Robustness of the Long-Range Structure in Denatured Staphylococcal Nuclease to Changes in Amino Acid Sequence†

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ABSTRACT: A nativelike low-resolution structure has been shown to persist in the Δ131Δ denatured fragment of staphylococcal nuclease, even in the presence of 8 M urea. In this report, the physical−chemical basis of this structure is addressed by monitoring changes in structure reflected in residual dipolar couplings and diffusion coefficients as a function of changes in amino acid sequence. Ten large hydrophobic residues, previously shown to play dominant roles in the stability of the native state, are replaced with polar residues of similar shape. Modest increases in the Stokes radius determined by NMR methods result from replacement of five isoleucine/valine residues with threonine, one leucine with glutamine, and oxidation of four methionines to the sulfoxides. Yet in the presence of all ten hydrophobic to polar substitutions and 8 M urea, the NMR signature of a native−like topology is still largely intact. In addition, removal of 30 residues from either the N-terminus (which deletes a three-strand β meander) or C-terminus (a long extended segment and the final α helix) produces only very small changes in long-range structure. These data indicate that both the general shape of the denatured state and the angular relationships of individual bond angles to the axes describing the spatial distribution of the protein chain are insensitive to large changes in the amino acid sequence, a finding consistent with the conclusion that the long-range structure of denatured proteins is encoded primarily by local steric interactions between side chains and the polypeptide backbone.

The denatured state plays a central role in both experimental and theoretical considerations of protein folding. For equilibrium folding experiments, it is one of two states of the polypeptide chain that is stable in solution and that interconverts in an all-or-nothing reaction. For kinetic folding experiments, the denatured state is the point of departure for the polypeptide chain as it proceeds to accrue more structure along the folding pathway. And from a theoretical standpoint, the denatured state determines the entropy that must be lost on folding; hence it frames the magnitude of the "folding problem" faced by real proteins.

Relatively little experimental work has been directed toward a more complete picture of the structure and dynamics of this complex ensemble of conformations. Because of the variety of parameters and time scales on which NMR experiments operate, NMR spectroscopy can provide a considerable amount of information about the averaged structure of denatured proteins (*1*, *2*). Although the interpretation of NMR parameters in detailed structural terms is often not possible, they usually reflect ensemble-averaged properties and thus contain information about the full range of conformations populated in solution.

Until recently, NMR data provided information only about local structural features, such as backbone angle preferences (scalar coupling constants and secondary chemical shifts), and qualitative information on the fractional population of turns and α helices (short- and medium-range NOEs[1]). However, with the development of methods for conferring a small alignment on proteins, a new, long-range structural parameter entered the armamentarium of the NMR spectroscopist—the residual dipolar coupling (*3*−*5*). When a nonspherical protein in solution is forced to diffuse and tumble in an asymmetric environment, incomplete cancellation of the dipolar coupling between magnetic nuclei connected by one or two covalent bonds may occur. The sign and magnitude of this coupling provide information on the orientation of an individual bond vector with respect to a unique molecular axis, which is determined by the axes of the moments of inertia and is referred to as the molecular alignment tensor. The resulting information regarding relative bond vector orientations is completely distance-independent and reflects nonrandom angular relationships of the bond vectors with respect to each other (*6*, *7*).

A previous study of the Δ131Δ fragment of staphylococcal nuclease (residues 10−140) found a correlation between residual dipolar couplings measured in 0 M and in 8 M urea, indicating the presence of long-range, nativelike structure in the absence of defined secondary structure (*8*). Additional NMR experiments on full-length wild-type staphylococcal nuclease denatured in urea or in low pH buffer revealed that the residual structure in these denatured states is very similar to that present in the Δ131Δ fragment, demonstrating that

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[1] Abbreviations: NOE, nuclear Overhauser effect; IPAP-HSQC, in-phase, antiphase heteronuclear single-quantum coherence; TEMED, tetramethylethylenediamine; water-sLED, water-suppressed longitudinal encode, decode.

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three different denatured forms of this protein share a common low-resolution structure that is independent of the mechanism used to destabilize the native state (*9*). Residual dipolar couplings have also been observed for the acid plus urea-denatured states of ubiquitin and eglin C (Ohnishi, Briggman, and Shortle, unpublished observations).

Once a denatured protein has expanded to fill a volume several times that of the native conformation, very short range attractive forces such as hydrogen bonds or dispersion forces are unlikely to play an important role in determining what structure persists. Electrostatic interactions are also unlikely to be important, since the long-range structure of Δ131Δ is little changed at pH 3.0 (*9*), conditions under which the protein has a net charge of approximately +20 or greater and few negatively charged groups remain. Hydrophobic interactions and local steric interactions between side chains and the backbone appear to be the most likely forces responsible for this persistent structure. Although the residual structure in denatured nuclease is only modestly perturbed upon addition of 8 M urea, hydrophobic interactions are not completely disrupted at these concentrations, as witnessed by the persistence of lipid bicelles in 8 M urea and the observation of small bits of local structure in the 434 repressor protein (*10*). In this report, a series of mutations are introduced into denatured nuclease to assess the roles played by interactions involving the largest hydrophobic groups and by long-range interactions of any type.

EXPERIMENTAL PROCEDURES

Recombinant DNA and Protein Expression. All mutants were constructed using the Kunkel method of oligonucleotide-directed mutagenesis (*11*) with the Δ131Δ gene cloned into the phage vector M13mp18 (*12*). After transformation of mutagenized phage into competent XL1-Blue cells, phage DNA was prepared from individual plaques, and the complete gene sequence was determined. The mutant was then transferred into the T7 vector pET11A for expression in the *Escherichia coli* strain BL21(DE3).

All protein samples were uniformly labeled with ^{15}N by growing cells in MOPS minimal medium supplemented with 1 g/L $^{15}NH_4Cl$ as the sole nitrogen source. For samples to be oxidized, L-methionine with a ^{13}C label on the ϵ-methyl group was added to the cells at a final concentration of 25 mg/L approximately 1 h before induction. Proteins were purified as described previously (*13*). After 18−24 h of dialysis against H_2O, protein samples were lyophilized and dissolved in 10% D_2O, 1 mM sodium azide, and 20 mM acetic acid titrated to pH 5.0 with sodium hydroxide.

Protein Chemistry. Figure 1 shows the sites of the altered hydrophobic residues mapped onto the crystal structure of staphylococcal nuclease and lists the combinations of mutations and modifications used in this report. To oxidize the four methionines in the sextuple mutant to the sulfoxide form, protein samples at approximately 1 mM in 6 M urea, 25 mM Tris-HCl, pH 8.0, and 0.4 M sodium chloride were made 100 mM in H_2O_2 and incubated for 1 h at room temperature (*14*). The reaction was quenched by addition of β-mercaptoethanol to a final concentration of 1 M, and the sample was dialyzed extensively against H_2O before lyophilization. Quantitative analysis of the first slice of a $^1H-^{13}C$ HSQC spectrum indicated that more than 95% of the ϵ-methyl protons of methionine (~2.1 ppm) had been oxidized to the sulfoxide (~2.8 ppm).

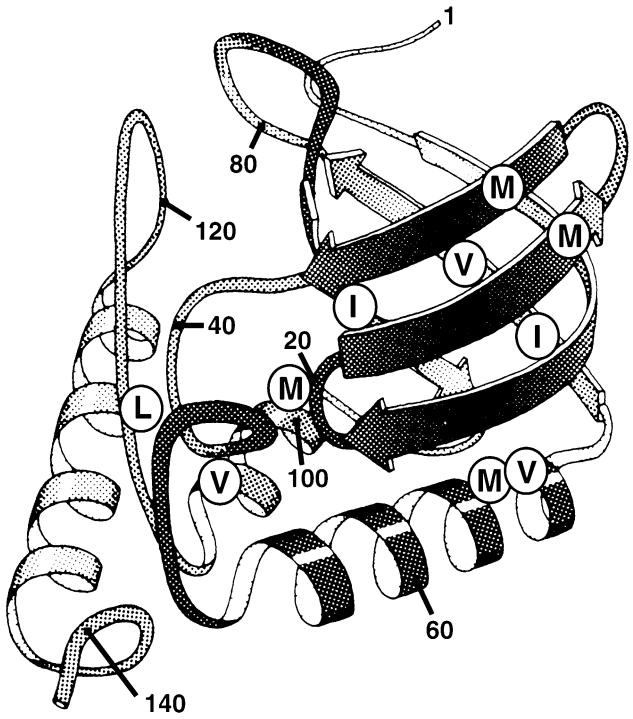

FIGURE 1: Ribbon diagram showing the ten sites of hydrophobic substitution and modification relative to the native structure of staphylococcal nuclease. (This figure is copyrighted by Dr. Jane Richardson and is used with her permission.) The substitution mutants used are as follows: double = I92T + L125Q; triple = I72T + I92T + L125Q; quadruple = I72T + V74T + I92T + L125Q; quintuple = V66T + I72T + I92T + V104T + L125Q; sextuple = V66T + I72T + V74T + I92T + V104T + L125Q. The hydrogen peroxide oxidized sextuple = sextuple + M26oxo + M32oxo + M65oxo + M98oxo.

Sample Alignment. Strained polyacrylamide gels were used as the alignment medium in all experiments as described previously (*9*, *15*, *16*). Cylindrical 12% polyacrylamide gels (1:20 bisacrylamide by weight) are cast in segments of 4.0 mm inside diameter Tygon tubing with one end sealed with parafilm. After polymerization with 0.15% TEMED and 0.15% ammonium persulfate, the gel cylinder is washed extensively in H_2O, with the last wash in the appropriate final concentration of buffer and urea. The gel cylinder is then cut to 21 mm in length, placed into a 1.5 mL microfuge tube with 250−300 μL of 1.2 mM protein samples, and incubated overnight to allow the protein to diffuse into the gel. The protein concentration after diffusion is typically around 0.5−0.6 mM. The protein-containing gel cylinder is then slid down a 5 mm Shigemi NMR tube (4.5 mm inner diameter) and compressed with the glass plunger to a length of 16−17 mm.

NMR and Data Processing. All samples were 0.5−0.6 mM protein in 20 mM sodium acetate, pH 5.0, with 10% D_2O. NMR spectra were collected at 32 °C on a Varian Unity-Plus 600 spectrometer with the IPAP-HSQC pulse sequence (*17*) obtained from Dr. Lewis Kay, with 128 complex points and a spectral width of 1400 Hz in the ^{15}N dimension. Each dimension was zero-filled once to a final of 1024 complex points in the ^1H dimension and 256 complex points in the ^{15}N dimension. Residual dipolar couplings were calculated by subtracting the value of the scalar coupling measured in

an isotropic sample from the total coupling measured in an oriented sample. Errors in dipolar coupling values have been previously estimated to be ±0.22 Hz from measurements of the same sample on three different days (*9*). Between 50 and 70 H_N peaks that were not overlapped and Lorentzian in shape were used in the correlation analysis. Scatter plots of residual dipolar couplings from these three data sets resulted in correlation coefficients that varied by ±0.01. All correlation coefficients analyzed in this study correspond to *p*-values of less than 0.0001.

Diffusion Measurements. To estimate the hydrodynamic (Stokes) radius of denatured nuclease, pulsed-field gradient NMR methods were used to measure the diffusion coefficient, using a water-sLED pulse sequence (*18*) with presat for water suppression, kindly provided by Dr. Scott Kennedy of the University of Rochester. Translational diffusion during a fixed delay in the pulse sequence attenuates the observed signal intensity I according to the equation:

$$I = I_0 \exp[(-\gamma\delta G)^2(\Delta - \delta/3)D] \quad (1)$$

where I_0 is the initial intensity in the absence of a pulsed-field gradient, γ is the ^{1}H gyromagnetic ratio, δ is the pulsed-field gradient duration, G is the gradient strength, Δ is the time between field gradient pulses, and D is the diffusion coefficient. With all delays held constant, the signal intensity depends on G and D. A series of 24 spectra were collected with δ = 6 ms and Δ = 120 ms and with the gradient strength varied from 0 to 47 G/cm. ^{1}H one-dimensional spectra were acquired with 4096 complex points, a spectral width of 8000 Hz, and 320 transients. Each experiment required about 6 h of data collection. Sample conditions consisted of 0.1 and 0.3 mM protein, 5 mM sodium acetate, ~99% D_2O, pH* 5.0, not corrected for the isotope effect, and 0.04% 1,4-dioxane as a hydration radius standard, with the temperature set to 32 °C. The protein diffusion coefficient was obtained by measuring peak heights of the five most intense peaks in the methyl and methylene proton region and fitting the resulting intensities to eq 1. Using the approach of Wilkins et al. (*19*), the value of D was averaged over these five peaks and standardized to dioxane to determine the Stokes radius (R_S), using the empirical relationship:

$$R_S^{\text{protein}} = (D^{\text{dioxane}}/D^{\text{protein}})R_S^{\text{dioxane}} \quad (2)$$

where the R_S of dioxane is 2.12 Å. Repeat experiments gave a standard deviation of approximately 0.4 Å for R_S.

RESULTS

Hydrophobic Substitutions of Δ131Δ: Residual Dipolar Couplings. To reduce the contribution of hydrophobic interactions to denatured state structure, a series of substitution mutants of Δ131Δ were constructed that replace large hydrophobic side chains with polar groups of similar shape. The β-branched residues isoleucine and valine were replaced with threonine, and leucine was replaced with glutamine. Chemical modification of methionines with hydrogen peroxide allowed this amino acid to be converted to the highly polar sulfoxide form. The sites chosen for substitution were selected on the basis of involvement in long-range hydrophobic contacts within the major hydrophobic core of folded nuclease and importance in maintaining the stability of the native state, as demonstrated by measurement of $\Delta\Delta G$ after mutation to alanine (*12*). Since preliminary results indicated that single mutations had very small effects on dipolar couplings, multiple mutants were constructed with increasing numbers of substitutions. The positions of the ten mutant sites in native nuclease are shown schematically in Figure 1: three valines (V66T, V74T, V104T), two isoleucines (I72T, I92T), one leucine (L125Q), and four methionines (M26oxo, M32oxo, M65oxo, and M98oxo).

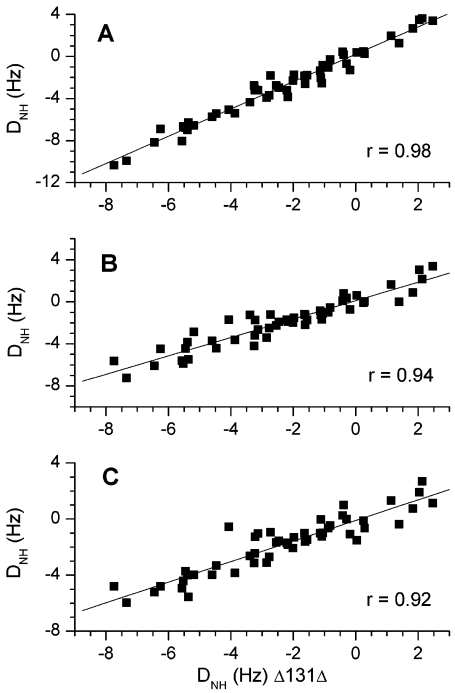

FIGURE 2: Scatter plots of H_N residual dipolar couplings for the (A) triple, (B) sextuple, and (C) oxidized sextuple mutants plotted against Δ131Δ in 12% compressed gels, 20 mM sodium acetate, pH 5.0, 32 °C, and no urea. *r* is the Pearson correlation coefficient.

Table 1: Correlation Coefficients between Sets of H_N Residual Dipolar Couplings as a Function of Urea Concentration[a]

[urea] (M)	no. of modifications						
	0	2	3	4	5	6	10
0		0.98	0.98	0.96	0.96	0.94	0.92
2	0.94	0.94	0.93	0.91	0.90	0.88	0.90
4	0.92	0.88	0.88	0.86	0.88	0.84	0.86
6	0.88	0.86	0.86	0.84	0.85	0.82	0.84
8	0.82	0.81	0.80	0.80	0.81	0.79	0.80

[a] All samples are in 20 mM sodium acetate, pH 5.0, at 32 °C. Proteins are designated by the number of hydrophobic modifications they contain. 0 is Δ131Δ and multiple mutants are described in Figure 1.

Between 50 and 70 H_N dipolar couplings were measured for ^{15}N-labeled Δ131Δ and five mutant forms using strained polyacrylamide gels. To compare the long-range structure in two different denatured proteins, the values of H_N couplings for corresponding residues were plotted in two dimensions, and the Pearson correlation coefficient was calculated. Examples of such scatter plots are shown in Figure 2 for a triple mutant, a mutant with six substitutions, and a mutant with ten modifications. The correlation coefficients for each modified form of Δ131Δ in the absence of urea compared to unmodified Δ131Δ in 0−8 M urea are listed in Table 1.

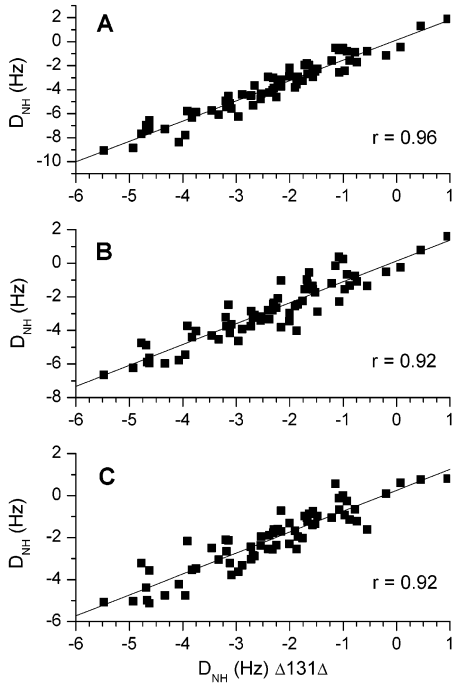

FIGURE 3: Scatter plots of H_N residual dipolar couplings measured in 8 M urea for the (A) triple, (B) sextuple, and (C) oxidized sextuple mutants plotted against the values for Δ131Δ. Except for urea, the conditions are the same as in Figure 2.

The most important feature in these data is the surprisingly small magnitude of the changes in structure that accompany these reductions in chain hydrophobicity. As can be seen in Figure 2, the correlation coefficients for Δ131Δ with three, six, and ten modifications do decrease monotonically, but only from 0.98 to 0.94 to 0.92. While it is not yet possible to convert the numerical values of the dipolar couplings into specific structural constraints, these data strongly imply that the ensemble-average structure of Δ131Δ is undergoing only modest changes in overall shape and in orientation of individual bond vectors relative to axes that describe this overall shape.

When the residual structure of Δ131Δ at different urea concentrations is compared to that of the modified forms in the absence of urea, a comparison can be made of structural changes that accompany these two perturbations of hydrophobic interactions. Inspection of the correlation coefficients in Table 1 reveals that, in every case, a modified form of Δ131Δ shows the best correlation with Δ131Δ in the absence of urea. In other words, substitution of several large hydrophobic residues does not produce the same structural changes as addition of urea, a finding which supports the conclusion that reduction of hydrophobic interactions is not the only mechanism underlying urea's action as a denaturant. Some of its denaturing activity may involve direct binding to the polypeptide backbone (*20*).

In addition, replacement of several large hydrophobic side chains produces a detectable change in structure even in the presence of 8 M urea. The scatter plots in Figure 3 show that, for three, six, and ten modifications, the correlation coefficients with Δ131Δ in 8 M urea decrease from 0.96 to 0.92 and 0.92, respectively. Thus, the changes in structure produced by these hydrophobic modifications in 8 M urea are almost as large as in the absence of urea, an observation

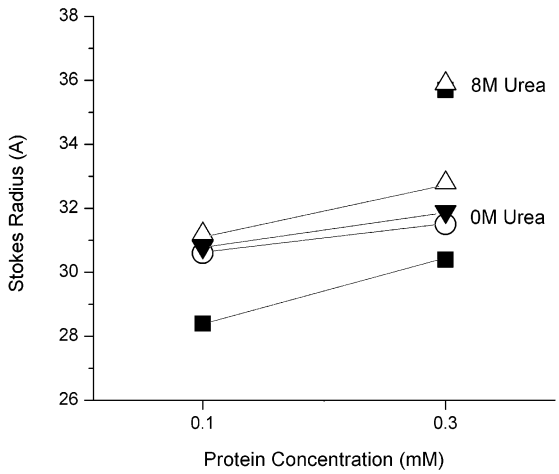

FIGURE 4: Estimated Stokes radius of Δ131Δ and three mutant forms: (■) Δ131Δ, (○) triple, (▼) sextuple, and (△) oxidized sextuple. The water-sLED pulsed-field gradient NMR method was used with dioxane as an internal standard to correct for changes in viscosity. Repeat experiments resulted in a standard deviation of 0.1 Å at 0.1 mM, 0.4 Å at 0.3 mM, and 0.9 Å in 8 M urea. The solid lines between the 0.1 and 0.3 mM data points are for visual guidance only.

that provides additional support for urea acting by a mechanism other than reduction of hydrophobic interactions.

Hydrophobic Substitutions of Δ131Δ: Diffusion Coefficients. The modest changes in long-range structure reflected in residual dipolar couplings suggest that hydrophobic substitutions and urea can only slightly alter the structure of the denatured state. Yet one of the fundamental principles of polymer physical chemistry is that the solution properties of a polymer must change as the solvent improves in quality and interacts more favorably with the backbone and side chains (*21*). So one would expect replacement of large hydrophobic side chains with polar groups to lead to an increase in the Stokes radius.

An increase in the radius of gyration with increasing urea concentration has been shown for staphylococcal nuclease and other denatured proteins by SAXS (*22*). To demonstrate the expected changes in size resulting from the hydrophobic substitutions, NMR methods employing pulsed-field gradients were used to measure the diffusion coefficients, and thus the Stokes radii, of several molecules. The data for Δ131Δ with zero, three, six, or ten modifications are shown graphically in Figure 4. Because of the nonideality displayed by Δ131Δ at concentrations above 0.4 mM (*23*) arising from electrostatic repulsion in the absence of added salt (Wang and Shortle, unpublished observations), measurements were made at 0.1 and 0.3 mM concentrations. Increasing the number of amino acid perturbations to ten increases the estimated Stokes radius from 28.4 Å (Δ131Δ) to 31.2 Å (oxidized sextuple mutant) at 0.1 mM (Figure 4). This corresponds to an increase in volume of approximately 30%, a significant expansion in size but one that leads to only small changes in ensemble-average bond vector orientations.

Upon the addition of 8 M urea, there is a significant expansion of the polypeptide chain. At 0.3 mM, the Stokes radius increases from 30.4 to 35.7 Å for Δ131Δ, approximately a 60% increase in hydrodynamic volume. For the oxidized sextuple mutant, the Stokes radius increases from 32.8 to 35.9 Å, which is about a 30% increase in volume. Since both proteins have similar Stokes radii in 8

M urea, the data indicate that there may be an upper limit to how far the denatured polypeptide chain can expand (*24*). These data suggest that the highly expanded denatured state cannot be regarded as a simple homopolymer coil, since the principles of polymer chemistry indicate that for a polymer chain the volume will continue to increase as the solvent−solute interactions become more favorable (*21*).

Truncations of Δ131Δ. The dipolar coupling data for Δ131Δ in urea and with multiple hydrophobic substitutions imply that hydrophobic interactions may not play a major role in encoding that component of the long-range structure reflected in residual dipolar couplings. Yet 8 M urea does not eliminate all hydrophobic interactions, and Δ131Δ with ten hydrophobic changes still contains approximately 25 large hydrophobic residues. Thus the possibility has not been excluded that a specific subset of residues, hydrophobic or otherwise and acting through transient long-range contacts, might play a critical role in establishing the long-range structure that persists in Δ131Δ. Therefore, instead of adding still more substitutions, a more drastic modification of sequence was employed, namely, truncation of large blocks of sequence from the ends of the molecule. This approach has the advantage that it makes no assumptions about the types of residues or interactions that may determine the long-range structure.

Since previous work has shown that fragments of nuclease smaller than 90−95 amino acids fail to accumulate in overexpressing cells (*25*), truncations were limited to the first 30 or last 30 residues, yielding two 101 amino acid fragments. Fragment 40−140 removes the first three β strands, which comprise three of the five strands of an antiparallel β barrel and approximately half of the major hydrophobic core of the molecule. Thus fragment 40−140 cannot form a complete hydrophobic core. Fragment 10−110 removes a hydrophobic extended segment (residues 111−115), a *cis*-proline turn, and the last and longest α helix. This molecule, on the other hand, contains most of the sequence needed to form an OB domain (*26*) and could form the principal hydrophobic core.

Yet, as shown in Figure 5, the dipolar coupling data very clearly indicate that those residues common to Δ131Δ and each of the fragments display the same long-range structure, with the correlation coefficients for both fragments being 0.97 or greater. While the combined effect on the sequence of Δ131Δ of these two truncations is to remove 45% of its residues, the net effect on structure is surprisingly small and argues convincingly that long-range contacts involving the first or last 30 residues play no discernible role in encoding the long-range structure reflected in the residual dipolar couplings. Even in 6 M urea, these two shortened fragments display nearly the same structure as Δ131Δ in urea (data not shown).

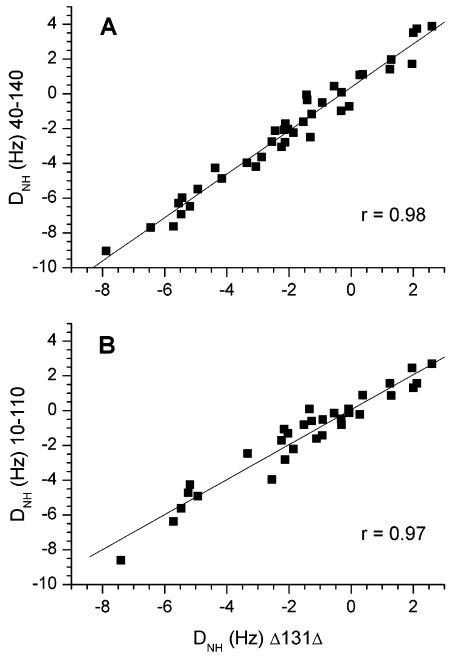

FIGURE 5: Scatter plots of H_N residual dipolar couplings for staphylococcal nuclease fragments (A) 40−140 and (B) 10−110 plotted against the values for Δ131Δ in 12% compressed gels, 20 mM sodium acetate, pH 5.0, 32 °C, and no urea.

DISCUSSION

In view of the data presented above, the long-range structure in denatured staphylococcal nuclease must be viewed as remarkably robust to changes in amino acid sequence. Replacement of ten large hydrophobic residues known to play important roles in stabilizing the native state results in only small changes in structure, even though the average volume occupied by the polypeptide chain increases by as much as 30%. Addition of 8 M urea to these modified denatured state proteins leads to further changes in structure; yet these changes too must be considered modest in their extent. Even removing either the first or the last 30 residues leads to changes in structure that are very small.

What inferences, then, can be made about this highly robust residual structure? Because dipolar couplings have a simple geometric interpretation, the structural features that must be preserved in order to retain essentially the same relative couplings can be stated with confidence. (1) The ensemble-average shape must remain the same. (2) The set of axes that describe this shape, namely, the axes of the moments of inertia tensor, must remain the same. (3) The ensemble-average orientation of individual bond vectors with respect to these axes must also remain the same. Thus, while there are several mechanisms by which a correlation between dipolar couplings from two molecules of similar structure could be lost, when a high statistical correlation is retained, these three structural features must also be retained.

The conversion of dipolar couplings into one or more conformations consistent with the NMR data is a difficult computation, requiring many more couplings per residue than presented here (*27*−*29*). And because the individual dipolar couplings reflect the ensemble-average bond vector orientation, it is not clear that structure calculations can in all cases give physically realistic conformations. For example, consider a chain segment that forms an α helix in 50% of conformations and is fully extended in the remaining 50%. Bond vector orientations in the α helix, which are parallel to the helix axis, would be averaged with orientations in the extended conformation, which are perpendicular to the direction of the backbone. Dipolar couplings would report an average bond vector orientation of approximately 45° relative to the average direction of the chain, a situation that does not correspond to any physically realistic backbone conformation. Thus at least for now, only qualitative conclu-

sions can be drawn from these data, but several general inferences follow directly.

First, the denatured state of staphylococcal nuclease is not a random coil (*30*). By definition, a random coil permits each monomer to assume an orientation in space that is independent of a large fraction of other monomers (*21*). There would be no fixed asymmetry of overall shape; there would be no physical distinguishable set of directions; and bond vectors would orient randomly with respect to all sets of axes. For the denatured state of nuclease to display relatively large residual dipolar couplings under alignment conditions similar to those that align folded proteins, the denatured state, like the native state, must exhibit relatively fixed orientation and spatial positioning (topology) of different chain segments with respect to each other and specific angular relationships between the ensemble-average bond vectors and the rest of the molecule. Thus, if the persistence length of a polypeptide chain is interpreted as the number of residues that must separate two residues before orientations in space are uncoupled and independent (*21*), it follows that denatured nuclease has a persistence length greater than the length of the chain itself.

In view of this extreme robustness to urea and changes in sequence, the conclusion seems inescapable that most of the long-range structure reported by dipolar couplings does not arise from long-range interactions. In our opinion, the most probable physical−chemical basis for this persistent long-range structure is local steric interactions between side chains and the protein backbone, a conclusion supported by recent observations of residual dipolar couplings in peptides 2−15 amino acids in length (*31*). Some of these peptides lack any hydrophobic amino acids, and most contain only a small fraction. Thus the polypeptide chain should be viewed as inherently stiff and only deformable in a limited number of directions. Like a bicycle chain that has been twisted out of the plane, the backbone of a denatured protein must have position/sequence-specific restraints on where it can flex and where, on average, the bends and turns will form.

The modest expansion that results from hydrophobic substitutions and from addition of high concentrations of urea occurs with relatively little alteration of the angular and spatial positioning of chain segments relative to one another. This observation suggests that long-range contacts between hydrophobic residues in the expanded denatured state act to pull chain segments together by a few angstroms, and in their absence, these segments diffuse apart with few concomitant changes in structure.

The observation that resists a ready explanation is the high correlation of dipolar couplings shown by the two 101 amino acid fragments compared with Δ131Δ. The shape of the denatured state of staphylococcal nuclease and the position of the moment of inertia axes must undergo almost no alteration upon removing approximately 25% of the chain from either end! While a long cylindrical shape (e.g., a long helical structure) might have the requisite robustness to truncation and thus could explain this observation, two lines of evidence suggest that the overall shape of Δ131Δ is not that of a cylinder or a long prolate ellipsoid. (1) The low-resolution solution structure of Δ131Δ determined by paramagnetic relaxation enhancement revealed a roughly spherical shape (*32*, *33*). (2) The concentrations of polyacrylamide or bicelles required to achieve alignment are significantly higher than those required by folded nuclease. Since folded nuclease does not deviate far from being spherical in shape, it suggests the ensemble-average shape of Δ131Δ is not highly asymmetric. Thus, the possibility exists that some overlooked or unknown principle or some technical issue is at work here, one that might render the simple geometric interpretation of residual dipolar couplings incomplete.

We propose that the persistent structure reflected in dipolar couplings for the denatured state of staphylococcal nuclease arises from two sources. First, and most importantly, local steric and hydrogen-bonding interactions between side chains and adjacently backbone segments severely restrict the allowed values of ϕ, ψ, and χ, a conclusion supported by analysis of the residue-specific ϕ/ψ propensities (*34*) and by analysis of conformational biases inferred from computer simulations (*35*, *36*). While these effects are probably sufficient to account for the dipolar couplings displayed by short peptides, it may be necessary to invoke steric exclusion between more distant chain segments to account for some of the orientational order in polypeptide chains as long as Δ131Δ. This possibility can be tested experimentally in at least two ways. Substitution of surface polar groups with glycine may alter the local steric interactions between adjacent stiff segments and thus modify the long-range positioning of chain segments with respect to each other. Alternatively, long (∼100 amino acids) polypeptides of random sequence might display more heterogeneity in segment−segment orientations and positions. For such arbitrary sequences of amino acids, there would have been no opportunity for fine-tuning of local interactions by evolutionary pressures to optimize patterns of long-range interactions, if such interactions are required for rapid folding and/or stability of a native state.

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