Thermally Denatured Ribonuclease A Retains Secondary Structure As Shown by FTIR†

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ABSTRACT: Fourier transform-infrared (FTIR) spectroscopy has been used to test for the presence of nonrandom structure in thermally denatured ribonuclease A (RNase A) at pH* 2.0 (uncorrected pH measured in D_2O). The amide I spectral region of the native and thermally denatured protein was compared. A substantial decrease in the amount of β-sheet and α-helix and a corresponding increase in the amount of turn and unordered structure was observed on thermal denaturation. The results indicate that thermally denatured RNase A contains significant amounts of secondary structure (11% helix and 17% β-sheet), consistent with previous results reported for circular dichroism, and with a relatively compact structure, as revealed by dynamic light scattering. These results are in contrast to those of amide protection experiments reported recently [Robertson, A. D., & Baldwin, R. L. (1991) *Biochemistry 30,* 9907–9914] which indicated no stable hydrogen-bonded structure under these experimental conditions. Possible explanations for this apparent discrepancy are given.

Since most of the functional properties of proteins arise from their native conformations, most studies have been done on the native state rather than on nonnative states; however, during the past few years more attention has been focused on intermediate and denatured states due to their involvement in three important phenomena: (1) protein folding and stability, (2) transport across membranes, and (3) proteolysis and protein turnover (Dill & Shortle, 1991).

In order to understand the structural basis for the thermodynamic stability of proteins, one needs structural information about both the native and denatured states. Normally for most proteins only two states, native and denatured, are in equilibrium. The free-energy difference between these states determines the stability of the protein. For RNase A, structural data for the native state have been obtained from diffraction and spectroscopic methods, but detailed structural information regarding the denatured state is largely lacking. Considerable evidence suggests that denatured states do not always behave like random-coil polypeptides. Both theoretical and experimental evidence exists for the presence of nonrandom structure in thermally denatured proteins. Various spectroscopic studies have suggested that thermally denatured RNase A has a nonrandom structure (Aune et al., 1967; Matthews & Westmoreland, 1975; Howarth, 1979; Labhardt, 1982; Privalov *et al.*, 1989). Circular dichroism (CD)[1] data suggest that there may be a significant amount of structure in thermally denatured RNase A under aqueous conditions (Labhardt, 1982) and in the presence of aqueous methanol (Lustig & Fink, 1992). On the other hand, calorimetric data indicate the absence of extensive hydrophobic contacts of the type seen in the native state and that all the hydrophobic groups are solvated (Privalov et al., 1989), whereas hydrodynamic data for thermally denatured RNase A are consistent with a relatively compact conformation (Nicoli & Benedek, 1976; Sosnick & Trewhella, 1992).

CD and hydrogen exchange experiments were used by Robertson and Baldwin (1991) to test for the presence of nonrandom structure in thermally denatured RNase A. Quenched-flow and 2D ^1H NMR spectroscopy showed that the exchange is approximately that predicted for a disordered polypeptide. In contrast, far-UV CD studies showed the presence of residual helix, which was found to disappear upon addition of Gdn·HCl. Robertson and Baldwin (1991) raised the question of whether the residual structure suggested by the CD spectrum of thermally denatured RNase A may be an artifact. In order to resolve this apparent paradox, we have used an alternate technique to characterize the secondary structure of the thermally denatured state, namely, FTIR.

MATERIALS AND METHODS

Highly purified bovine pancreatic ribonuclease A (homogeneous by PAGE) was purchased from Calbiochem. D_2O was obtained from ICN biomedical; DCl was from MSD Isotopes. The native sample was suspended in D_2O (pH *7.0) (uncorrected pH measured in D_2O), and hydrogen–deuterium exchange was achieved by heating the sample to 62 °C for 2 h and then cooling to room temperature. This led to complete H/D exchange as judged by the absence of the amide II band (which reflects, predominantly, N–H deformation vibrations, but not N–D). RNase A (D-exchanged) was denatured by dissolving it in D_2O (pH* 2.0) and heating it to 65 °C for 40 min. The sample was then immediately injected into a cell which was maintained at 65 °C.

FTIR data were acquired on a Nicolet 800 spectrophotometer equipped with a mercury–cadmium–telluride detector. A demountable liquid flow cell with CaF_2 windows and 50 μm Teflon spacer was used. The sample chamber was purged with nitrogen for 30 min between samples. Data were collected with the aperture open to the fullest setting to allow maximum throughput of optical energy to the detector; 2000 scans were collected for each set with a 2-cm^{-1} resolution. The signal produced by protein solutions at 6–24 mg/mL resulted in amide I bands with S/N ratio greater than 500 for the native

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[1] Abbreviations: CD, circular dichroism; FTIR, Fourier Transform infrared spectroscopy; FSD, Fourier self-deconvolution; SVD, singular value decomposition.

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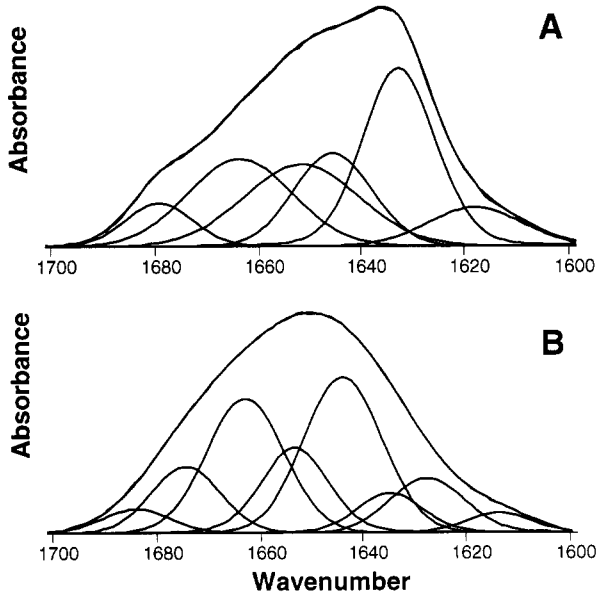

FIGURE 1: Comparison of the amide I′ spectra of native RNase A with that of thermally denatured RNase A. The curve-fit amide I′ spectra and the contribution of the different secondary structures for native (A) and thermally denatured (pH* = 2.0, 65 °C) (B) RNase A are shown. The pathlength was 50 μm, and the IR cell windows were CaF_2.

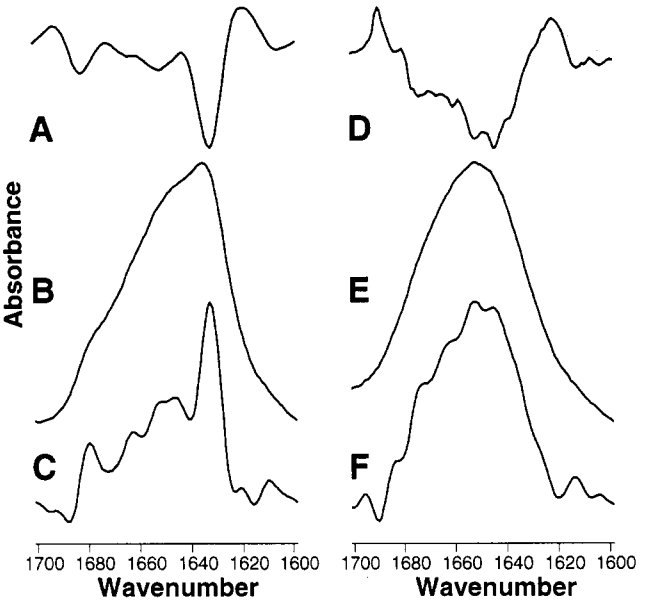

FIGURE 2: Resolution-enhanced FTIR spectra of RNase A. Comparison of the second-derivative (A and D) and the FSD (C and F) deconvoluted spectra for the native (A–C) and thermally denatured (D–F) RNase A.

and the thermally denatured protein. The experiments were done in triplicate at various protein concentrations. The spectra were superimposable regardless of concentration.

The software used for data collection and analysis included Nicolet SX software version 4.4 and LabCalc (Galactic Industries Corp.). Protein spectra were ratioed against spectra of buffer alone. The position of component peaks in the amide I band were determined using second-derivatives and Fourier self-deconvolution. Savitsky–Golay (1964) second derivatives were taken with 11–17 convolution points. The Fourier self-deconvolution program supplied with LabCalc was used with a γ factor of 2.5–5 (Griffiths and Pariente, 1986) and a filter that ranged from 0.2–0.25. Great care was taken to correctly subtract any contribution from residual water vapor in the sample chamber. The quality of the water vapor subtraction was evaluated by comparing the protein spectra second derivative with the second-derivative water-vapor spectrum.

Curve-fitting was performed using a modified version of the program provided with LabCalc. Initial peak positions, widths, and intensities were determined from the second-derivative and FSD spectra. To generate initial parameters for fitting the raw spectrum, the FSD was initially fit with a linear baseline and all other parameters free. When the reconstructed spectrum was indistinguishable from the actual FSD spectrum, iterations were stopped. The FSD peaks were then widened by γ and used as initial parameters for fitting the raw spectrum. For this procedure, fixed peak positions and baseline were used. After 200 iterations the peak centers were freed, and 50 more iterations were performed. There was less than 1-cm^{-1} change in the peak position during this final stage. The fit was not improved by increasing this number of iterations.

CD spectra were obtained using an AVIV model 60DS instrument, with a thermostated cell holder. A 0.01-mm path length cell was used with a 20 mg/mL protein concentration. Rayleigh light scattering experiments were performed at 330 nm using 12 and 24 mg/mL RNase A, pH* 2.0, 65 °C.

Table 1: Band Positions (cm^{-1}) and Secondary Structure Assignments for the Amide I′ Band of RNase[a]

	α	β	turns	unordered
native	1652	1632, 1620	1680, 1663	1645
denatured	1653 ± 0.1	1635 ± 1.0, 1628 ± 1.3	1684 ± 0.4, 1674 ± 0.6, 1662 ± 1.1	1644 ± 0.4

[a] The band positions were determined by second-derivative and fast Fourier self-deconvolution (see text).

RESULTS

Previous studies have shown that no further CD spectral changes occur in the *thermal* unfolding of RNase A at temperatures above 65 °C at pH* 2.0, and these were the conditions used in the amide protection experiments (Robertson & Baldwin, 1991). We used identical conditions for the FTIR and CD experiments reported here. The amide I region of the FTIR spectra of native and thermally denatured RNase A (pH* 2 at 65 °C) is shown in Figure 1. Visual inspection of the amide I envelope of the native and denatured states revealed a striking difference in the band shape between them. For the native state, the band is fairly asymmetric and has a peak maximum at 1635 cm^{-1} which corresponds to β structure. In contrast, the denatured protein has a broad symmetric peak centered around 1649 cm^{-1}, indicative of the predominance of unordered structure.

More detailed information is found in the resolution-enhanced spectra. Figure 2 shows the FSD deconvoluted amide I spectra and the second derivatives of the spectra for the native and denatured protein. The agreement of peak positions for these two resolution-enhancement techniques was good. The peak positions are given in Table 1, along with secondary structure assignments based on the analyses of Byler and Susi (1986), Prestrelski et al. (1991), and the normal mode calculations of Krimm and Bandekar (1986). Deconvolution of the amide I envelope was performed using the peak positions determined as described above. The curve-fit spectra are shown in Figure 1. The band at 1613 cm^{-1} is due to aromatic side-chain frequencies and was not included in the secondary structure calculations. However, it was nec-

Table 2: Comparison of FT-IR, X-ray, and CD Secondary Structure Content of the Native State and the Secondary Structure Content of the Thermally Denatured State

	%α	%β	% turns	% unordered
		Native		
FTIR	20	38	26	16
X-ray[a]	23	46	21	10
CD[b]	21	25	16	38
CD[c]	19	37		
CD[d]	22	(29)	(16)	(32)
CD[e]	26	28	21	22
CD[f]	25	29	14	32
		Denatured		
FTIR	11.4 ± 2.4	16.6 ± 1.8	42 ± 2.2	30 ± 2.3
CD[c]	13			
CD[g]	14	14		
CD[d]	12			

[a] Levitt and Greer (1977). [b] Yang et al. (1986). [c] Labhardt (1982). [d] This study. [e] Sarver and Krueger (1991). [f] Chang et al. (1978). [g] Lustig and Fink (1992).

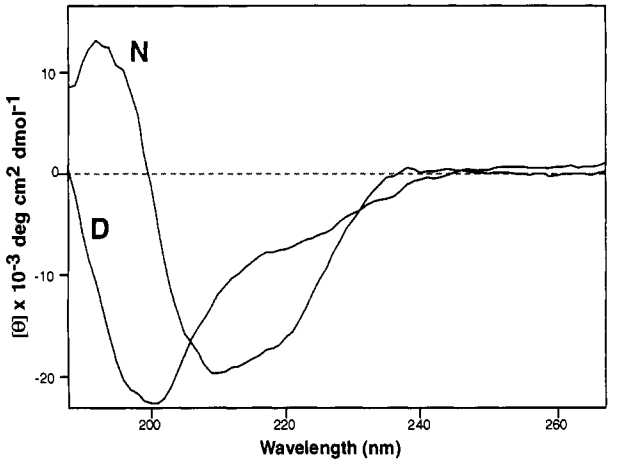

FIGURE 3: Circular dichroism spectra for native and thermally denatured RNase A. Conditions were the same as in Figure 1.

essary to include it for curve-fitting in order to avoid distortions of the amide I bands. The contributions of the different types of secondary structure for the native and the denatured states were determined from the relative areas of the individual band contributions and are given in Table 2. Comparison of the secondary structure content of the native state obtained by FTIR, X-ray, and CD is also shown in Table 2. Good agreement is seen between the X-ray and the FTIR data for the native state, whereas the CD data are in poorer agreement with the X-ray results. We also examined the CD spectra of RNase A in the native and thermally denatured states under conditions identical to those used in the FTIR experiments (Figure 3). The spectra were deconvoluted using the singular value decomposition (SVD) method of Johnson (Compton & Johnson, 1986). The results for the native state were in good agreement with those reported by Yang et al. (1986), Chang et al. (1978), and Sarver and Krueger (1991) (using combined IR and CD data). It is worth noting that the reported secondary structure of native RNase A, determined by different deconvolution methods, varies substantially except for the helix content (Venyaminov et al., 1991). The unfolded state did not deconvolute well using the SVD method. Thus, whereas the results for helix content are probably accurate, the breakdown by secondary structure type for the remaining classes probably has a large error (Hennessey & Johnson, 1982; Venyaminov et al., 1991); this may reflect the nature of the structures in the basis set.

Comparison of the secondary structure content by FTIR for the native and thermally denatured protein indicates that thermal denaturation results in the loss of two thirds of the β-sheet structure and half of the helix structure. The structural changes are also reflected in the appearance of additional bands which may indicate increased conformational freedom in the denatured state. However, the denatured protein retains significant secondary structure in that it has 11% helix and 17% β-sheet. The lost secondary structure is replaced predominantly by a large increase in turns and unordered structure. The CD data, in agreement with previous reports, also indicated the presence of 12% helix in the thermally unfolded protein (Table 2).

It should be noted that the published FTIR spectrum of native RNase A by Byler and Susi (1986) shows some differences from that reported here. We believe the reason for the discrepancies is lack of complete D/H exchange in their sample. This conclusion is based on the position of their amide I component bands, which are at positions corresponding to those between the fully deuterated and fully protonated species (Haris et al., 1986), and the difficulty in obtaining complete D/H exchange for native RNase A. In preliminary experiments, for example, in which the sample of ribonuclease was dissolved in D_2O and left under native or only slightly denaturing conditions, we also observed only partial D/H exchange (i.e., a spectrum similar to that reported by Byler and Susi).

DISCUSSION

Tanford (1968) showed that in the presence of 6 M guanidinium hydrochloride several proteins exhibited the hydrodynamic properties of a random coil. Although this result has sometimes been misinterpreted to imply that the denatured state is a random coil, Tanford's definition of the denatured state could include a whole spectrum of nonnative states ranging from those having significant secondary structure to those approaching a random-coil. It is now clear that many denatured proteins may retain ordered secondary structure (Dill & Shortle, 1991; Goto et al., 1990).

The FTIR data show that the thermally denatured state of RNase has about half the helix and β-sheet content of the native state. Interestingly, although the amount of unordered structure has doubled, there is more turn than unordered structure in the thermally denatured state. This finding agrees with molecular dynamics simulations which also show substantial turn content in "denatured" polypeptides [e.g., Soman et al. (1991)]. The residual 11% helix measured by FTIR in thermally denatured RNase is in agreement with CD studies which indicate 12–14% helix remaining (Labhardt, 1982; Yang et al., 1986; and the present study). The addition of Gdn·HCl to the thermally unfolded RNase A led to a noncooperative loss of the remaining helix structure. Similar results were observed for thermally denatured RNase A in the presence of 50% methanol monitored by CD (Lustig & Fink, 1992). FTIR studies of the amide III region (Anderle & Mendelsohn, 1987) have qualitatively shown the presence of α-helix and β-sheet in thermally denatured RNase A. Recently Sosnick and Trewhella (1992) also used FTIR to examine thermally denatured RNase (under different conditions from those used in the amide protection experiments, namely, at pD = 5.7). They also observed spectral evidence for residual secondary structure, although a quantitative analysis was not done.

Light scattering data on thermally denatured RNase A (Nicoli & Benedek, 1976) showed an increase of as little as 7% in the hydrodynamic radius, compared to that of the native

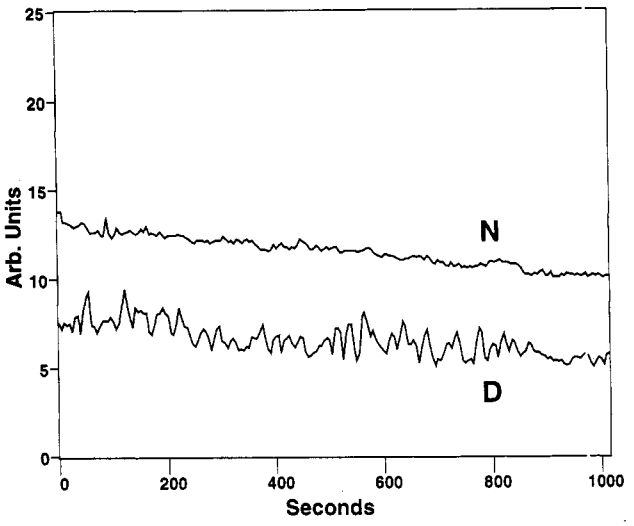

FIGURE 4: Rayleigh light scattering traces to monitor for aggregation. N is the native state; D is the thermally denatured state. Conditions were the same as in Figure 1. Protein concentration was 24 mg/mL. The trace for the denatured state is noisier due to the higher temperature. The small time-dependent changes seen in both traces reflect instrumental drift at the high sensitivity used to monitor the samples.

state, depending on the salt concentration. In the presence of 50% methanol the hydrodynamic radius was found to increase by 50% upon thermal denaturation, whereas Gdn·HCl, pH 2 denaturation led to a 100% increase in hydrodynamic radius (Lustig & Fink, 1992). Thus the thermally denatured protein both in aqueous and 50% methanol is relatively compact. This was confirmed in a recent report using small-angle X-ray scattering, in which a 30% increase in radius of gyration was observed at pD 5.7 on thermal denaturation (Sosnick & Trewhella, 1992) (compared to the greater than 100% increase expected for a random coil conformation).

In principle, the residual structure observed in the thermally denatured protein could also be due to aggregation of the unfolded state. The presence of aggregation can be detected by FTIR. An increase in the intensity of the amide I band around 1615–1620 cm^{-1} is frequently indicative of the formation of structure due to *inter*molecular hydrogen bonding, especially in thermally unfolded proteins (Clark et al., 1981). The absence of this feature in our data indicates that the residual structure detected was not due to aggregation. The band in the vicinity of 1613 cm^{-1} is due to contributions from the side chains of tyrosine and phenylalanine and is seen in the spectra of both native and denatured protein. It has also been reported previously that RNase A does not aggregate on unfolding at high temperatures (Clark et al., 1981). This was further confirmed by using Rayleigh light scattering to monitor the sample under conditions and concentrations identical to those used in the FTIR experiment, which showed no evidence of any aggregation or other scattering phenomena to concentrations at least as high as 24 mg/mL (Figure 4).

The results of the dynamic light scattering experiments (Nicoli & Benedek, 1976) and the small-angle X-ray scattering experiments (Sosnick & Trewhella, 1992), in conjunction with CD data (Labhardt, 1982), along with the present FTIR results, indicate that thermally denatured RNase A is in a compact conformation with some residual secondary structure. Proton NMR (Kutyshenko et al., 1989) and ^{13}C NMR (Howarth & Yun Lian, 1981) studies are also consistent with the presence of residual structure in thermally denatured RNase A. Unfortunately, the strong absorbance of urea and guanidine hydrochloride in the amide regions of IR absorbance precluded an experiment to monitor the effect of adding denaturant on the residual structures determined by FTIR.

Talluri and Scheraga (1990) have reported, using NMR H/D exchange, that thermally denatured RNase has residual structure in the vicinity of the C-terminal region, residues 105–119. They also showed that the exchange was faster from peptides from this region than the protein itself, indicating the presence of short- and long-range interactions in the thermally denatured protein which slow the exchange. These results have been questioned by Robertson and Baldwin (1991), who showed that the amide exchange rates for valine and isoleucine residues are slower than previously believed and could be responsible for the observed slower exchange rates seen by Talluri and Scheraga (1990).

Since both FTIR and CD results indicate that thermally denatured ribonuclease A retains a significant amount of regular structure, the question thus arises as to why the amide protection experiments of Robertson and Baldwin (1991) indicate a fully disordered structure. Only 36 of the backbone amide protons in RNase A exchanged slowly enough to show sufficient 2D NMR cross-peak intensity to allow measurement of their degree of protection. These 36 amides are scattered throughout the sequence, in helix, sheet, turn and other regions. There are no obvious reasons why there should be some flaw in the analysis of the amide protection experiments, although if there is it is important to determine it, since amide protection experiments are now an important tool in the study of nonnative states of proteins.

The calorimetric data on thermally unfolded RNase A (Privalov et al., 1989) suggest significant solvent penetration of the native hydrophobic core, also consistent with limited remaining ordered structure. On the other hand, the CD, FTIR, and light scattering data clearly suggest a relatively compact conformation with significant helix and sheet. A partial resolution of this apparent paradox may lie in the nature of the conformation and dynamics of the thermally denatured protein. Rapidly interconverting conformations with major solvent penetration could account for the calorimetric observations. Similarly, any secondary structure would be in rapid equilibrium with the corresponding disordered conformation. It is reasonable to assume that the residual helix and sheet structure detected by CD and FTIR arise from a relatively few residues spending significant amounts of time as helix and sheet, rather than many residues spending small amounts of time as helices or sheets. It is possible that the secondary structure observed by FTIR and CD occurs in regions of the polypeptide chain not involving the 36 amides which were measured in the amide protection experiments, although this seems very unlikely. In fact, it is most likely that the observed helix and sheet in thermally denatured RNase A correspond to regions which are helix and sheet in the native state.

An important point to note is that the NMR amide protection experiments and the FTIR and CD measurements involve quite different probes, the former essentially dynamic, the latter static. Consequently the different probes report on different aspects of the structure of the denatured state. One way to reconcile the amide protection results with those from CD and FTIR is as follows. If we assume that the secondary structure present in the denatured state is the same as that in the native, but is only marginally stable, then it will be rapidly (probably on a microsecond or faster time scale) interconverting between the disordered and fully solvent-exposed state and the folded state in which the amides are protected from exchange. Our FTIR data suggest that half

of the native helix and one-third of the native β-sheet remain in the thermally denatured state. This could be construed to mean that all of the native ordered secondary structure is present as much as half of the time. This would correspond, in the extreme, to an amide protection factor of only 2, not much larger than the average of 1.6 for all sites observed by Robertson and Baldwin (1991). Thus if the rate constants for amide proton exchange are in error by a factor of less than 2, then the data could be reconciled with a model of the thermally denatured protein in which much of the native secondary structure is rapidly flickering back and forth between ordered and disordered structure. Thus static techniques such as CD and FTIR see the time-average structure, in contrast to dynamic methods such as the NMR-measured amide exchange.

Consequently the thermally denatured state of ribonuclease A is rather compact, with substantial *unstable* native-like secondary structure. This is consistent with a model in which the denatured protein is much more dynamic than the native state, in the sense that it is in rapid equilibrium with a more substantially unfolded conformation, and in which the equilibrium lies in favor of the compact form. This is presumably a reflection that the driving forces for unfolding are not particularly strong, relative to the internal interactions which favor folding, under the experimental conditions used. One can picture the compact form consisting of relatively native-like regions of secondary structure which are in less intimate contact with each other than in the native state and, in fact, where the side chains are not maximally close packed, but separated by some solvent penetration. Such a denatured state may be a reasonable model for an intermediate formed fairly late in folding. The underlying reason for the residual structure is most likely that the various forces favoring folding (disulfide bonds, hydrophobic interactions, intramolecular hydrogen bonding) are only marginally offset by those favoring unfolding (kinetic energy, intermolecular hydrogen bonding to the solvent, electrostatic repulsion).

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