistry 30*, 1193−1199.
10. Antonino, L. C., Kautz, R. A., Nakano, T., Fox, R. O., and Fink, A. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7715−7718.
11. Hynes, T. R., and Fox, R. O. (1991) *Proteins: Struct., Funct., Genet. 10*, 92−105.
12. Eftink, M. R., and Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199−227.
13. Glatter, O., and Kratky, O. (1982) in *Small-angle X-ray scattering*, pp 515, Academic Press, London.
14. Uversky, V. N., Karnoup, A. S., Khurana, R., Segel, D. J., Doniach, S., and Fink, A. L. (1999) *Protein Sci. 8*, 161−173.
15. Evans, P. A., Dobson, C. M., Kautz, R. A., Hatfull, G., and Fox, R. O. (1987) *Nature 329*, 266−268.
16. Kuwajima, K., Okayama, N., Yamamoto, K., Ishihara, T., and Sugai, S. (1991) *FEBS Lett. 290*, 135−138.
17. Nakano, T., Antonino, L. C., Fox, R. O., and Fink, A. L. (1993) *Biochemistry 32*, 2534−2541.
18. Kalnin, N. N., and Kuwajima, K. (1995) *Proteins: Struct., Funct., Genet. 23*, 163−176.
19. Ikura, T., Tsurupa, G. P., and Kuwajima, K. (1997) *Biochemistry 36*, 6529−6538.
20. Wallenhorst, W. F., Green, S. M., and Roder, H. (1997) *Biochemistry 36*, 5795−5805.
21. Fink, A. L. (1995) *Methods Mol. Biol. 40*, 343−360.
22. Fink, A. L. (1995) *Subcell. Biochem. 24*, 27−53.
23. Fink, A. L. (1995) *Annu. Rev. Biophys. Biomol. Struct. 24*, 495−522.
24. Goto, Y., and Fink, A. L. (1990) *J. Mol. Biol. 214*, 803−805.
25. Calciano, L. J., Escobar, W. A., Millhauser, G. L., Miick, S. M., Rubaloff, J., Todd, A. P., and Fink, A. L. (1993) *Biochemistry 32*, 5644−5649.
26. Yang, A. S., and Honig, B. (1994) *J. Mol. Biol. 237*, 602−614.
27. Nakamura, H. (1996) *Q. Rev. Biophys. 29*, 1−90.
28. Kay, M. S., and Baldwin, R. L. (1998) *Biochemistry 37*, 7859−7868.
29. Yang, A. S., and Honig, B. (1993) *J. Mol. Biol. 231*, 459−474.
30. Pace, C. N., Grimsley, G. R., Thomson, J. A., and Barnett, B. J. (1988) *J. Biol. Chem. 263*, 11820−11825.
31. Kaushik, J. K., and Bhat, R. (1999) *Protein Sci. 8*, 222−233.
32. Meeker, A. K., Garcia-Moreno E., B., and Shortle, D. (1996) *Biochemistry 35*, 6443−6449.
33. Kuhlman, B., Luisi, D. L., Young, P., and Raleigh, D. P. (1999) *Biochemistry 38*, 4896−4903.
34. Tan, Y. J., Oliveberg, M., Davis, B., and Fersht, A. R. (1995) *J. Mol. Biol. 254*, 980−992.
35. Loladze, V. V., Ibarra-Molero, B., Sanchez-Ruiz, J. M., and Makhatadze, G. I. (1999) *Biochemistry 38*, 16419−16423.
36. Spector, S., Wang, M., Carp, S. A., Robblee, J., Hendsch, Z. S., Fairman, R., Tidor, B., and Raleigh, D. P. (2000) *Biochemistry 39*, 872−879.
37. Collins, K. D., and Washabaugh, M. W. (1985) *Q. Rev. Biophys. 18*, 323−422.
38. Fink, A. L., Calciano, L. J., Goto, Y., Nishimura, M., and Swedberg, S. A. (1993) *Protein Sci. 2*, 1155−1160.
39. Uversky, V. N., Gillespie, J. R., and Fink, A. L. (2000) *Proteins: Struct., Funct., Genet. 41*, 415−427.

40. Su, Z. D., Arooz, M. T., Chen, H. M., Gross, C. J., and Tsong, T. Y. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2539−2544.
41. Nishimura, C., Riley, R., Eastman, P., and Fink, A. L. (2000) *J. Mol. Biol. 299*, 1133−1146.
42. Bieri, O., Wildegger, G., Bachmann, A., Wagner, C., and Kiefhaber, T. (1999) *Biochemistry 38*, 12460−12470.
43. Uversky, V. N., Gillespie, J. R., Millett, I. S., Khodyakova, A. V., Vasiliev, A. M., Chernovskaya, T. V., Vasilenko, R. N., Kozlovskaya, G. D., Dolgikh, D. A., Fink, A. L., Doniach, S., and Abramov, V. M. (1999) *Biochemistry 38*, 15009−15016.
44. Makhatadze, G. I., Lopez, M. M., Richardson, J. M., and Thomas, S. T. (1998) *Protein Sci. 7*, 689−697.
45. Leberman, R., and Soper, A. K. (1995) *Nature 378*, 364−366.
46. Melander, W., and Horvath, C. (1977) *Arch. Biochem. Biophys. 183*, 200−215.
47. Przybycien, T. M., and Bailey, J. E. (1991) *Biochim. Biophys. Acta 1076*, 103−111.
48. Arakawa, T., Bhat, R., and Timasheff, S. N. (1990) *Biochemistry 29*, 1914−1923.
49. Qu, Y., Bolen, C. L., and Bolen, D. W. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9268−9273.
50. Arakawa, T., and Timasheff, S. N. (1984) *Biochemistry 23*, 5912−5923.
51. Kella, N. K., and Kinsella, J. E. (1988) *Int. J. Pept. Protein Res. 32*, 396−405.
52. Kendrick, B. S., Chang, B. S., Arakawa, T., Peterson, B., Randolph, T. W., Manning, M. C., and Carpenter, J. F. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 11917−11922.
53. Liu, Y., and Bolen, D. W. (1995) *Biochemistry 34*, 12884−12891.

BI000861K