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Characterization of a ribonuclease S refolding intermediate

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During ribonuclease S (RNase S) refolding, two peptide fragments recognize each other, and bind together to form a refolding intermediate which slowly converts to the native state. We have characterized this refolding intermediate using absorbance, circular dichroism (CD), and nuclear magnetic resonance (NMR) spectroscopies. These techniques reveal significant amounts of both secondary and tertiary structure; the intermediate differs from a molten globule in being packed and native-like, but it resembles a molten globule in having no near-ultraviolet (UV) CD spectrum. Final refolding is slow and accompanies proline isomerization. The results show that at least two separate stages are observed in the formation of the tertiary structure of RNaseS.

1. Introduction

The refolding reactions of small proteins are often modelled by a mechanism with just one principal intermediate in refolding: $U \rightleftharpoons I \rightarrow N$ (U = unfolded, I = intermediate, N = native). The intermediate is formed rapidly in refolding experiments and the rate-limiting step in refolding is the slow $I \rightarrow N$ reaction; I is not observed in unfolding experiments. This model was developed by Kuwajima (1977) to represent the refolding kinetics of bovine α-lactalbumin, and it has been used by Fersht and co-workers to represent detailed studies of the refolding pathway of barnase (Kuwajima 1977; Matouschek *et al.* 1992). As applied to α-lactalbumin, an important feature of the model is that the early kinetic intermediate is identified with the equilibrium molten globule intermediate (Ikeguchi *et al.* 1986; Kuwajima 1977). The model has proven useful as a guide in interpreting pulsed hydrogen/deuterium (H/D) exchange studies of the refolding pathways of several small proteins (Baldwin 1993).

When circular dichroism (CD) is used as an additional probe in conjunction with pulsed H/D exchange, the results for cytochrome *c* (Elöve *et al.* 1992) and hen lysozyme (Radford *et al.* 1992; Chaffotte *et al.* 1992) indicate that at least one additional stage in the refolding process can be observed: much of the secondary structure of the native protein is formed, according to CD data, before significant protection against H/D exchange is acquired. The hen lysozyme results of Radford *et al.* (1992) also suggest that additional later refolding intermediates can be observed. These results raise the possibility that, when the refolding reactions of other small proteins are examined more thoroughly by using multiple probes,

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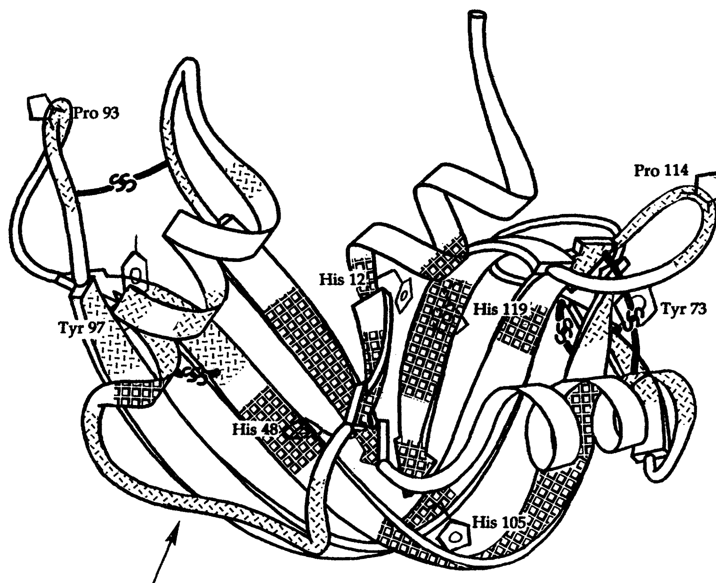

Figure 1. The structure of pI_2. Histidine residues and residues that histidines contact are folded in the intermediate and are shown in square hashing. Tyrosine and proline residues, and the residues they contact are unfolded and are shown in dashed hashing. Most of the remaining side-chains are likely to be folded, as indicated qualitatively by chemical shift dispersion in the first 1D ^1H-NMR refolding spectrum. The S-protein β-sheet is fully formed in the intermediate, although the S-peptide helix is destabilized. An arrow indicates the bond at which RNase A is cleaved by subtilisin to give RNase S. The region surrounding this cleavage is disordered in the crystal structure (Wyckoff *et al.* 1970). This figure was adapted from a ribbon diagram of RNase A by Jane Richardson.

additional stages in the refolding process may be discovered. They also focus interest on the nature of the rate-limiting step in refolding: to understand this step, it is necessary to characterize the intermediate preceding the rate-limiting step.

We report here the results obtained by studying a late step in the refolding kinetics of RNase S. RNase S is the product of specific proteolysis of ribonuclease A (RNase A) by subtilisin (Richards & Vithayathil 1959). Despite this cleavage, the resulting fragments, S-peptide (residues 1–20) and S-protein (residues 21–124) can recombine to form active ribonuclease. When a concentrated, unfolded solution of S-protein and S-peptide is allowed to refold, most of the S-protein molecules partially refold to form an early intermediate I_1. This species then binds S-peptide, abbreviated p, forming a partially folded complex, pI_2.

Folded RNase S contains two *cis* peptide bonds associated with prolines 93 and 114 (Wyckoff *et al.* 1970) (figure 1). After unfolding, these peptide bonds are free to isomerize, resulting in a population of molecules containing a mixture of *cis* or *trans* peptide bonds. About 20% of unfolded S-protein molecules have both proline peptide bonds in the native *cis* isomeric form; the refolding of these molecules, termed U_F, is fast and occurs in the dead time of the manual mixing experiments reported here (Labhardt & Baldwin 1979). Using a fluorescein probe attached to Lys 7 of S-peptide to monitor specific binding to S-protein during refolding, Labhardt *et al.* (1983) showed that the remaining 80% of unfolded molecules, termed U_S, refold rapidly to form a partially folded intermediate, pI_2. The subsequent and final refolding step is slowed by the presence of a non-native proline bond:

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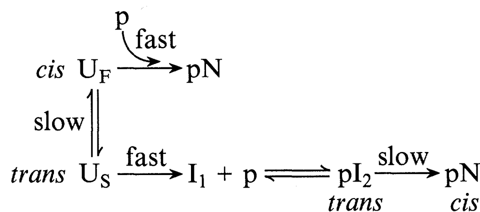

The persistence and abundance of the intermediate complex pI_2 make it an attractive subject to study; its structure and properties, and the nature of the final step in refolding, $pI_2 \rightarrow pN$, are the subjects of this paper. We show here that it is possible to characterize pI_2 by real-time, one-dimensional proton NMR (1D ^{1}H-NMR). This technique has been used by Blum *et al.* (1978) to monitor the refolding of heat-denatured RNase A.

The role of the S-peptide α-helix in forming the pI_2 complex has aroused considerable curiosity because earlier studies failed to find evidence for the presence of this α-helix in pI_2, despite the widespread belief that recognition between the S-peptide and S-protein during refolding must involve this S-peptide helix. Brems & Baldwin (1984) studied the stability of peptide NH protons in the S-peptide and S-protein moieties of the pI_2 intermediate by tritium pulse labelling, followed by HPLC separation of S-peptide from S-protein. They concluded that the S-peptide helix, if it is formed in pI_2, can have only marginal stability in their refolding conditions (pH 6.0, 10 °C). Labhardt (1984) studied refolding at a high temperature (32 °C) by circular dichroism and found that the S-peptide helix is formed only in a late stage of refolding, coincident with the shielding of tyrosine side-chains from solvent. In our work, protonated S-peptide is allowed to combine with deuterated S-protein during refolding. The 1D NMR spectrum of S-peptide amide protons in pI_2 is then used to answer the question of whether or not the S-peptide helix is present in pI_2 and, if so, how stable it is.

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2. Results

(*a*) *Refolding monitored by near UV-CD, absorbance and proline isomerization*

The final refolding of RNase S monitored by near UV-CD, UV absorbance and proline isomerization is shown in figure 2. The rate of proline isomerization was determined by measuring the fraction of molecules which have non-native proline isomers after an initial refolding step (Cook *et al.* 1979). At 10 °C, the observed refolding rate is about 0.0028 s^{-1} for all these probes, indicating that some tyrosine side-chains are fixed concomitantly with proline isomerization in the final slow step ($pI_2 \rightarrow N$). This slow step accounts for approximately 80% of the amplitude seen for the complete equilibrium folding transition; the missing 20% is caused by $U_F \rightarrow pN$, which is fast and occurs in the mixing dead time (see above scheme). The amplitude and rate of refolding monitored by tyrosine absorbance remain relatively constant over a wide range of pH (4–7) (data not shown). The rate, but not the amplitude, is strongly affected by temperature: $k_{obs} = 8.7 \times 10^{-4}\ s^{-1}$ at 0 °C and increases to $8.9 \times 10^{-3}\ s^{-1}$ at 20 °C. The calculated activation enthalpy is 18 kcal mol^{-1} over this temperature range. Similar values, 19 ± 3 kal mol^{-1} have been reported for proline isomerization in unstructured peptides (Brandts *et al.* 1975).

Both the amplitude and rate of refolding, monitored by tyrosine absorbance or near UV-CD, are affected by sodium sulphate, a general stabiliser of protein structure.

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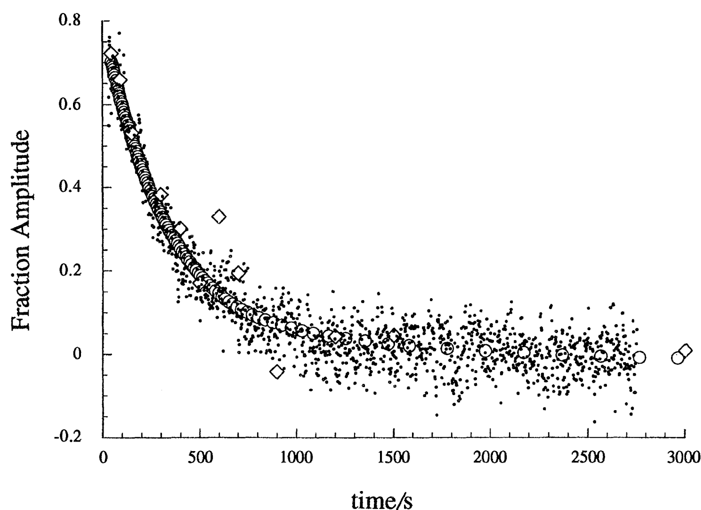

Figure 2. RNase S refolding monitored by near UV-CD ●; absorbance change at 287 nm ○; and proline isomerization ◇. Refolding was carried out in 50 mM sodium cacodylate. 0 M sodium sulphate, pH 6.0 at 10 °C. The observed rates were 2.9×10^{-3} s^{-1}, 3.2×10^{-3} s^{-1} and 2.4×10^{-3} s^{-1} respectively. The fractional amplitude was computed by dividing the observed amplitude by the total equilibrium unfolding change. The amplitude of the proline monitored reaction was decreased by a small amount to correct for back isomerization in the second unfolding step of the assay. The fit of the absorbance monitored refolding experiment revealed a second slow phase, $k_2 = 2.9 \times 10^{-4}$ s^{-1}, of minor amplitude, 9%. This second phase probably arises from RNase S species analogous to U_SI of RNase A (Schmid *et al.* 1986).

The observed slow amplitude decreases approximately linearly with increasing sodium sulphate concentration; for absorbance, $\Delta\epsilon$ at 287 nm equals 1600 cm^{-1} M^{-1} at 0 M sodium sulphate and 700 cm^{-1} M^{-1} at 1 M sodium sulphate. The log of the rate increases linearly with increasing sodium sulphate; from $k_{obs} = 0.0028$ s^{-1} at 0 M to $k_{obs} = 0.0165$ s^{-1} at 1 M sodium sulphate.

(*b*) *1D ^{1}H-NMR monitored refolding*

Refolding was also monitored by 1D ^{1}H-NMR. In heat or solvent denaturant unfolded S-protein and S-peptide, the C2 protons of His 12, 48, 105 and 119 share a common solvent environment and resonate as a barely resolved doublet at 8.6 ppm (Kuwajima & Baldwin 1983*a*). During refolding, these protons acquire unique environments and show distinct lines in a 1D ^{1}H-NMR spectrum (Meadows *et al.* 1969; Kuwajima & Baldwin 1983*a*). The increase in area of these distinct peaks can be used to monitor the refolding of this part of the protein. The first spectrum acquired after the initiation of refolding, at pH* 5 (the pH meter reading uncorrected for the deuterium isotope effect), 0 °C, 1.07 mM S-protein, contains the four histidine peaks at their native chemical shifts (when corrected for the effect of residual GdmCl) and at full amplitude (figure 3). Neither the chemical shifts nor the amplitudes show any additional changes in later spectra.

Some buried alkyl protons, particularly CγH of Val 63 and also CγH of Val 57 and Thr 45, of folded RNase S are shielded and show resolvable lines in a 1D ^{1}H-NMR spectra (Rico *et al.* 1989; Robertson *et al.* 1989). These distinct lines are also seen in the first 1D ^{1}H-NMR spectrum taken after the initiation of refolding. All portions of the first refolding spectrum, in fact, show significant chemical shift dispersion which

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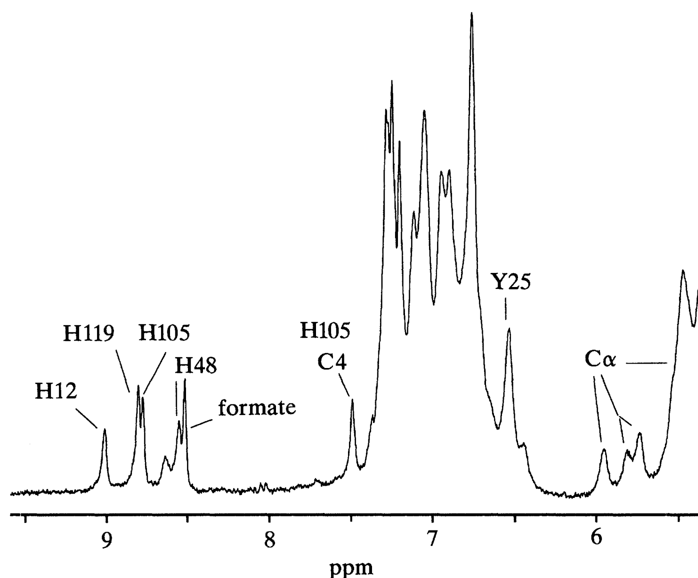

Figure 3. 1D ^{1}H-NMR spectrum of refolding RNase S. The downfield region of the first spectrum of RNase S recorded after initiation of refolding in 50 mM sodium acetate, pH* 5.0 at 0 °C. The four histidine C2 proton peaks, the His 105 C4 proton peak, the peak due to the side-chain protons of Tyr 25 and three native-like Cα proton resonances are labelled. A small peak with the chemical shift of an unfolded His C2 proton is observed; it arises from the His 12 in the small excess (30%) of S-peptide which is unbound and largely unfolded (Kuwajima & Baldwin 1983*a*). An additional peak is due to residual formate remaining from the purification procedure.

is not seen in the unfolded spectrum. Three distinct lines are present in the α carbon protein region, between 5.7 and 6.0 ppm. A line with the native chemical shift for the Tyr 25 side-chain, which is hydrogen bonded to His 48 in folded RNase S, is also observed. These lines, like those of the histidine and alkyl side-chains, show little or no change in peak height or chemical shift over the time range at which tyrosine monitored refolding occurs. The peaks for the tyrosine side-chains which give rise to spectroscopic changes, Tyr 73 and Tyr 97 (Horwitz & Strickland 1971), are in the crowded aromatic region of the spectrum and cannot be resolved. These peaks are further masked by the resonance of the residual protonated GdmCl.

To investigate the formation of the active site during the refolding of RNase S, we monitored refolding by 1D-NMR in the presence of the competitive inhibitor 2′CMP. The binding of 2′CMP to native RNase A is known to cause a striking change in the C2H chemical shift of His 12 (Meadows *et al.* 1969). The first spectrum acquired after refolding initiation shows a shift of the C2H resonance of His 12 of 0.2 ppm downfield (data not shown). In addition, the linewidths of the 2′CMP C6 and C5 protons are broadened in this first spectrum, although their chemical shifts equal those of 2′CMP in the absence of ribonuclease (data not shown). There are several qualitative changes in the first refolding spectrum, compared to RNase S refolding in the absence of 2′CMP. Notably, there are spectral changes at about 0.70 ppm that may arise from changes in the environment of Thr 45, which lies in the inhibitor binding site of folded RNase S. No significant changes are observed in subsequent spectra.

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(c) S-peptide H/D exchange

To determine the conformation of S-peptide during refolding, we measured the chemical shifts and protection factors of S-peptide amide protons during refolding by 1D-NMR. Normally, the amide proton region of a protein 1D ^{1}H-NMR spectrum is crowded, and individual resonances are impossible to observe. In RNase S, it is, however, possible to observe individual peaks for S-peptide amide protons by deuterating the amides of S-protein (Kuwajima & Baldwin 1983*a*). When deuterated S-protein was refolded with protonated S-peptide, six or seven amide proton peaks were observed in the first spectrum recorded (data not shown). These peaks are spread over a broad range of chemical shift (7.97–9.02 ppm) (data not shown). The H/D exchange rates of these amide protons are only slightly lower than those observed for unfolded S-peptide (data not shown); the calculated protection factors range from 6 to 35.

3. Discussion

The results provide a fairly detailed structural picture of the intermediate pI_2 (figure 1). pI_2 is not fully native. The lack of wild type signals for tyrosine absorbance or near-UV CD, which are regained only with the slow conversion of pI_2 to native RNase S (pN) (figure 2), indicates that the tyrosine residues giving rise to these changes are not fixed in pI_2. Of six tyrosine residues in RNase S, only two, Tyr 73 and Tyr 97, are buried in the native state (Lee & Richards 1971) and contribute to the observed spectroscopic changes (Horwitz & Strickland 1971). The burial of Tyr 73 and Tyr 97 occurs at the same rate as proline isomerization; these events are coupled in the final, rate-limiting step of refolding.

In contrast, 1D ^{1}H-NMR spectra of pI_2 show that all four histidine residues are present in conformations that produce the final native chemical shifts after only 120 s of refolding. At this time, 57 % of molecules still contain non-native tyrosine and proline residues. Because of the sensitivity of NMR chemical shift to environment, the presence of native histidine chemical shifts suggests that residues which pack against the histidines are also folded. This implies that a large amount of tertiary structure is present in pI_2 (figure 1).

The changes observed in the 1D-NMR spectrum of pI_2 with addition of 2′CMP indicate that the active site is present in pI_2. The downfield shift of the His 12 C2 proton has been observed previously in a complex of native RNase A and 2′CMP, and indicates hydrogen bond formation between His 12 and the phosphate group of 2′CMP (Meadows *et al.* 1969). The observed changes near 0.70 ppm may arise from binding of Thr 45 to the nucleotide (Hahn *et al.* 1985). In addition, the broadening of the 2′CMP C6 and C5 proton resonances shows that 2′CMP is tumbling more slowly in solution, as is expected if the nucleotide were bound to the intermediate. Binding of 2′CMP will stabilize the active-site structure of pI_2. It is likely, however, that this structure is present in the absence of 2′CMP, since two active site residues, His 12 and His 119, show native chemical shifts when RNase S is refolded without inhibitor.

The broad dispersion of S-peptide amide proton chemical shifts (8–9 ppm) in pI_2 indicates that the S-peptide backbone is structured and is likely to be helical. In native RNase S, the amide protons of the S-peptide α-helix give rise to seven distinct peaks over a similarly wide chemical shift range (7.8–8.8 ppm) (Kuwajima & Baldwin, 1983*a*). In contrast, unfolded S-peptide amide proton peaks are compressed

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together over a narrow range of chemical shift (8.1–8.5 ppm) (Kuwajima & Baldwin 1983*a*). When bound to native S-protein, the helical S-peptide amide protons have high protection factors, ranging from 500 to 5000 (Kuwajima & Baldwin 1983*b*). In pI_2, these amide protons are only weakly protected, indicating that the α-helix is rather unstable. Low protection factors for the S-peptide α-helix were also obtained by Brems & Baldwin (1984). This low stability likely explains why the S-peptide helix is absent when refolding is carried out at high temperature (Labhardt 1984). The secondary structure in the S-protein moiety of pI_2 is well formed, as indicated by CD and tritium exchange (Labhardt 1984; Brems & Baldwin 1984). Protection factors for the S-protein β-sheet in pI_2 are quite high (about 10^4) (A. J. Doig & R. L. Baldwin, unpublished results).

During refolding, most small proteins rapidly fold, on a millisecond timescale, to an intermediate which has the properties resembling an equilibrium molten globule (Baldwin 1993). Conversion of the molten globule intermediate to native protein is the rate-limiting step for these proteins. pI_2 is clearly not a molten globule because it possesses significant regions of native-like tertiary structure. A similar, structured intermediate has been extensively studied in the refolding of ribonuclease T1 (Kiefhaber *et al.* 1992; Mullins *et al.* 1993). The conformation of the earlier intermediate I_1 (scheme above) remains to be characterized.

In RNase A, site-directed mutagenesis has been used to show that proline isomerization occurs in the final step of refolding (Schultz *et al.* 1992). As this also appears to be the case for RNase S, it is interesting to consider how the presence of non-native proline peptide bonds may prevent the formation of native-like structure in portions of pI_2. Two models may account for the observed behaviour. In the first, or 'direct disruption' model, the presence of a non-native peptide bond directly interferes with the formation of a particular native structure. For example, *trans* Pro 93 might alter the local structure to prevent the packing of Tyr 97. This disruption might be transmitted through the C40–C95 and C26–C84 disulphide bonds to the second α-helix (residues 24 to 34) of RNase S. This helix makes many contacts with S-peptide in native RNase S; its disruption might account for the low affinity of S-protein for S-peptide in the intermediate. In native RNase S, the hydroxyl groups of Tyr 115 and Tyr 73 are hydrogen bonded (Wyckoff *et al.* 1970). A *trans* peptide bond at Pro 114 will likely disrupt this hydrogen bond, causing the observed non-native spectroscopic behaviour of Tyr 73.

A second model, termed 'global destabilization' proposes that the presence of non-native proline isomers produces a general weakening of native structure that affects all parts of the molecule equally. This model assumes that some portions of the protein, in this case, Tyr 73 and Tyr 97, are less stable than others and so remain unfolded in pI_2. Global destabilization predicts that if refolding was carried out under conditions which more strongly favour the native state, the less stable portions of the molecule might fold before proline isomerization. The loss of slow refolding amplitude observed by tyrosine absorbance or near UV-CD, in the presence of the stabilizer sodium sulphate shows that tyrosine residues can fold before proline isomerization, providing support for this model. The replacement of the *cis* Pro 114 peptide bond with a *trans* bond by site-directed mutagenesis decreases the protein's stability (Schultz & Baldwin 1992). Molecular dynamic simulations have shown that while non-native proline bonds can be accommodated in a structured protein, they disrupt local structure and destabilize the entire molecule (Levitt 1981).

In conclusion, we have shown that RNase S refolding can be followed by real time

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1D 1H-NMR. With this technique, we show that the pI_2 is not a typical molten globule, but a refolding intermediate with portions of both native and non-native structure. In studies of molten globule refolding intermediates and also of the kinetics of refolding, the near-UV CD spectrum is commonly taken as the 'signature' of tertiary structure formation. These results demonstrate separable kinetic stages in the formation of tertiary structure in RNases S as well as the failure of a conventional probe (near-UV CD) to detect tertiary structure when it is first formed.

4. Materials and methods

(a) *Materials and reagents*

RNase S was prepared from RNase A by cleavage with subtilisin using a procedure based on that of Richards & Vithayathil (1959). S-peptide and S-protein were separated by the gel filtration procedure of Hearn *et al.* (1971). The concentration of RNase S or S-protein was determined by absorbance at 280 nm using an extinction coefficient of 9560 cm^{-1} M^{-1} (Connelly *et al.* 1990). S-peptide concentrations were determined approximately by weight, and by absorbance at 258 nm using an extinction coefficient of 320 M^{-1} cm^{-1} (Richards & Logue 1962). GdmCl solutions were prepared from BRL ultra pure grade material, as described by Pace *et al.* (1991). Heavy water, 99.9% D, was from Cambridge Isotope Laboratories. Sodium acetate and sodium sulphate were obtained from Baker. The sodium cacodylate and 2′CMP used here were from Sigma. Trimethylsilypropionate (TSP) from ICN Biomedicals was used as a chemical shift standard.

(b) *Procedure*

S-peptide and S-protein were unfolded in 2.25 M GdmCl, pH 2. Unfolded protein was incubated at room temperature for at least 30 min to achieve an equilibrium mixture of fast and slow refolding molecules, then cooled before initiation of refolding. A range of refolding temperatures, between 0 and 20 °C, was used. A slight molar excess (30%) of S-peptide was used in all experiments, except in the S-peptide H/D exchange experiment, where a 30% molar excess of S-protein was used. Refolding was initiated by ten-fold dilution of unfolded material into refolding buffer. The dead time was about 30 s for the near UV-CD and absorbance experiments, and 2 to 3 min for the NMR experiments. Refolding buffer contained either 50 mM sodium acetate (pH 4–5) or 50 mM sodium cacodylate (pH 6–7). In some experiments, refolding buffer also contained sodium sulphate. The isomerization of proline residues during refolding was measured by the assay of Cook *et al.* (1979). In this procedure, unfolded RNase S containing an equilibrium mixture of U_F and U_S species was refolded at pH 6, 10 °C in 0.23 M GdmCl. Before refolding was complete, the sample was rapidly unfolded at pH 2, 0 °C in 5.10 M GdmCl for 60 s. This unfolding step produces a mixture of U_F and U_S species. Native protein unfolds to U_F; longer refolding times, therefore, yield more U_F. The solution was finally refolded at pH 6, 10 °C in 0.90 M GdmCl. The slow amplitude of this last refolding step arises from U_S; by varying the length of the initial refolding step it is possible to determine the rate of proline isomerization. Deuterated solvents were used for the NMR experiments. To observe histidine C2 proton resonances, S-peptide and S-protein amide protons were replaced by deuterons by three cycles of heating in D_2O and freeze drying before unfolding. Only S-protein amide groups were so deuterated for the S-peptide H/D exchange experiment. In this experiment, only S-protein was unfolded in GdmCl,

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this solution was added to protonated, freeze dried S-peptide just before addition to refolding buffer. The decrease in the height or area of the amide proton peaks with time was fit to an exponentional function to give the rate of H/D exchange. Some NMR experiments contained 2′CMP, at a concentration slightly below that of S-protein. Upon initiation of refolding, NMR samples were rapidly shimmed and multiple successive 1D ^{1}H-NMR spectra were recorded.

(c) *Instrumentation*

Changes in absorbance were monitored at 287 nm using a Cary 118 spectrometer. An Aviv model 60 DS instrument was used to follow changes in the near UV-CD at 275 nm. Kinetic data were fit to an exponentional decay function using Kaleidagraph from Synergy. 1D ^{1}H-NMR spectra were acquired on a 500 MHz General Electric GN-Omega spectrometer. The FID was the sum of 48 or 64 scans and took about 90 s to 120 s to acquire. NMR data were processed using the Felix program of Hare Research on a Silicon Graphics Iris workstation. The FID was multiplied by an exponential function to improve signal to noise.

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