Molecular Alignment of Denatured States of Staphylococcal Nuclease with Strained Polyacrylamide Gels and Surfactant Liquid Crystalline Phases†

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ABSTRACT: Residual dipolar couplings reflect the orientation of vectors between pairs of magnetic nuclei relative to a unique set of molecular axes. Thus, unlike NOEs and scalar couplings, dipolar couplings provide access to long-range structural information. A prerequisite for measurement of these NMR parameters is imposition of a weak net alignment, most simply by forcing the macromolecules to tumble in an asymmetric environment that restricts some orientations more than others. In this report, several denatured forms of staphylococcal nuclease are aligned by using compressed and stretched polyacrylamide gels, a nonionic type of lipid bilayer disk or bicelle, and a liquid crystalline phase formed by a cationic lipid. All three types of media can be used at high urea concentrations. While polyacrylamide gels and bicelles produce similar alignment tensors through steric interactions, a liquid crystalline phase of cetylpyridinium bromide aligns denatured nuclease along a different set of axes, presumably through electrostatic effects. The analysis of residual dipolar couplings collected with two different alignment tensors may permit the calculation of ensembles of conformations. The dipolar couplings observed for staphylococcal nuclease denatured with urea, by low pH or by deletion of residues from both termini, suggest that all denatured forms share a common "topology", one which has been shown previously to be nativelike. Although SDS/nuclease complexes give sharp and disperse $^1H-^{15}N$ correlation spectra, only small couplings are observed in strained polyacrylamide gels.

During the past 10 years, NMR methods have been applied to the challenging task of analyzing proteins in the denatured state. Although side chain and α protons lose most of their dispersion when the native state breaks down, the amide protons remain reasonably well dispersed in both ^1H and attached-^{15}N dimensions. Consequently, NMR characterization of a protein in the denatured state typically begins with assignment of the amide protons, followed by collection of 3D spectra in which scalar coupling constants, secondary chemical shifts of C_α, C_β, and C′, and NOEs[1] are read out from the basic $^1H-^{15}N$ correlation spectrum (*1*).

All three of these NMR parameters report only on local structural features. Scalar coupling constants depend on single dihedral angles, usually ϕ, ψ, or χ_1. Similarly, the secondary chemical shifts of these three carbon atoms reflect the values of ϕ and ψ (*2*). In folded proteins, the NOE has proven to be invaluable for detecting long-range interactions between protons far apart along the sequence that are brought to separations of less than 5 Å in the folded conformation. However, in only a limited number of denatured proteins have long-range NOEs been reported (*3*). More commonly, short- and medium-range NOEs are detected that demonstrate the persistence of local turns or segments of α helices (*4*−*6*). Extensive NOE characterization of Δ131Δ, a 131 residue fragment of staphylococcal nuclease that serves as a denatured state under nondenaturing conditions, has revealed only a small number of medium-range NOEs (*7*).

Residual dipolar couplings, on the other hand, can provide information about the angular relationships between bond vectors in pairs of amino acid residues that is entirely independent of the distance separating the residues (*8*, *9*). Thus their observation demonstrates the persistence of long-range structure of proteins in the denatured state. To observe dipolar couplings in a denatured protein, this dynamic and heterogeneous ensemble of conformations must satisfy several physical conditions. Its time-averaged structure must have sufficient constancy for there to be a meaningful fixed frame of reference that defines a set of molecular axes. In addition, the denatured protein must be orientable in solution. With conventional media in which alignment depends almost entirely on steric interactions (*10*), this essentially means that the distribution of mass in the ensemble-averaged conformation, as described by the inertial tensor, must not be spherically symmetric. And finally, some fraction of residues must maintain a net time-averaged orientation with respect to these molecular axes.

In a recent publication (*11*), we reported the observation of dipolar couplings in one denatured form of the small protein staphylococcal nuclease oriented in strained polyacrylamide gels. Here we address the practical issues arising with strained polyacrylamide gels and report the use of two additional media for aligning urea-denatured proteins. All three media are compatible with urea to concentrations of

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[1] Abbreviations: CPBr, cetylpyridinium bromide; IPAP-HSQC, in-phase, antiphase heteronuclear single-quantum coherence; alkyl-PEG, alkyl poly(ethylene glycol); NOE, nuclear Overhauser effect; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine.

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at least 8 M and low pH conditions. In addition, we present data suggesting that the low-resolution ensemble-averaged structure of denatured staphylococcal nuclease is independent of the mechanism used to destabilize the native state. This common denatured state "topology" is highly robust to perturbation by urea or pH.

EXPERIMENTAL PROCEDURES

Alignment Media. All gels were cast in segments of $^5/_{32}$ in. (4.0 mm) inside diameter Tygon tubing. The oxygen gas absorbed in the walls of Tygon tubing inhibits acrylamide polymerization. If the tubing is not degassed prior to gel casting, small variations in gel diameter can be achieved by adjusting the time of polymerization and/or the amounts of TEMED and ammonium persulfate used to initiate polymerization.

Typically, 1.0 mL of 8−14% acrylamide (1:20 bisacrylamide by weight) in H_2O was made 0.1% in TEMED and 0.1% in ammonium persulfate, mixed, and then pipetted into a segment of tubing sealed at one end with parafilm. After 3−15 min, the tubing was inverted over a 16 × 150 mm glass test tube containing 15 mL of water. Removal of the parafilm should allow the gel to slide freely under its own weight into the test tube. The test tube is sealed with parafilm and very gently rocked in a horizontal position with changes of water every hour for 3−8 h. The last incubation is carried out with a solution of the appropriate final urea and/or buffer concentration. The gel cylinder is transferred to a large square of parafilm on a light box, blotted dry, and sliced into two 21 mm segments. Each cylinder is transferred to a microfuge tube and approximately 300 μL of protein solution at the appropriate urea/buffer concentrations is added. The microfuge tube is incubated horizontally for 4−16 h, with occasional mixing by gentle rolling.

Protein-containing gel cylinders to undergo compression are transferred to a large square of parafilm and nudged into a 5 mm Shigemi NMR tube (4.5 mm i.d.) containing 50 μL of buffer. After being pushed slowly to the bottom with the glass plunger, the gel is compressed with pressure from the index finger, typically to a length of 17 mm with displacement of all surrounding air bubbles. The position of the plunger is secured with a vertically oriented strip of Scotch tape pressed tightly against both the NMR tube and plunger.

Cylinders to undergo radial compression (stretching) are forced into a short segment of $^5/_{32}$ in. Tygon tubing with a beveled end. A 30 mm segment of 4.0 mm Wilmad NMR tube (3.2 mm i.d.) is inserted into the beveled end against the gel cylinder. A 4.0 mm plunger is inserted in the opposite end, and the gel is firmly and slowly pushed out of the Tygon tubing into the glass tube. Tears usually are confined to the first 4−8 mm of the stretched gel, so the gel-containing glass tube is dropped "good end first" into a 5.0 mm Shigemi NMR tube (4.2 mm i.d.). The NMR tube should be seated in the spinner so that the defect-free gel matrix is positioned within the probe coil. The thin layer of water between the two NMR tubes gives rise to an artifactual splitting of the water line, with the component from this outer layer displaying a shorter 90° pulse than the water inside the gel.

The instructions of Ruckert and Otting (*12*) were followed with care to obtain bicelles with polyoxyethylene 5 octyl ether (C8E5) and 1-octanol. Glass capillary pipets were used for addition of 1-octanol. As the correct ratio of 1-octanol to C8E5 is approached from below, the solution goes from clear to turbid to a final clearing with a faint blue tinge when the liquid crystalline state forms.

The protocol of Barrientos et al. (*13*) was modified to include concentrated urea. An 8.1% (w/v) stock solution of liquid crystalline phase (CPBr/1-hexanol, 3/4 w/w) was prepared containing 7.5 M urea and 12.5% deuterium oxide. After 1 min of vortexing, 4 mL of solution was incubated at 70 °C for 5 min and then allowed to cool over 10 min to room temperature. To remove a small amount of excess hexanol, the solution was spun at 10000 rpm for 5 min, resulting in a clear bottom phase and the hexanol-rich phase on top. For a 400 μL sample, 12 μL of 1 M sodium bromide and 8 μL of 1 M sodium acetate, pH 5.0, was added to 320 μL of the stock surfactant solution. Labeled Δ131Δ protein and H_2O were added to a final concentration of 0.4 mM protein.

NMR and Data Processing. Samples were typically 0.4−0.6 mM ^{15}N-labeled Δ131Δ in 20 mM sodium acetate, pH 5.0, with 10% D_2O. The IPAP-HSQC pulse sequence (*14*) obtained from Dr. Lewis Kay was used for all spectra, with 128 complex points and a spectral width of 1200−1400 Hz in the ^{15}N dimension. Each dimension was once zero-filled to a final of 1024 complex points in the ^{1}H dimension and 256 complex points in the ^{15}N dimension. All spectra in strained polyacrylamide gels were collected at 32 °C, except those with anionic detergents, which were collected between 10 and 25 °C. All spectra in alkyl-PEG bicelles and CPBr were collected at 20 °C. Data were processed and peaks picked automatically using the NMRPipes software. Residual dipolar couplings were calculated by subtracting the value of the scalar coupling in isotropic solution from the total coupling measured in an oriented solution.

RESULTS

Strained Acrylamide Gels. To measure dipolar couplings for structural analysis, a weak net alignment must be conferred on a protein, usually by placing it in an asymmetric environment where random tumbling cannot lead to all possible orientations. Typically, liquid crystalline phases are established that orient in the magnetic field of the NMR spectrometer. These phases can be comprised of disks of lipid bilayers or bicelles (*15*), filamentous bacteriophage (*16*), the lamellar liquid crystalline phase (*13*), or fragments of purple membrane containing bacteriorhodopsin (*17*). Solutions of Δ131Δ, a 131-residue unfolded fragment of staphylococcal nuclease, with phospholipid bicelles, with or without the cationic additive cetyltrimethylammonium bromide (*18*), failed to achieve the liquid crystalline state due to phase separation.

Two recent papers reported the use of strained polyacrylamide gels to orient folded proteins for NMR analysis (*19*, *20*). If the gel is strained by compression, the protein's environment is no longer isotropic, and weak alignment can be achieved on the basis of steric interaction with the gel cavities. To obtain measurable couplings with a minimum of line broadening, the optimal acrylamide composition must be determined by trial and error. For protein G (57 residues), an 8% acrylamide with 1:20 bisacrylamide cross-linker was used (*19*), leading to approximately 4 Hz of line broadening.

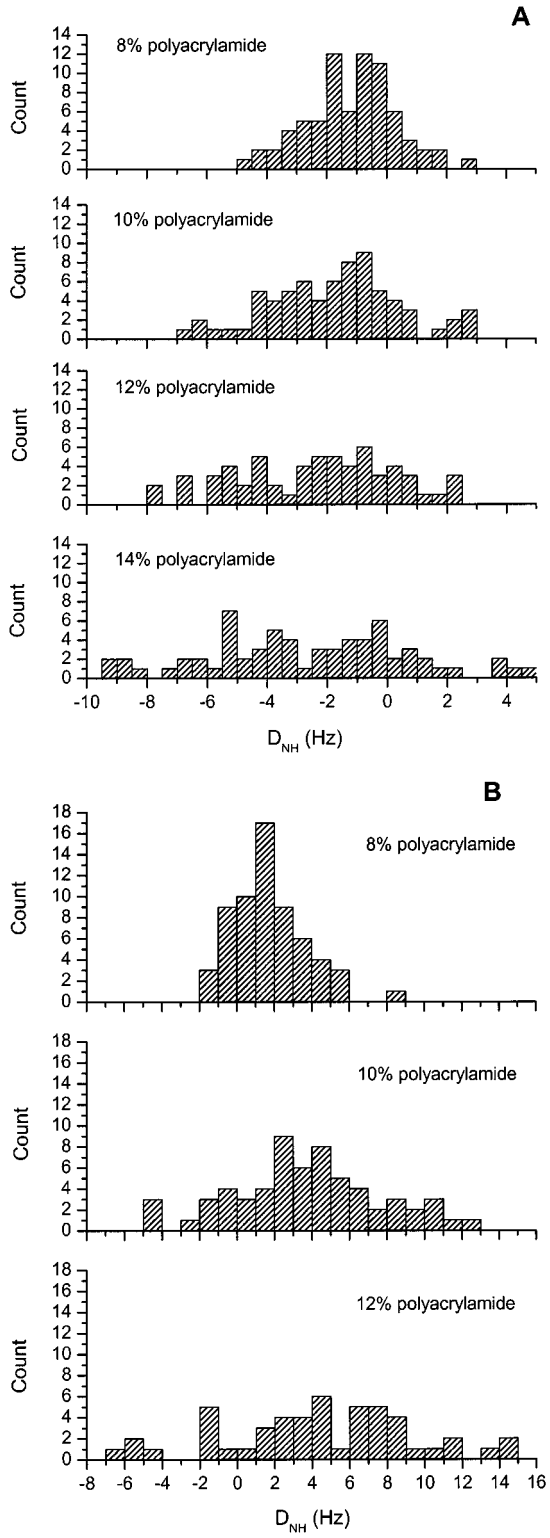

FIGURE 1: (A) Histograms of the NH residual dipolar couplings, in hertz, measured for ^{15}N-labeled Δ131Δ in compressed polyacrylamide gels. (B) Histograms of NH residual dipolar couplings measured in radially compressed (stretched) polyacrylamide gels.

For ubiquitin (76 residues), strained gels from 4% to 10% were found to give a range of dipolar couplings with only modest line broadening (*20*). A surprisingly high concentration of acrylamide is required to orient Δ131Δ. As shown in Figure 1A, on applying a more or less constant compression (length/diameter in mm 21/4.0 → 17/4.5) to polyacrylamide gels of 8%, 10%, 12%, and 14% acrylamide (1:20 bisacrylamide), the observed NH dipolar couplings increase in magnitude, from a range of +2 to −5 Hz to a range of +5 to −9 Hz. Line widths increase monotonically from an average value of 21 Hz in free solution to 26 Hz in the 14% gel. Measurement of dipolar couplings for the same compressed gel sample on three separate days gave an average standard deviation of ±0.22 Hz, a reasonable estimate of the measurement error for most experiments. The finding that higher acrylamide concentrations are required to orient Δ131Δ than folded ubquitin and protein G suggests that this denatured state is only slightly asymmetric in shape, having ratios of its inertial tensor axes close to 1.0.

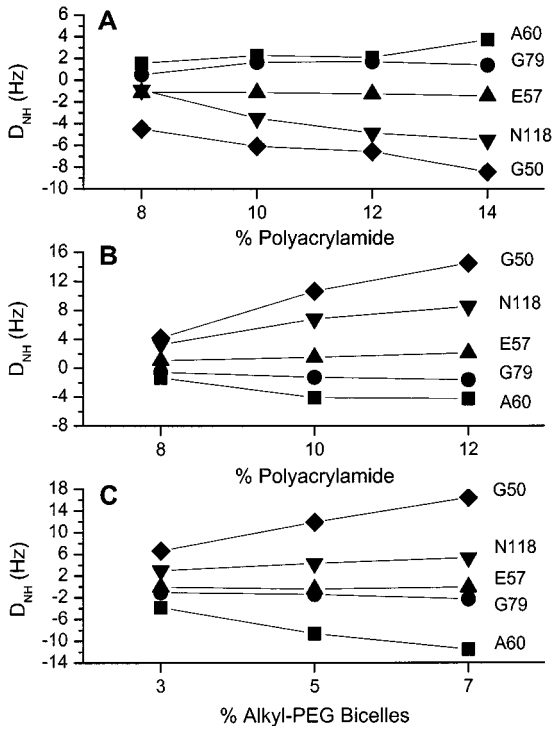

FIGURE 2: Graphs of the NH residual dipolar couplings for five residues in Δ131Δ plotted as a function of alignment conditions. (A) Percent polyacrylamide with the gel cylinder in compression. (B) Percent acrylamide with the gel cylinder in radial compression (stretch). (C) Concentration of C8E5/1-octanol bicelles as percent C8E5 by weight.

In Figure 1B, histograms show the dipolar couplings measured for radially compressed gels (o.d. 4.0 mm → 3.2 mm) of 8%, 10%, and 12%. This method of inducing strain is less well tolerated by gel cylinders, with tears usually developing when the gel is stretched to more than 1.4−1.5 times its original length. However, the magnitudes of the couplings induced can be significantly larger for an individual gel, with the range of couplings increasing to +15 to −7 Hz in 12% acrylamide. With care, one gel cylinder can be subjected to both types of strain.

In Figure 2A,B, the dipolar couplings for five residues are tracked as a function of acrylamide concentration and compression/stretch. In addition to changes in magnitude, the signs of the couplings invert between gels in compression and in stretch mode. Within the oblate ellipsoids of the compressed gel, the long axis of Δ131Δ should be aligned perpendicular to the field, whereas in the stretched gel, the long axis should be aligned with the field. As expected, this change in orientation leads to an inversion in sign.

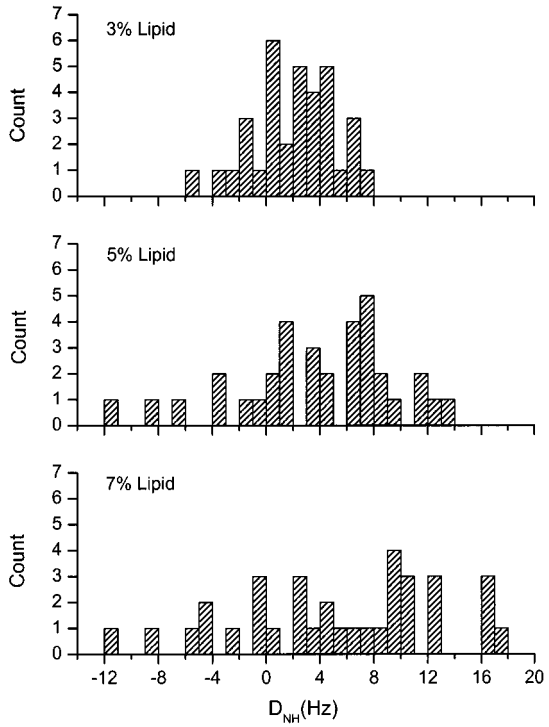

FIGURE 3: Histograms of the NH residual dipolar couplings, in hertz, measured for ^{15}N-labeled Δ131Δ in bicelles consisting of C8E5/1-octanol in a molar ratio of 0.87. The bicelle concentration is expressed as percent C8E5 lipid.

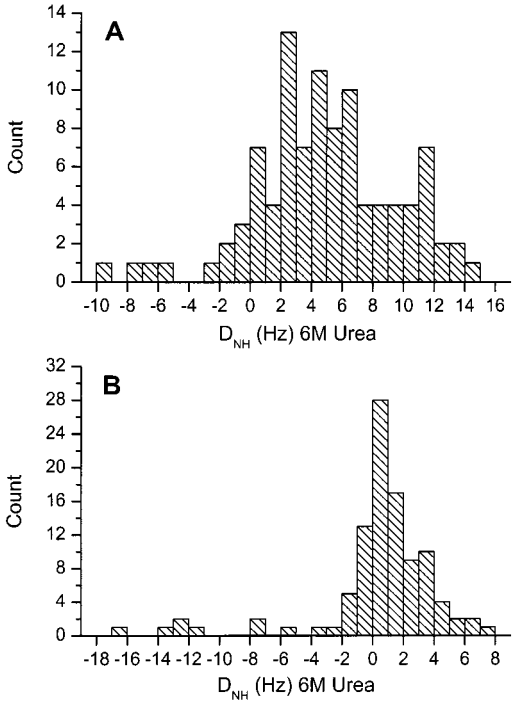

FIGURE 4: Histograms of D_{NH} for Δ131Δ in 6 M urea (A) in 5% C8E5/1-octanol bicelles and (B) in 6.5% cetylpyridinium bromide/hexanol liquid crystalline phase.

Unfortunately, dipolar couplings from samples oriented in strained polyacrylamide gels are not sufficient for use in a de novo structure determination. While compression and stretching change the sign and value of the dipolar couplings, the two data sets are different manifestations of the same alignment axes. To break the degeneracy in allowed orientational restraints consistent with the dipolar couplings, a second, significantly different alignment mechanism that utilizes different molecular axes must be found. Attempts to modulate the alignment tensor (*18*) by incorporation of negatively and positively charged monomers or hydrophobic monomers into the gel matrix or by distorting gel cavities into triaxial ellipsoids all failed to break the tight correlation with dipolar couplings measured in unmodified gels.

Alkyl-PEG Bicelles. While polyacrylamide is expected to be an inert matrix for denatured proteins, the lipid components in standard bicelle media could potentially bind to denatured proteins and thereby perturb their structure. Although phase separation was observed with all preparations of Δ131Δ in phospholipid bicelles, reasonable behavior was displayed with bicelles formed by lipids with a neutral poly(ethylene glycol) headgroup (*12*), especially upon addition of urea. Of the several alkyl-PEG/alcohol mixtures characterized by Ruckert and Otting (*12*), the C8E5/1-octanol mixture was chosen in order to avoid the more soluble alcohol 1-hexanol, which is used in the other formulations.

As the concentration of bicelles is increased from 3% to 5% to 7% (expressed as the weight percent of alkyl-PEG), the alignment efficacy increases in an approximately linear manner, as reflected in the increase in quadrupolar splitting of solvent D_2O from 17.0 to 30.8 to 45.3 Hz, respectively. Figure 3 shows histograms of the distributions of dipolar couplings as a function of percent lipid. Not included in these histograms are the couplings for the two C-terminal residues, I139 and W140. Both peaks are broadened by the bicelle media. While their dipolar couplings cannot be measured accurately, these two residues exhibit large negative couplings of less than −20 Hz, suggesting that the protonated C terminus of Δ131Δ binds transiently to the lipid bilayers. To reduce this effect, subsequent studies were carried out in the presence of urea (Figure 4A).

When the pH of the sample is lowered below the standard value of 5.3, the couplings of residues near the C terminus become larger and the lines significantly broader. Below pH 4.0, more than one-half of all peaks disappear from the ^{1}H−^{15}N HSQC spectrum. As mentioned below, full-length nuclease, which includes another nine residues at the C terminus, does not show this phenomenon, even at pH 3.0. These findings support the conclusion that, when protonated, the C terminus, which has the sequence KKEKLNIW-COOH, partitions into the bicelles' lipid interface. Presumably only the terminal isoleucine-139 and tryptophan-140 are responsible, but since the other residues in this sequence cannot be unequivocally identified in these spectra, binding may also include asparagine at position 138 and leucine at position 137. At modest urea concentrations, the extent of binding can be significantly reduced. As shown in Figure 5A, the dipolar couplings measured in this bicelle media show a tight correlation with those obtained in strained polyacrylamide gels.

Cetylpyridinium Bromide/1-Hexanol Liquid Crystalline Phase. To align denatured nuclease by a mechanism other than steric interactions, the liquid crystalline phase formed by cetylpyridinium bromide and 1-hexanol (*13*) was evaluated. At low concentrations of urea, NH peaks for the 10−20 residues nearest the carboxy terminus were broadened and displayed large negative dipolar couplings, suggestive of transient binding of Δ131Δ to this cationic lipid phase in a manner similar to that described for alkyl-PEG bicelles.

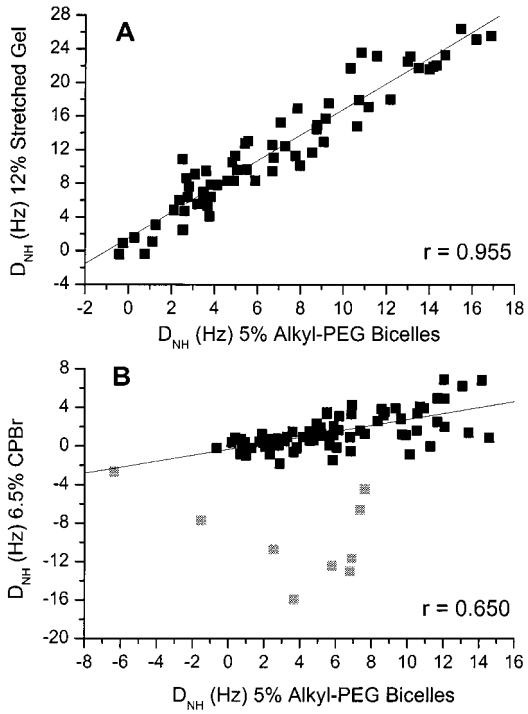

FIGURE 5: (A) Scatterplot of the dipolar couplings D_{NH} measured for Δ131Δ in 6 M urea in a 12% stretched gel versus the couplings measured in 5% C8E5/1-octanol bicelles. *r* is the Pearson correlation coefficient. (B) Scatterplot of D_{NH} in 6 M urea in 6.5% cetylpyridinium bromide phase gel versus the couplings measured in 5% C8E5/1-octanol bicelles. The gray-shaded points are omitted from the calculation of *r*.

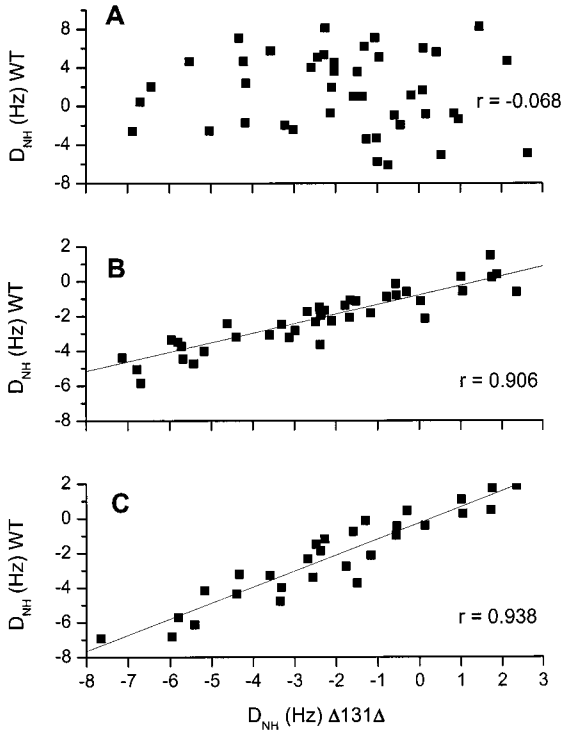

FIGURE 6: Scatterplots of D_{NH} for Δ131Δ in 6 M urea in a compressed 12% gel plotted against couplings for three states of wild-type, full-length nuclease. (A) Folded nuclease at pH 5.3 in a compressed 8% gel. (B) Nuclease denatured in 4 M urea, pH 5.3, in a compressed 12% gel. (C) Nuclease denatured by pH 3.0, 25 mM sodium citrate in a compressed 12% gel. *r* is the Pearson correlation coefficient.

However, in the presence of 6 M urea, the amide protons displayed nearly normal line widths, and the couplings from C-terminal residues were less severely perturbed, as shown in Figure 4B. This set of dipolar couplings is much less correlated with those obtained in polyacrylamide gels and with alkyl-PEG bicelles (Figure 5B). When residues showing abnormally large negative couplings are omitted from the CPBr/hexanol data (squares shown in gray), the correlation coefficient *r* is reduced to 0.65, suggesting that the axes of the alignment tensor produced by this surfactant mixture are significantly different from those gels and bicelles. This finding raises the prospect of converting the structural information in dipolar couplings into ensembles of conformations compatible with derived angular restraints.

Urea, Acid, and SDS Denatured States of Full-Length WT Nuclease. The data presented above describe dipolar couplings for the Δ131Δ large fragment of nuclease. While these and other studies of this large fragment support the conclusion that Δ131Δ represents a reasonable model of the denatured state under physiological conditions, it remains an experimental issue as to how representative it is.

Figure 6A shows the dipolar couplings measured for folded full-length nuclease under the standard conditions used for Δ131Δ, namely, pH 5.3 with no added salt or urea. No correlation is demonstrable between dipolar couplings in the folded nuclease and Δ131Δ fragment. However, a structural analysis of Δ131Δ based on paramagnetic relaxation enhancement from 14 extrinsic spin label probes revealed that this denatured fragment has, at low resolution, the same general arrangement of chain segments as does the folded state (*21*, *22*). Until more structural information is obtained, we cannot resolve this discrepancy. The simplest explanation for why we see no correlation between folded and unfolded nuclease is that the overall shape has changed sufficiently to completely alter the axes of the inertial tensor. Whereas folded nuclease is quite asymmetric and easy to orient, Δ131Δ appears to be more nearly symmetric, since high concentrations of acrylamide are required to orient it. Any large change in the directions or lengths of the axes of the inertial tensor would reduce or eliminate a correlation between the two sets of dipolar couplings.

However, on addition of 4 M urea to full-length nuclease (Figure 6B), which converts more than 99% of molecules to the denatured form, a very good correlation with Δ131Δ is found, suggesting that urea-denatured nuclease (149 residues) and Δ131Δ (131 residues) exhibit approximately the same solution structure. Removal of nine residues from each end of nuclease has not modified the residual structure that persists in these two distinct denatured states.

Full-length nuclease denatured by lowering the pH to 3.0 (*23*), without addition of neutral salts, also forms an expanded denatured state with less residual structure than Δ131Δ at pH 5.3 (*24*). Yet, as shown in Figure 6C, the dipolar couplings from this denatured form of nuclease show a similar, strong correlation with the couplings for Δ131Δ. These data suggest that there is a common topology to the denatured state of staphylococcal nuclease that is relatively insensitive to the details of how denaturation is achieved.

To examine the structure of staphylococcal nuclease denatured with anionic detergents such as SDS, a screen for dipolar couplings was carried out with a variety of detergents at different concentrations. Using 0.4 mM Δ131Δ at pH 5.3 and compressed 6% or 8% polyacrylamide gels, the follow-

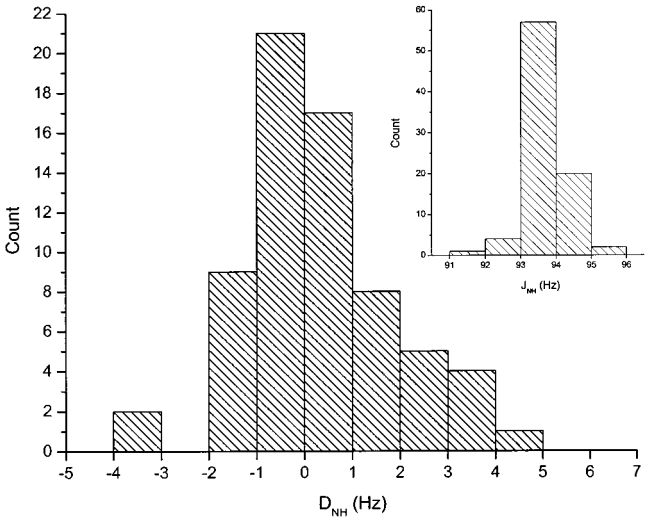

FIGURE 7: Histogram of D_{NH} for Δ131Δ in micelles formed from 42 mM sodium decanesulfonate, 8 mM sodium dodecyl sulfate, pH 5.3, and 20 mM sodium acetate, pH 5.3, at 25 °C in a compressed 6% polyacrylamide gel. Insert: Total couplings under the same conditions but in free solution.

ing detergents were evaluated: sodium dodecyl sulfate or SDS (10−50 mM); sodium decanesulfonate (10−50 mM); 1:1, 3:1, and 5:1 mixtures of sodium decanesulfonate and SDS; 1:10 and 1:20 mixtures of dodecanol with SDS; sodium octylbenzenesulfonate (50 mM); 1:3, 1:1, and 3:1 mixtures of octylbenzenesulfonate and SDS; and 1:10 to 1:20 mixtures of the dodecylpentaethylene oxide with SDS. In all cases, only small dipolar couplings were observed. Figure 7 shows the largest range of couplings measured in any of these experiments, with sodium decanesulfonate and SDS at a 5:1 ratio. It is noteworthy that, in most cases, the $^1H-^{15}N$ correlation peaks were quite sharp and reasonably disperse, with the greatest dispersion occurring when octylbenzenesulfonate was used. These observations could indicate that there may be little or no residual structure in the detergent-denatured state. Alternatively, and more likely, the time-averaged structures of nuclease/detergent micelles are nearly spherically symmetric and therefore cannot be readily aligned by steric interactions.

DISCUSSION

The physical explanation for $^1H-^{15}N$ residual dipolar couplings in a large polymer is that individual amide bond vectors must retain a relatively fixed orientation with respect to an arbitrary set of axes passing through the time-averaged structure of the polymer. The presence of both a fixed set of molecular axes and persistent bond orientations unequivocally demonstrates the persistence of unique long-range structure in the Δ131Δ fragment of staphylococcal nuclease. The fact that the denatured state can be oriented by either acrylamide gels or lipid bicelles demonstrates that the overall shape of the ensemble average conformation must not be spherically symmetric. One or more of the ratios of the axes of the inertial tensor must differ from 1.0.

It is important to note that the failure to detect dipolar couplings cannot be interpreted as evidence for the absence of long-range structure. While this may be the correct explanation, the possibility that the denatured state cannot be oriented because of a high symmetry of its time-averaged structure must be considered. Thus, the amount of residual long-range structure in the SDS-denatured state of nuclease remains an open question.

The formal possibility that a small fraction of highly structured conformations is responsible for the observed dipolar couplings is unlikely for several reasons. (1) This subset of compact conformations would have to be extremely resistant to urea, since 8 M urea reduces the magnitude of the couplings by only one-third. (2) To give rise to small averaged couplings in the context of very sharp lines, this structured subset of conformations would have to be in dynamic equilibrium with the much larger subset of random conformations, with an improbably high interconversion rate in 8 M urea of greater than 1/ms. (3) The residual dipolar couplings of this structured subset would have to be quite large to withstand a significant dilution by the large fraction of random conformations. Dipolar couplings larger than 30 Hz are typically not observed for folded proteins because the excessive orientation required leads to very severe line broadening. Therefore, the hypothetical structured subset could not have couplings much larger than 40−50 Hz and thus could only give rise to averaged couplings of 10−15 Hz by constituting a relatively large fraction of all conformations. Nothing in the data points to a bimodal distribution of conformations with respect to size or structure.

Polyacrylamide gels, alkyl-PEG bicelles, and cetylpyridinium bromide liquid crystals can be used to achieve sufficient alignment of denatured nuclease to obtain dipolar couplings in the ±5 to ±20 Hz range needed for structural work. From a practical standpoint, bicelles are simpler to use, and the measurement of the quadrupolar splitting of D_2O provides a simple quantitative measure of aligning potency of a given preparation. Polyacrylamide gels, on the other hand, can be assumed to be more inert and less prone to perturb the denatured state. Compression is technically simpler than stretching, because of the ease with which stretched gels tear when submitted to excessive strain, yet stretching (radial compression) can provide a larger net alignment.

Comparison of the dipolar couplings for nuclease denatured by removal of nine amino acids from both termini (Δ131Δ), by addition of urea, or by lowering the pH suggests that, in all three cases, the polypeptide chain forms very similar ensembles of conformations. Even the overall shape must be quite similar, because changes in the inertial tensor axes through the time-averaged structure would rotate bond vectors into new angular relationships with the magnetic field and lead to a weaker, or possibly the loss of, correlation between sets of dipolar couplings.

In addition to the angular relationship between bond vectors and the magnetic field, residual dipolar couplings also depend on the generalized order parameter *S*. The general equation (*25*) for the dipolar coupling D_{NH} between the amide proton and attached nitrogen is

$$D_{NH} = S\gamma_N\gamma_H/r^3_{NH}[A_a(3\cos^2\theta - 1) + (3/2)A_r(\sin^2\theta\cos 2\phi)]$$

where γ_N and γ_H are the gyromagnetic ratios of ^{15}N and ^1H, r_{NH} is distance between the two nuclei, A_a and A_r are the axial and rhombic components of the alignment tensor, and

θ and ϕ are the angles relating the orientation of the NH bond vector to the alignment tensor. The dipolar coupling scales linearly with *S*, which varies from 0 to 1.0. *S* is a measure of the amount of variation or disorder in the orientation of the bond vector and can be interpreted roughly in terms of the average normalized projection of the bond vector along its average orientation.

While the numbers of couplings plotted are small, the histograms in Figure 1, especially those for 8% gels, look like a "powder pattern" in solid-state NMR. In effect, the frequency and distribution of dipolar couplings is close to that expected for a set of randomly oriented bond vectors all with the same order parameter. For Δ131Δ, the order parameters involved in ^{15}N relaxation have been measured and vary from 0.4 to 0.85 (*26*). However, ^{15}N relaxation order parameters report motional averaging that occurs in less than 1 ns, whereas the order parameter relevant to dipolar couplings covers a time scale 6−7 orders of magnitude longer. Thus, the general appearance of the histograms in Figure 1 suggests that, on the time scale of milliseconds, all residues in Δ131Δ may have approximately the same order parameter (*27*, *28*), even though they have quite different values on the nanosecond time scale. If this should prove to be a good approximation, the structural interpretation of residual dipolar couplings in denatured proteins will be greatly simplified.

To calculate ensembles of conformations consistent with the angular restraints implied by sets of measured residual dipolar couplings, the degeneracy of allowed angles relative to the magnetic field must be broken by obtaining data derived from one or more different alignment tensors. The data shown in Figure 5 demonstrate that these three media provide the alignment tensor variation required to use sets of dipolar couplings in structure calculations. Attempts are in progress to identify consistent patterns in the structure of short peptide segments of Δ131Δ from dipolar couplings obtained in 6 M urea, by searching for fragments of folded proteins with similar patterns of dipolar couplings (*29*).

NOTE ADDED IN PROOF

Ubiquitin and eglin C denatured in 8 M urea, pH 3.0, and aligned in alkyl-PEG bicelles give a range of NH residual dipolar couplings similar to those shown in Figure 3 (Briggman and Shortle, unpublished observations).

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