Accelerated Publications

Direct Demonstration of Structural Similarity between Native and Denatured Eglin C†

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ABSTRACT: To characterize the long-range structure that persists in the unfolded form of the 70-residue protein eglin C, residual dipolar couplings (RDCs) for HN−N and HA−CA bond vectors were measured by NMR spectroscopy for both its low pH, urea denatured state and its native state. When the data sets for the two different structural states were compared, a statistically significant correlation was found, with both sets of dipolar couplings yielding a correlation coefficient of $r = 0.47$ to 0.51. This finding directly demonstrates that the denatured state of eglin C has a nativelike global structure, a conclusion reached indirectly for staphylococcal nuclease by combining two different types of NMR data. A simple computer simulation showed that the degree of variation in phi and psi angles that yields the RDC correlation of $r = 0.5$ was inversely dependent on the statistical segment length, ranging from ±6 to ±30 degrees at the upper limit. Stable nativelike topologies that persist on unfolding would explain the rapid refolding kinetics displayed by many proteins and might provide a natural barrier against amyloid fibril formation.

The denatured states of proteins play important roles in folding and in numerous biological and pathological phenomena. In addition to being the starting point for experimental analysis of the kinetics of folding, the denatured state can interact with intracellular chaperones, proteases, and the cell's transport machinery; in some cases, it readily aggregates to form insoluble amyloid fibrils. While protein chemists have long presumed that denatured proteins are more or less random, experimental data over the past 10−20 years has shown that significant residual structure often persists (*1*−*7*). Obviously, the details of this persistent structure could provide important clues to a more complete understanding of how proteins are able to fold so rapidly and how they avoid the formation of insoluble aggregates.

Multiple technical problems limit the utility of experimental methods in probing the residual structure in denatured proteins, many being a direct consequence of the fact that denatured proteins do not have a single conformation. Instead, they populate many diverse, rapidly interconverting conformations in a complex and dynamic ensemble. Consequently, experimental data always represent ensemble-averaged parameters. NMR[1] methods can provide useful information about local structure, permitting identification of persistent turns and segments of alpha helices (*3*−*7*). By detecting segments undergoing slower or smaller structural fluctuations, spin relaxation methods can identify regions with residual structure in denatured proteins (*8*−*11*), although the interpretation of the relaxation data is often problematic when the molecule is very dynamic (*12*, *13*). While NMR studies have identified persistent local structure in a reasonable number of denatured proteins, such studies have not led to serious reconsideration of random-coil models for describing the statistical properties of denatured proteins.

A more complete picture of the denatured state requires characterization of long-range structure. One NMR method that has found some application, paramagnetic relaxation enhancement, attaches spin labels at sites on the protein surface, permitting distances between residues of 10−20 Å to be measured (*14*−*17*). Unfortunately, these data cannot be converted to precise constraints because many of the assumptions used in the calculation of intramolecular distances are violated to varying degrees by the denatured state ensemble (*15*).

Measurement of residual dipolar couplings (RDCs) in folded proteins is providing a new source of information for

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[1] Abbreviations: NMR, nuclear magnetic resonance; RDC, residual dipolar coupling; HSQC, heteronuclear single quantum coherence; IPAP, inphase antiphase.

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FIGURE 1: Schematic representation of the three-dimensional structure of folded eglin C (1cse) (*24*).

refining protein structure (*18*, *19*). Because this NMR parameter reflects the orientation of bond vectors relative to a fixed set of molecule axes, it reports on global or long-range structure in a distance-independent manner. Recently, denatured proteins have been demonstrated to orient under the same conditions used for folded proteins and to display similar ranges of residual dipolar couplings (*20*). In the case of staphylococcal nuclease, the denatured states attained by three different denaturants—genetic mutations, low pH, and high urea concentration—have been shown to display quite similar dipolar coupling and therefore similar structures (*21*). More importantly, studies of a large denatured fragment of nuclease by paramagnetic relaxation enhancement (*14*, *15*) and residual dipolar couplings led to the surprising conclusion that a nativelike topology persists, even when nuclease is denatured in 8 M urea (*22*).

To address the generality of this surprising conclusion to other soluble proteins, studies were undertaken of the 70-amino acid protein eglin C. This small monomeric protein is a serine protease inhibitor whose structure has been determined at high resolution by NMR methods (*23*) and X-ray crystallography (*24*). Its structure consists of a four-strand beta sheet flanked on one side by an alpha helix and on the other by an exposed loop involved in protease binding (Figure 1). Eglin C is soluble at high concentrations and displays rapid two-state folding behavior. In this report, we demonstrate that denatured eglin C maintains a nativelike topology by directly comparing dipolar couplings measured for the native and denatured states. In addition, simple computer simulations are used to obtain a semiquantitative measure of the structural similarity of these two very different states of eglin C.

MATERIALS AND METHODS

Sample Preparation. In this study, the mutant F10W/V14A was used to facilitate denaturation by lowering stability, and is referred to as "eglin C". Eglin C was purified by ion exchange and size exclusion chromatography as described previously (*25*). The protein was dissolved in 20 mM KH_2PO_4 (pH 7.0) with 50 mM KCl for the characterization of the native state. For analysis of the denatured state, 20 mM citrate buffer (pH 3.0) with 8 M urea was used.

NMR Spectroscopy. All NMR data were collected on a Varian Inova 600 spectrometer, at 20 °C for the denatured state and at 37 °C for the native state. ^{15}N-^{1}H HSQC spectra were recorded with 0.5 mM ^{15}N enriched protein with 1024 complex points and 128 increments. The backbone ^{15}N and ^{1}H resonances of eglin C denatured form were assigned using the HNCACB pulse sequence (*26*), and the resonance assignments are provided in the supplementary material. Those of its native form were assigned as described previously (*25*). Eight-percent and 12% cylindrical polyacrylamide gels (1:20 bis-acrylamide) were used for the measurement of dipolar couplings of native form and denatured forms, respectively. Typically a 21-mm-long gel cylinder loaded with protein by overnight soaking was compressed to approximately 17 mm as described previously (*20*, *22*). HN−N dipolar couplings were measured using the ^{15}N-^{1}H IPAP-HSQC pulse sequence (*27*) with 12 Hz/point resolution in the ^{15}N dimension, and the final spectra were processed to about 0.2 Hz/point after zero-filling. Eglin C labeled with ^{15}N and ^{13}C was used for the HA−CA dipolar coupling measurements, employing the HNCO-based pulse sequence (*28*) in which HA−CA couplings are transferred onto the carbonyl ^{13}C resonance. Data were collected with 19 Hz/point resolution in the ^{13}C dimension, and processed to about 0.3 Hz/point after zero-filling.

Computer Simulations. The three-dimensional coordinates of all atoms of 1cse (*24*), a 1.2-Å resolution X-ray crystal structure of eglin C, were loaded from the pdb file. After addition of alpha hydrogens to all alpha carbons and amide hydrogens to all nonprolines, segments of the structure of length *N* amino acids were loaded into a separate matrix. Starting at the amino terminus of the segment, the dihedral angle psi of residue 1 was rotated by plus or minus X degrees (with ± X/4 variation), moving each residue after 1 until the carboxy terminus was reached. Such random psi rotations were applied to residues 1 to *N*. Then starting with residue *N*, phi was varied by plus or minus X degrees (± X/4), moving each residue until the amino terminus was reached, with random phi rotations applied to every residue from *N* to 1. This bidirectional approach allows a segment of length *N* to be varied in isolation, without a stationary point. To modify the entire protein, successive nonoverlapping segments of length *N* were varied across the entire chain. To retain the same alignment tensor as the native state, HN−N and HA−CA bond vectors of residue i in the native state were replaced by the corresponding vectors from one "randomized" fragment of length *N*. Residual dipolar couplings were calculated from three-dimensional coordinates using the program PALES (*29*).

RESULTS

In 8 M urea at pH 3.0 and 20 °C, eglin C appears to be in an expanded denatured state as reflected by the expected number of sharp peaks in the ^{15}N-^{1}H correlation spectra, with chemical shift dispersion in both dimensions limited to random coil values (Figure 2B,D). In contrast, eglin C in the native form at pH 7.0 and 37 °C shows ^{15}N-^{1}H correlation spectra with well-dispersed peaks as characterized previously (Figure 2A,C) (*23*). HN−N dipolar couplings and HA−CA

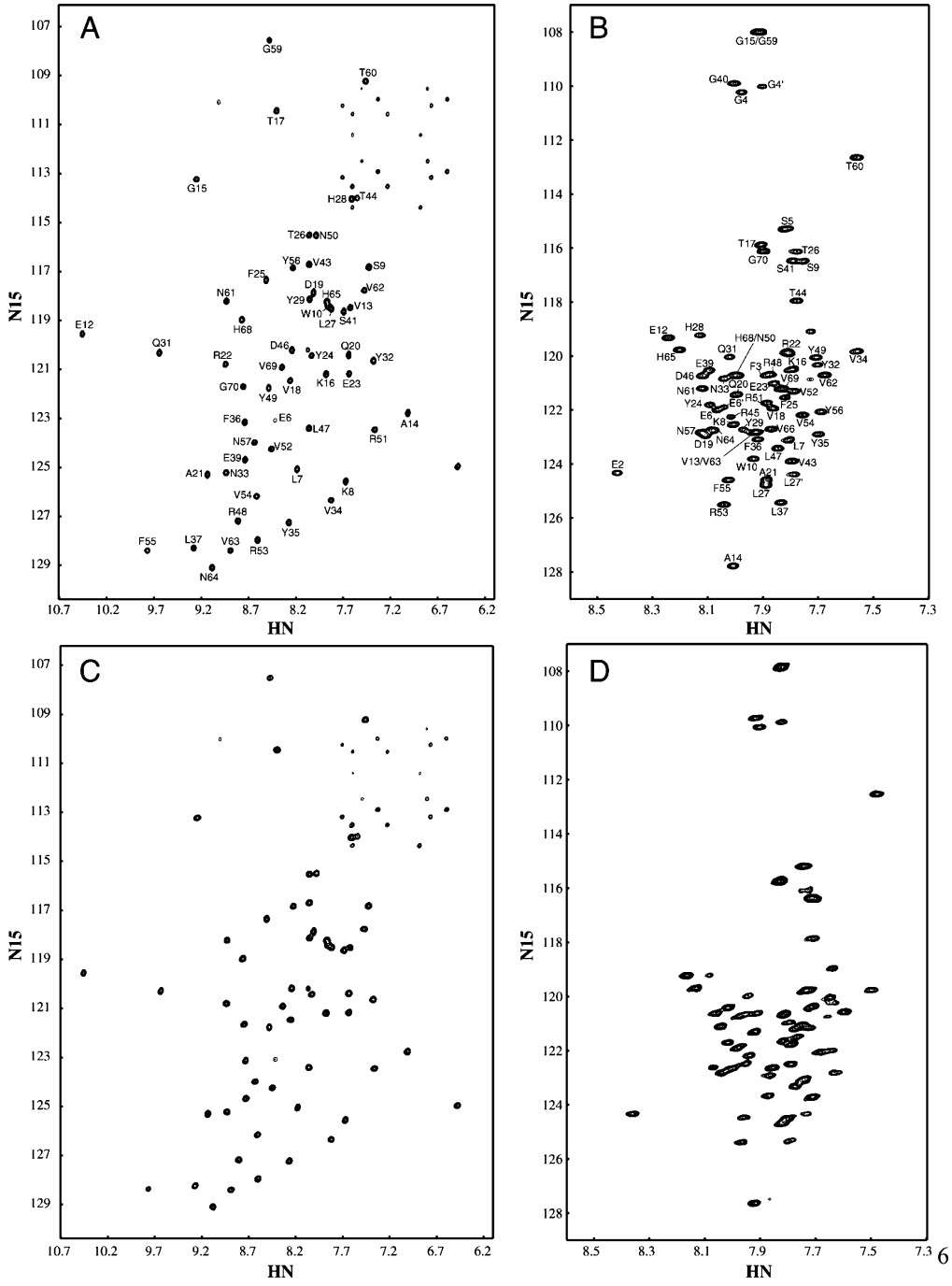

FIGURE 2: ^{1}H-^{15}N correlation spectra (HSQC) of the native (A, C) and denatured (B, D) eglin C under isotropic (A, B) and strained polyacrylamide gel constrained (C, D) conditions.

dipolar couplings were measured in strained polyacrylamide gels (*30*, *31*) for both native and denatured forms using the conditions described above. NMR spectra in polyacrylamide gels were essentially identical to those obtained in free solution, suggesting that no major structural changes are induced by entrapment in the gel (Figure 2).

The native form shows a large distribution in dipolar couplings for both HN−N and HA−CA nuclei, while the unfolded form shows a smaller but significant range of values (Figure 3). By plotting the values of RDCs for both forms as function of sequence position, a suggestion of a similar pattern of variation can be seen. The large ranges of dipolar couplings for the denatured form is distinctly different from the monotonic distribution predicted for a hypothetical random coil chain (*32*), further confirming that the denatured eglin C is not a random coil.

A modest correlation is seen between HN−N couplings measured in the native and denatured states (Figure 4A), and a similar correlation is seen between pairs of HA−CA couplings (Figure 4B). Standard correlation analysis on these two scatter plots yields a Pearson correlation coefficient $r = 0.51$ for the HN−N couplings and $r = 0.47$ for the HA−CA couplings. From statistical tables that assume a normal distribution of values in both dimensions, the p values are estimated to be $p < 0.002$ and $p < 0.008$, respectively. Contributing to the evidence that these correlations are not occurring by chance are the findings that both correlations are positive and that both correlation coefficients are similar

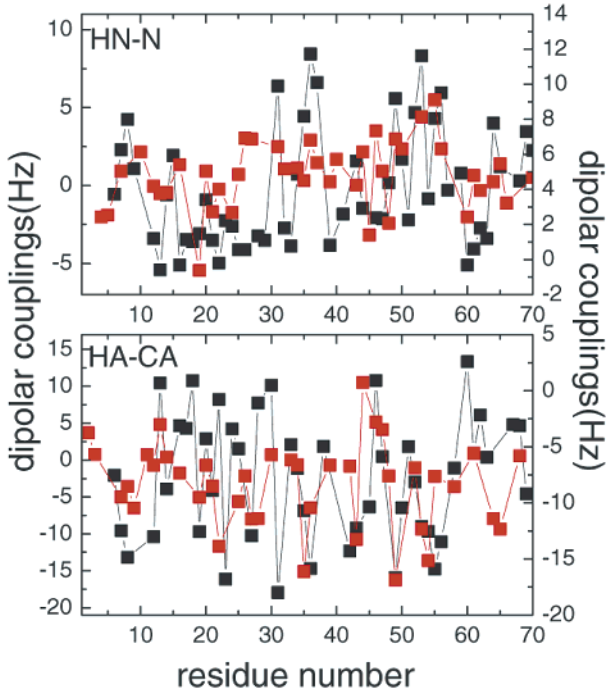

FIGURE 3: Dipolar coupling of native (black) and denatured (red) eglinC for HN−N (top) and HA−CA (bottom) nuclei plotted as a function of residue position. Left axes are for the native form and right are for the denatured form. Only nonoverlapped peaks with Lorentzian line shapes were analyzed.

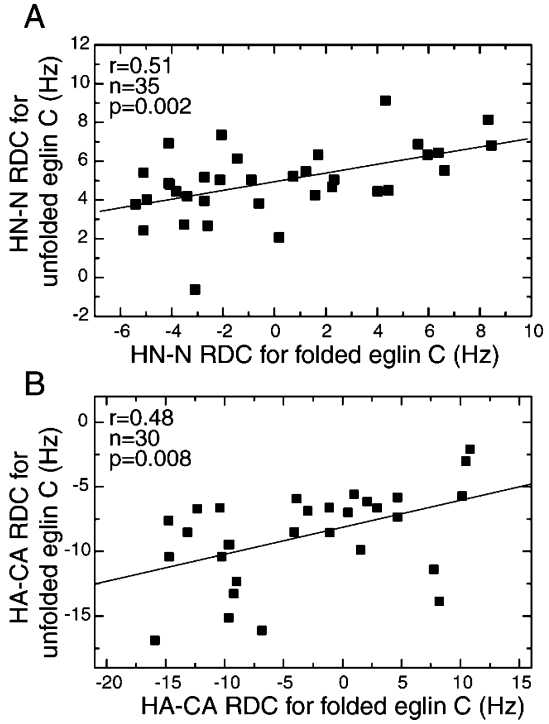

FIGURE 4: Scatter plots comparing HN−N (A) and HA−CA (B) dipolar couplings between native and denatured eglin C. *r*, *n*, and *p* represent the Pearson correlation coefficient, number of points, and standard *p* value, respectively.

in value, as expected if the data reflect structural similarity between the two states.

To address the concern that the level of significance implied by the Pearson correlation coefficients and standard *p* values may not be appropriate in the present case, we employed two nonparametric analyses of the data. Simple

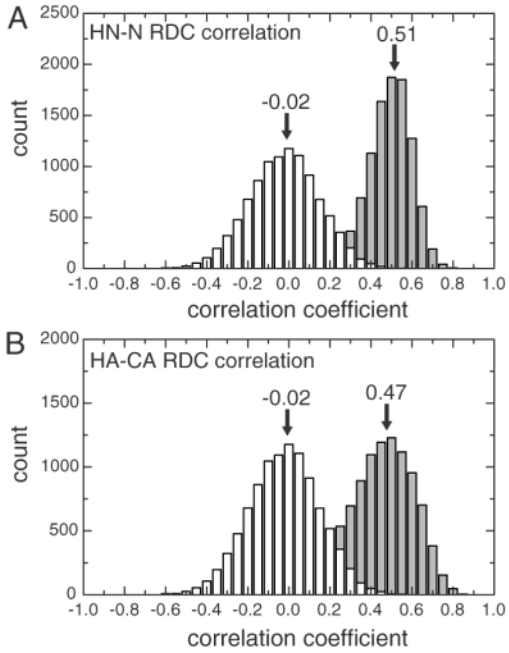

FIGURE 5: Nonparametric statistical testing on the HN−N (A) and HA−CA (B) dipolar coupling datasets. Distributions of correlation coefficients from the randomization testing are shown as white histograms, and those from balanced bootstrap resampling of the original data are shown in gray. Arrows indicate the mean values.

randomization testing of significance (*33*) was applied by randomly swapping values of native and denatured couplings for the paired data points and then calculating the resulting correlation coefficient. Ten thousand trials for the HN−N and HA−CA data sets gave mean values of *r* near zero (Figure 5). The frequencies with which the correlation coefficient of randomized data reached the observed values of 0.51 and 0.47 were 0.0013 and 0.005, respectively.

To search for biases in the data and to estimate the standard deviation of the correlation coefficient in the two cases, balanced bootstrap resampling was employed (*33*). Ten thousand resampling trials produced essentially the same mean value of *r*, 0.514 and 0.469 for the HN−N and HA-CA couplings, suggesting the correlation does not result from a small number of "atypical" data points (Figure 5). In addition, the estimated standard deviations of the two bootstrap means (0.11 and 0.16) suggest that 97% of the time, a hypothetical resampling of HN−N data would yield a correlation with a *p* value of 0.04 or less, and 84% of the time the same would hold for the HA−CA data.

Since calculation of physically realistic conformations consistent with observed dipolar couplings requires much more data collected with different alignment tensors (*34*), the structural similarity between the native and denatured states cannot yet be described in physical terms. The only recourse at present is to employ computer simulations in an effort to identify what range of structural perturbation could convert the native structure into a structure whose dipolar couplings would yield correlation coefficients around 0.5. Perhaps the simplest such simulation is to randomly change phi and psi angles in the native structure by some average amount, recalculate the dipolar couplings for this modified conformation, and correlate these couplings with the experimental data for the native state. To avoid the change in shape that must accompany any change in structure, the modified

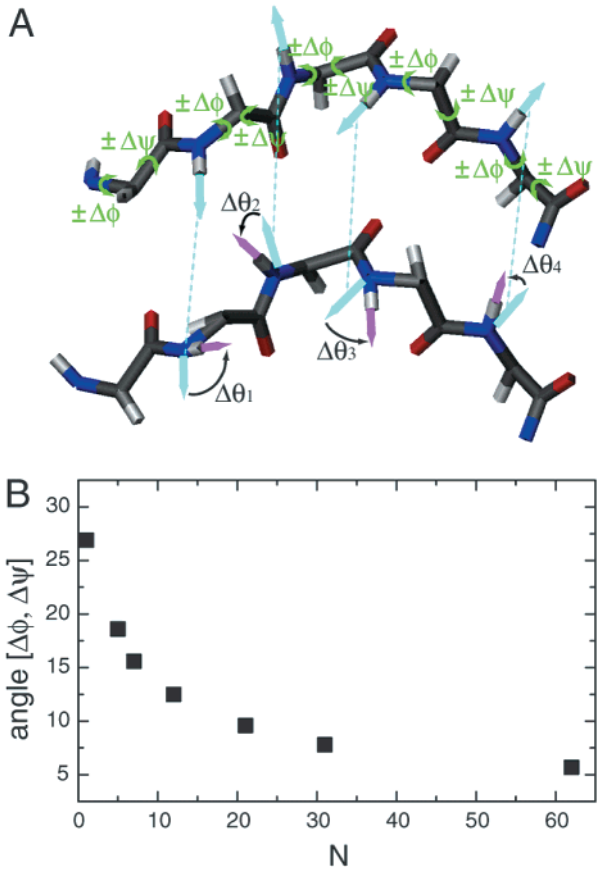

FIGURE 6: Simulation of a "virtual" denatured state. (A) A segment of eglin C *N* residues in length underwent random variations in phi/psi by plus or minus (X degrees ± X/4). The altered HN−N and HA−CA bond vectors were then translated back onto the native structure. As the length of the segment increases, variation in bond vector orientation ($\Delta\theta$) becomes significantly larger than variation in phi/psi ($\Delta\phi$, $\Delta\psi$). (B) For segment length *N* and variation in ($\Delta\phi$, $\Delta\psi$), 250 model structures of the "virtual denatured state" were generated. HN−N dipolar couplings of all structures were calculated and correlated with the experimental values for the native structure. Variations in ($\Delta\phi$, $\Delta\psi$) that yielded a mean correlation coefficient of 0.5 were determined and plotted for various *N* values.

HN−N and HA−CA bond vector orientations were transferred back onto the native structure before calculation of the couplings, giving a "virtual denatured state" that is entirely native except for these two sets of bond vectors (Figure 6).

A grid search of different random variations in phi and psi that produced a correlation coefficient of 0.5 between native and virtual denatured states yielded a value of ± 6 degrees. Implicit in this calculation is the assumption that changes in phi and psi of each residue effectively rotate the remainder of the protein as a rigid body. In the denatured state, it is more reasonable to assume that the extent of structural change produced by phi/psi variations does not propagate to both ends of the chain but rather is limited to segments several residues in length, known in polymer physical chemistry as the statistical segment length (*35*). Figure 6 shows that as the statistical segment length is reduced from 62 residues, the amount of average variation in phi/psi that yields a correlation coefficient of 0.5 increases, reaching a value of ± 27 degrees in the case where each residue's changes in phi/psi are independent of the rotations produced by all others. One inference that can be drawn from these simulations is that a very high level of structural similarity is required to observe a direct correlation between native and denatured dipolar couplings.

DISCUSSION

A direct comparison between RDC values from the denatured and native eglin C provided a statistically significant correlation coefficient around 0.5. The simplest explanation for this correlation is that at the global, long-range level, there is an underlying structural similarity. In particular, these two states must have a similar overall shape, described by similar ratios of the moments of inertia tensor, and there must be similar ensemble-average bond vector orientations relative to these axes (*18*, *19*). Although the argument supporting this explanation is based entirely on statistical correlations, it is compelling nonetheless because the physical bases of residual dipolar couplings are well understood. There are at least three mechanisms by which the correlation between sets of dipolar couplings collected on very similar structures can be lost: (a) changes in shape, (b) changes in the average bond vector orientation, and (c) large variations in bond vector motion. However, to our knowledge no plausible physical mechanism has been proposed that could produce a spurious correlation.

While the RDC values for folded eglin C are more or less equally distributed among positive and negative values (Figures 3 and 4) as is typical of folded globular proteins (*36*), the values for denatured eglin C show a highly biased distribution, with mostly positive (HN−N) or negative (HA−CA) values. Similar distributions in RDC values have been observed for denatured proteins such as staphylococcal nuclease (*20*−*22*), protein G B1 domain (*37*), ubiquitin (Ohnishi & Shortle, unpublished data), intestinal fatty acid binding protein (Ohnishi et al., unpublished data), as well as short peptides (*38*) and the S-peptide of RNase A (*39*), suggesting this pattern may be common to most or all flexible polypeptides. Although the mechanism behind this asymmetric pattern is not clear, one possibility is that it is a consequence of dynamic averaging among many different orientations.

The formal possibility that a small fraction of highly structured conformations in the conformational ensemble of denatured eglin C is responsible for the weak correlation in dipolar couplings between the two states is unlikely for the following reasons. (1) 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. (2) 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 bimodal distribution of conformations with respect to size and structure.

As mentioned above, analysis of a large denatured fragment of staphylococcal nuclease by paramagnetic relaxation enhancement directly demonstrated the persistence of a nativelike topology in the absence of denaturant (*14*, *15*). Subsequent studies using dipolar coupling measurements revealed that this topology persists in the presence of high concentrations of urea (*22*), after replacement of 10 large hydrophobic core residues with similarly shaped polar side chains in 8 M urea (*21*), and after replacement of seven surface charged residues with glycines (Ackerman & Shortle, unpublished data). Unlike eglin C, however, no correlation between dipolar couplings measured for native and denatured nuclease could be demonstrated (*20*). Presumably in this case, either the shape of the denatured state is significantly different from that of the native state, or the ensemble-averaged bond vector orientations deviate to a larger extent from their native values.

It has been shown both theoretically (*40*) and experimentally (*41*) that RDC values for the same protein obtained in two different alignment systems may show no correlation. Since polyacrylamide gels establish a molecular alignment through steric restraining mechanisms, a significant change in the overall molecular shape, even though a nativelike topology is maintained, would cause a change in the molecular alignment tensor, leading to a significant loss in the RDC correlation. Thus, even if all proteins possess a denatured state with nativelike topology, it is likely that some will not show a direct correlation between native and denatured dipolar couplings.

Recently, Ding et al. showed a comparison of RDC values for the native and acid denatured states of protein G B1 domain, yielding a Pearson correlation coefficient $r = +0.37$ for a relatively small size of dataset ($n \sim 26$, $p \sim 0.05$) (*37*). Although the authors concluded that no structural similarity between these two states exists because of the low statistical significance, these data may represent yet another example of a persistent nativelike topology.

To date we have experimentally demonstrated nativelike ensemble averaged structure for the denatured states of staphylococcal nuclease by a combination of paramagnetic relaxation enhancement methods and RDCs (*22*) and of eglin C, by RDC measurements alone. Recently, molecular dynamics simulations have suggested that several small proteins display nativelike ensemble averaged structures in their unfolded states (*42*). Similar conclusions have been reached for human alpha-lactalbumin, both by computer simulations combined with NMR data (*43*) and by neutron-scattering methods (*44*). In addition, a nativelike arrangement of hydrophobic residues was inferred for denatured hen lysozyme on the basis of NMR relaxation data (*45*). Thus, the traditional concept of denatured proteins as random-coils has been seriously questioned for several proteins. Increasing evidence supports the conclusion that most, if not all, denatured proteins maintain some elements of nativelike structure.

While the idea that all denatured proteins might retain long-range structure resembling their native state may seem surprising, it would explain two otherwise puzzling phenomena common to many proteins. Antibodies raised against medium-length peptides often cross react at high affinity with the same sequence in folded proteins (*46*), a less surprising result if peptides typically sample a relatively limited conformation space around the native structure. Consistent with this conclusion is the finding that 6 out of 6 short peptides analyzed in strained gels could be oriented and displayed residual dipolar couplings (*38*). Many proteins, even some more than 200 amino acids long, can fold in milliseconds. Such rapid folding could be a consequence in part of prepositioning of the chain near the native state, greatly reducing the conformational search required to find the folded conformation. In effect, the free energy expended by the ribosome during protein synthesis pays much of the cost of reducing the chain entropy from that of a hypothetical random coil to a more modest value, making the entropy still to be lost on folding small enough to be overcome by the weak forces that stabilize the native state.

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