Nonnative Isomers of Proline-93 and -114 Predominate in Heat-Unfolded Ribonuclease A†

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ABSTRACT: The peptide bonds preceding both Pro-93 and Pro-114, which are in the cis conformation in native RNase A, are predominantly in the trans conformation in the heat-unfolded protein. The percentages are estimated to be 60% and 63%, respectively, with a standard deviation of ±7% in each quantity. These ratios are close to those found for corresponding sequences in X-Pro-Y peptides. The concentration of the trans proline species was determined from the integrated intensities of resonance peaks of the $C^{\alpha}H$ protons of Tyr-92 and Asn-113, which are well resolved in the 1D proton NMR spectrum of heat-unfolded RNase A. The assignments of the resonances were deduced from 2D NOESY and DQF-COSY spectra of unfolded RNase A in D_2O. Furthermore, the $C^{\alpha}H$ protons of both Tyr-92 and Asn-113 had an intense NOE cross-peak with the $C^{\delta}H$ and $C^{\delta'}H$ of the respective following prolines. For both Pro-93 and Pro-114, these NOE cross-peaks would arise only if the X–Pro peptide bond were in the trans conformation. It is generally believed that the rate of refolding of RNase A is considerably reduced by nonnative proline isomers, such as trans Pro-93. Two models for folding RNase A, that are consistent with these new results and the work of previous investigators, are presented here.

When bovine pancreatic ribonuclease A (RNase A)[1] is unfolded reversibly by chemical denaturants and/or heat, it forms a mixture of three slowly interconverting species called U_f, U_s^I, and U_s^{II}. The three species account for roughly 20%, 30%, and 50%, respectively, of the total protein, as judged by UV absorbance and inhibitor binding (Tsong et al., 1972; Garel & Baldwin, 1973; Garel et al., 1976; Hagerman et al., 1979; Cook et al., 1979; Schmid & Blaschek, 1981; Lin & Brandts, 1983a, 1987a). Once refolding is initiated, the three species behave very differently. U_f rapidly regains the native conformation (18–400 ms). The other two forms of the protein, U_s^I and U_s^{II}, refold on a time scale of seconds to minutes.

A third slow phase has been identified by Lin and Brandts (1987a). It accounts for 5% of the unfolded species as judged by UV absorbance. Because of its low concentration, it has not been detected in our experiments. Also, any effect that this phase has on our conclusions falls within the limits of accuracy (±7%); therefore, the third slow phase will not be discussed any further in this paper.

The experimental evidence indicates that unfolded RNase A is a mixture of three conformational isomers, of which two, U_s^I and U_s^{II}, contain at least one nonnative conformation around a key covalent bond. For example, the peptide bond between Tyr-92 and Pro-93 is 100% cis in the fully folded protein. When RNase A is unfolded, this bond undergoes reversible isomerization to form a mixture of cis and trans isomers (Lin & Brandt, 1983b, 1987b; Schmid, 1986; Schmid et al., 1986). It is generally believed (although not conclusively proven) that, once the protein is returned to conditions where the native form is thermodynamically stable, the molecules containing cis Pro-93 refold rapidly. The folding is retarded in the molecules containing the nonnative trans isomer of Pro-93. Previous results (Schmid & Baldwin, 1978) support the conclusion that both U_s^I and U_s^{II} contain nonnative isomer(s) of the four proline peptide bonds of which two, 93 and 114, are cis in the native protein and the other two, 42 and 117, are trans. Presumably, the 20% of the protein that folds in the fast phase, U_f, contains no nonnative isomers that inhibit the refolding.

RNase A also contains four disulfide bonds. The disulfide bond (C^{β}–S–S–C^{β}) has two stable conformations. The C^{β}–S bonds are mutually perpendicular. Therefore, both left- and right-handed conformations of the C^{β}–S–S–C^{β} moiety are possible. The internal rotational energy barrier about the S–S bond is estimated to be as high as 15.5 kcal mol^{-1} based on studies with model compounds (Kessler & Rundel, 1968; Fraser et al., 1971), and *might* be higher inside a globular protein. Once the protein is unfolded, the four disulfide bonds are free to isomerize into the nonnative conformation. It has been proposed that these nonnative isomers of the disulfide bond might give rise to slow-folding species (Mui et al., 1985).

The disulfide bonds themselves form a series of intertwined loops. Nall et al. (1978) have proposed that these loops may become tangled upon unfolding, thus giving rise to a slow-folding form(s) of the protein.

Given the large number of bonds that can isomerize into nonnative forms, the question becomes not why does 80% of RNase A refold so slowly, but why does 20% fold so quickly. There are nine separate arrangements (four prolines, four disulfides, and the pattern of disulfide loops), all of which could presumably adopt nonnative isomers in the unfolded state. The

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[1] Abbreviations: AMX, a three-spin system such as the α and β protons of Ser, Cys, Asp, Asn, His, Phe, Tyr, and Trp (these amino acids cannot be distinguished in a DQF-COSY spectrum); DQF-COSY, double quantum filtered correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; NMR, nuclear magnetic resonance; ppm, part per million; DSS, 2,2-dimethyl-2-silapentanesulfonate; Gdn·HCl, guanidine hydrochloride; RNase A, bovine pancreatic ribonuclease A; U_f, U_s^I, and U_s^{II}, single fast and two slow phases found when RNase A refolds.

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percentage of molecules that retain the native conformation of all nine bonds should be vanishingly small. To date, the isomers that give rise to the slow-folding forms have not been unambiguously identified, and the question remains unanswered.

Two theories are invoked in the literature to explain why so much of the protein folds in the rapid phase. One states that the protein refolds even in the presence of nonnative isomers. In staphylococcal nuclease (Fox et al., 1986; Evans et al., 1987, 1989) and in calbindin D_{9K} (Chazin et al., 1989), both the cis and trans isomers of proline are found in the fully folded protein. Furthermore, the cis and trans isomers of unfolded staphylococcal nuclease refold at roughly the same rate (Evans et al., 1989). The result shows that proteins can fold properly even if the molecule contains a nonnative conformational isomer.

Alternatively, there may be sufficient native structure in the unfolded protein to retain the native conformational isomer of the relevant bonds. Lin and Brandts (1983b, 1984, 1987b) have assayed the conformational states of three of the four prolines of denatured RNase A using isomer-specific proteases (ISP). Their results show that all three prolines are predominantly in the native form (see Discussion for more details). Their evidence indicates that short-range interactions stabilize the native isomers in the unfolded protein and reduce the population of slow-folding species.

Unfortunately, it has been difficult to measure the percentages of different isomers in the unfolded protein, and hence different conclusions based on different approaches have been reached. A direct way to measure the proportions of the native and nonnative isomers of the appropriate bonds is needed to resolve this question. Estimates of the amount of trans Pro-93 and Pro-114 are presented in this paper based on the 1D NMR spectrum of heat-unfolded RNase A. The results indicate that, in the heat unfolded protein, the nonnative trans proline is the dominant form for both Pro-93 and Pro-114. Various models for the refolding of RNase A are discussed in light of the new results.

Materials and Methods

Bovine pancreatic ribonuclease A (RNase A) type IIa (Sigma, St. Louis, MO) was further purified by the method of Taborsky (1959) and was a generous gift from D. M. Rothwarf. The exchangeable protons were removed by lyophilizing the sample 3 times from 99.6% D_2O (Aldrich, Milwaukee, WI). The sample concentration was approximately 5 mM. No buffer was used. The pH was adjusted to the acidic range using concentrated HCl before the first lyophilization, and then checked after use. Sample pH varied from 2 to 3 with no significant effect on the spectra other than variations in the melting temperature. The spectra were referenced to the most upfield resonance which in turn had been calibrated against DSS (Adler & Scheraga, 1988).

Measurements were made either on a General Electric GN500 (Figures 1 and 2) or on a Varian VXL-400 (Figure 3) NMR spectrometer. The 90° pulse width was 15 μs for the GE and 20 μs for the Varian. All experiments, except the DQF-COSY experiment, were carried out on both instruments. For the 2D measurements, approximately 512 t_1 values were measured, and 2048 (Varian) or 4096 (General Electric) t_2 data points were collected. Sine bell shifted by 60° was used in both directions for the DQF-COSY spectrum (Figure 3). A 60° squared sine bell was used in t_2 and a 90° squared sine bell in t_1 to process the NOESY spectrum (Figure 2).

The integrated intensities of the $C^{\alpha}H$ resonances were normalized to the peak areas of the 43 aromatic ring protons

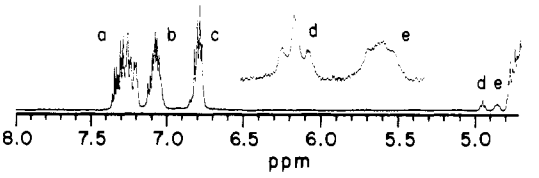

FIGURE 1: ^1H NMR spectrum of heat-unfolded RNase at 50 °C, pH 2. The peaks are labeled as follows: (a) the combined resonances of the 15 phenylalanine ring protons and 4 $C^{\delta}H$ histidines; (b) the 12 Tyr $C^{\delta}H$; (c) the 12 Tyr $C^{\epsilon}H$; (d) the $C^{\alpha}H$ of Asn-113 that precedes trans Pro-114; (e) the $C^{\alpha}H$ of Tyr-92 that precedes trans Pro-93. The inset shows peaks d and e more clearly.

that lie between 6.7 and 7.5 ppm. These protons fall into 3 resolved groups, 19 protons from the 15 Phe ring and 4 $C^{\delta}H$ His, 12 from the $C^{\delta}H$ Tyr, and 12 from the $C^{\epsilon}H$ Tyr. Each of the three groups of resonances was integrated separately. The carrier frequency was shifted to halfway in between the resonances of the aromatic ring protons and the $C^{\alpha}H$ of Tyr-92 and Asn-113, approximately 6 ppm, to minimize pulse width distortion between the resonances. A 10-s delay between pulses was used in order to avoid partial saturation. A total of 256 free induction decays were collected. Measurements were made over a range of temperatures from 50 to 75 °C. The relative intensities did not vary significantly with temperature. Distortions in the base line were removed by fitting selected points between the resonances to a third-order polynomial (Abramowitz & Stegun, 1968). At least two points for the base-line fit were selected from the upfield and downfield sides of both the Asn and Tyr $C^{\alpha}H$ resonances.

The RNase A was checked for covalent modification after the experiment by HPLC using a Mono S high-performane cation-exchange column at pH 7 (Pharmacia, Upsala, Sweden). There was no detectable degradation of the protein (<2%). However, the 1D NMR spectra of the samples at temperatures below the T_m showed that approximately 5–20% of RNase A did not refold. The second form of RNase A could not be detected in NMR spectra taken above the T_m, and none of the observed spectral properties of the unfolded protein, including the integrated intensities of the $C^{\alpha}H$ peaks, correlated with the amount of this second form. Similar problems with irreversible changes after prolonged heating have been observed for both bovine pancreatic trypsin inhibitor (Roder, 1978) and staphylococcal nuclease (Evans et al., 1989). Assuming that the nonfolding form of the protein contains all-trans isomers, the total percentage of trans Pro-93 and trans Pro-114 in the remaining intact protein would have been overestimated. The true percentage of trans proline in the intact protein would be roughly 50% instead of 60%. Similarly, if the nonfolding forms contained all-cis isomers, then the total percentage of trans Pro-93 and trans Pro-114 would be approximately 75%.

Results

Figure 1 shows the 1D NMR spectrum of heat-unfolded RNase at 50 °C, pH 2, between 8 and 5 ppm, which includes part of the $C^{\alpha}H$ region. Two sets of peaks, the triplet labeled d and the doublet of doublets labeled e, are resolved from the other $C^{\alpha}H$ resonances on the downfield side of the envelope. These resonances have been assigned to the $C^{\alpha}H$ of Asn-113 and Tyr-92, respectively. Proof of the assignments is presented below. Furthermore, the NOESY spectrum of this sample shows strong cross-peaks between each of the $C^{\alpha}H$ of Asn-113 and Tyr-92 with a corresponding pair of $C^{\delta}H$ and $C^{\delta'}H$ of different prolines (peaks e–h, Figure 2). NOE cross-peaks arise from distance-dependent dipole–dipole interactions, and

Table I: Chemical Shifts of Resonances in Selected Trans X-Pro Sequences

sample[a]	chemical shift (ppm) X(C$^{\alpha}$H)	X(C$^{\beta}$H)	X(C$^{\beta'}$H)	Pro(C$^{\delta}$H)	Pro(C$^{\delta'}$H)
RNase A at 50 °C, pH 2					
Lys-41-Pro-42	4.62	1.81	1.70	3.82	3.63
Tyr-92-Pro-93	4.85	3.02	2.80	3.73	3.60
Asn-113-Pro-114	4.95	2.79	2.68	3.74	3.63
Val-116-Pro-117	4.33	no[b]	na[b]	3.62	3.62
OT16[c,d] at 20 °C, pH 2.2					
Asn-113-Pro-114	4.97	2.83	2.67	3.75	3.62
Val-116-Pro-117	4.31	1.90	na	3.61	3.61
terminally blocked NPYVP[d] at 33 °C, pH 2.4					
Asn-113-Pro-114	4.94	2.81	2.64	3.79	3.64
Val-116-Pro-117	4.31	no	na	3.57	3.57
terminally blocked YPN[e] at 25 °C, pH 6.6					
Tyr-92-Pro-93	4.59	3.07	2.83	3.72	3.54

[a] The sequence numbers correspond to those of RNase A. [b] no, not observed; na, not applicable. [c] OT16 corresponds to the last 20 amino acids of RNase A. [d] From M. Adler, J. M. Beals, G. T. Montelione, and H. A. Scheraga unpublished results. [e] From Stimson et al. (1982). The difference in the chemical shifts of Tyr-92 C$^{\alpha}$H between YPN and RNase A may result from the positive charge of Lys-91.

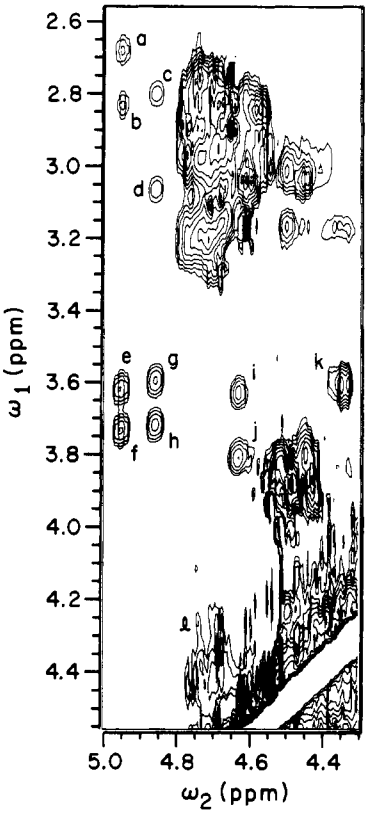

FIGURE 2: Section of the NOESY spectrum of heat-unfolded RNase A at 50 °C, pH 2. Peaks are labeled according to the following description. Peaks a–d are intraresidue cross-peaks between α- and β-protons of Asn-113 (a and b) and Tyr-92 (c and d). Peaks e–k are cross-peaks between δ- or δ'-protons of a trans proline and the C$^{\alpha}$H of the preceding residue. The pairs of residues are (e and f) Asn-113 and Pro-114, (g and h) Tyr-92 and Pro-93, (i and j) Lys-41 and Pro-42, (k) Val-116 and Pro-117. Peak ℓ represents unresolved C$^{\alpha}$H and C$^{\alpha}$H cross-peaks.

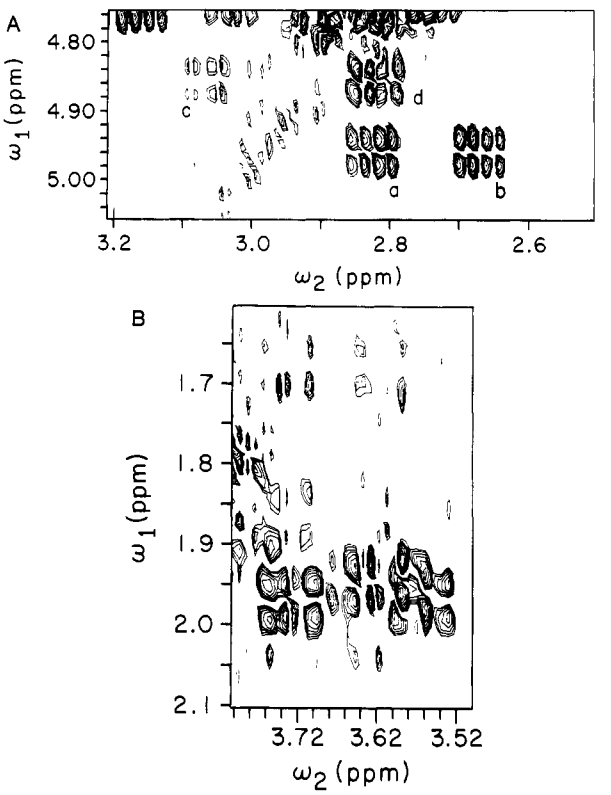

FIGURE 3: Selected sections from the proton DQF-COSY spectrum of heat-unfolded RNase A at 67 °C, pH 3. (Panel A) The cross-peak between the α- and β-protons (a and b) of Asn-114 and (c and d) of Tyr-92. (Panel B) The cross-peaks between the δ- and γ-protons of the four prolines.

are observable if the ^{1}H...^{1}H separation is $\leq$5 Å. Calculations have shown that the C$^{\alpha}$H to Pro C$^{\delta}$H and C$^{\delta'}$H NOE cross-peaks would be observable only when the proline peptide bond is trans instead of cis (Wüthrich et al., 1984; Montelione et al., 1986).

The assignments of the two C$^{\alpha}$H's are based on the NOESY and DQF-COSY spectra (Figures 2 and 3). The DQF-COSY spectrum (peaks a–d, Figure 3A) shows each C$^{\alpha}$H coupled to two C$^{\beta}$H's. The splitting pattern indicates that these are AMX spin systems, i.e., a single α-proton coupled to two β's. Several pieces of evidence support the hypothesis that these β-protons are not part of a longer side chain. First, there are no detectable cross-peaks to any additional γ-protons. Second, the individual peaklets are symmetric and show no evidence of any passive coupling partners, i.e., γ-protons, that are characteristic of longer side chains (Neuhaus et al., 1985). Finally, the chemical shifts of these two AMX systems are similar to those found for Asn and Tyr in a variety of small polypeptides, including fragments of RNase A (Table I).

The NOESY spectrum (Figure 2) shows that both C$^{\alpha}$H protons have intense cross-peaks to resonances between 3.5 and 3.9 ppm. These protons have been assigned to the C$^{\delta}$H and C$^{\delta'}$H of two prolines, based on the DQF-COSY spectrum. In the DQF-COSY spectrum (panel B, Figure 3), the resonances from 3.55 to 3.65 ppm, the Pro C$^{\delta'}$H, have cross-peaks only with protons from 1.7 to 2.0 ppm, the Pro C$^{\gamma}$H and C$^{\gamma'}$H.

The chemical shifts of the $C^{\delta}H$'s and $C^{\gamma}H$'s are characteristic of those seen for proline in short peptides (Bundi & Wüthrich, 1979), including fragments of RNase A (Table I). The chemical shifts of the $C^{\gamma}H_2$ and $C^{\delta}H_2$ cross-peaks are unique among amino acid in unstructured peptides, and the cross-peaks are well resolved from all other peaks in a simulated DQF-COSY spectrum of an unstructured peptide containing all 20 amino acids [unpublished data based on Bundi and Wüthrich (1979)]. Furthermore, as shown by the degeneracy of the aromatic resonances (Figure 1), the protein is substantially unfolded under these conditions. It would be unusual to observe any structural NOE's in the absence of detectable compact structure. Therefore, we would expect to see two types of NOE's at the frequencies of peaks e–k in Figure 2: intraresidue cross-peaks or the sequential $C^{\alpha}H$–$C^{\delta}H$ NOE's found for trans X–Pro sequences. There are no peaks in the DQF-COSY spectrum that correspond to NOE peaks labeled e–k in Figure 2 (data not shown); therefore, these peaks can be assigned as sequential NOE's from proline $C^{\delta}H$. It is worth noting that the sequential NOE's for Asn-113 and Tyr-92, e–h, are more intense than their intraresidue α–β cross-peaks, a–d (Figure 2).

We must consider the possibility that one of the resonances at 3.55–3.65 ppm is a $C^{\beta}H$ of a serine. In small peptides, the β-protons of serines have chemical shifts similar to those of the proline $C^{\delta}H$'s [3.88 ppm for serine vs 3.65 ppm for proline (Bundi & Wüthrich, 1979)]. However, the DQF-COSY spectrum shows no cross-peaks which might correspond to either the α–β or the β–β cross-peaks of serine at the appropriate frequencies (data not shown). The absence of such peaks tends to preclude this possibility.

There are no resolved NOE cross-peaks in Figure 2 between the Tyr-92 and Asn-113 $C^{\alpha}H$ and any peak which could correspond to $C^{\alpha}H$ of proline. Such cross-peaks would arise if the proline peptide bond were cis instead of trans. However, work with small fragments of RNase A (M. Adler, J. M. Beals, G. T. Montelione, and H. A. Scheraga, unpublished results) indicates that the $C^{\alpha}H$ of the Asn is shifted upfield, to lower ppm, when followed by a cis proline. Therefore, the peak labeled ℓ (Figure 2), which contains many overlapping resonances, may include these cross-peaks as well.

The evidence presented so far establishes that the two $C^{\alpha}H$ protons belonging to AMX spin systems precede trans proline residues. The four prolines of RNase A, listed along with the preceding residues, are Lys-41 and Pro-42, Tyr-92 and Pro-93, Asn-113 and Pro-114, and Val-116 and Pro-117, of which Tyr-92 and Asn-113 are the only AMX spin systems. Therefore, these AMX's must belong to Tyr-92 and Asn-113. The exact assignments of these two residues are deduced from further analysis of the NOESY spectrum. The $C^{\alpha}H$ and $C^{\beta}H$'s of Tyr-92 have NOE cross-peaks to an aromatic resonance at 7.1 ppm, the $C^{\delta}H_2$ of a tyrosine ring. Asn-113 does not have a similar set of NOE cross-peaks (data not shown). Thus, we can assign the two peaks d and e in Figure 1 to Asn-113 at 4.95 ppm and Tyr-92 at 4.85 ppm, followed by trans Pro-114 and Pro-93, respectively.

Figure 2 also shows some other interesting cross-peaks labeled i–k. The chemical shift of the cross-peaks i and k in ω_1 at 3.6 ppm is characteristic of the $C^{\delta'}H$ of proline. Therefore, the $C^{\alpha}H$ protons that appear at 4.62 ppm (peaks i and j) and 4.33 ppm (peak k) most likely belong to two residues that precede trans proline. Two possibilities are Lys-41 and Pro-42, and Val-116 and Pro-117. The Lys-41 $C^{\alpha}H$ resonances, peaks i and j, can be identified by the two cross-peaks with protons at 1.70 and 1.81 ppm in the DQF-COSY spectrum which correspond to its own $C^{\beta}H$ and $C^{\beta'}H$ (data not shown). The Val cross-peak is identified by the doublet shape in the ω_2 direction, indicating that the $C^{\alpha}H$ resonance is coupled to a single $C^{\beta}H$ proton. Furthermore, the Pro-117 $C^{\delta}H_2$ protons have an NOE cross-peak to a resonance at 0.88 ppm, presumably the Val $C^{\gamma}H_3$ (data not shown). The Val-116 $C^{\alpha}H$ and Pro-117 $C^{\delta}H_2$ resonances also match the chemical shift of these protons in fragments of RNase A (Table I). The intensities of peaks i–k indicate that the Pro-42 and Pro-117 are predominantly trans. More precise measurements of the percentages of isomers have not been made since neither the $C^{\alpha}H$'s of Lys-41 and Val-116 nor the $C^{\delta}H$'s of Pro-42 and Pro-117 are resolved in the 1D spectra.

Table II: Integrated Intensities of Selected Aromatic Resonances, Asn-113 $C^{\alpha}H$ at 4.95 ppm and Tyr-92 $C^{\alpha}H$ at 4.85 ppm

resonances	expected no. of protons	obsd no. of protons[a]	1σ[b]
Phe ring His $C^{\delta}H$	19	19.2	0.17
Tyr $C^{\delta}H$	12	12.1	0.07
Tyr $C^{\epsilon}H$	12	11.7	0.17
Asn-113 $C^{\alpha}H$	<1[c]	0.63	0.07
Tyr-92 $C^{\alpha}H$	<1[c]	0.60	0.07

[a] Intensities are normalized against the entire integrated intensity of the 43 aromatic ring protons between 6.7 and 7.5 ppm. [b] Statistics based on 11 spectra collected over a temperature range of 50–75 °C at pH 2 and 3. [c] This value would equal 1 if Pro-114 and Pro-93, respectively, were 100% in the trans conformation and/or the chemical shift of the $C^{\alpha}H$ was the same for both the cis and trans conformations of the peptide bond. The observed number of protons is less than 1. This indicates that 37% of Pro-114 is cis and 40% of Pro-93 is cis. Furthermore, the chemical shifts of the $C^{\alpha}H$'s of Asn-113 and Tyr-92 have been altered by the cis peptide bond.

Taken together, the evidence strongly supports the assignment of the resonances as presented here. There are four prolines in ribonuclease, and there are four intense peaks in the NOESY spectrum (e, g, i, and k in Figure 2) which seem to correspond to sequential $C^{\alpha}H$–$C^{\delta}H$ NOE's which are characteristic of trans X-Pro sequences. Therefore, these assignments represent the simplest explanation of the data, and given the lack of evidence for the existence of compact structure in RNase A under these conditions, it is safe to assume that the simplest explanation is correct. However, in the absence of the full sequential assignments, some ambiguity inevitably remains. Unfortunately, it is not possible to extend the assignments due to problems with overlapping resonances.

The percentages of the trans Pro-93 and trans Pro-114 were determined by comparing the integrated intensities of the resolved $C^{\alpha}H$ resonances of Tyr-92 and Asn-113 to those of the 43 aromatic ring protons that lie between 6.7 and 7.5 ppm. The carrier frequency was shifted by approximately 6 ppm to minimize distortions from variation in pulse width with frequency. (The intensities of the four $C^{\epsilon}H$ His at 8.6 ppm were 10% lower than expected and were not used in the calculations.) The results from 11 integrations from independent data sets collected over a range of temperatures from 50 to 75 °C (Figure 1 and data not shown) are presented in Table II. No significant variation in the relative intensities was observed at different temperatures.

The intensities for the $C^{\alpha}H$ of Tyr-92 and Asn-113 preceding trans prolines integrate as 0.60 and 0.63 protons, respectively, with a single standard deviation of 0.07 proton. Comparisons between the numbers of protons in the three resolved groups of aromatic resonances (Table II) and the expected values indicate that there was no significant systematic deviations. The measured intensities showed less than a 2% variation when the limits of integration were changed

to include the tailing edges of the peaks (data not shown).

Discussion

Percentages of Nonnative Trans Isomers. Our data indicate that the peptide bonds preceding Pro-93 and Pro-114 are 60% and 63% trans (1σ = 7%) in reversibly heat-unfolded RNase A at pH 2 or 3. These results differ from those of Lin and Brandts (1983b, 1984, 1987a). They estimated the amount of trans isomer at Pro-93 and Pro-114 to be 30% and 5%, respectively. The percentage of trans proline was measured by using isomer-specific proteases (ISP), trypsin for Pro-93 and chymotrypsin for Pro-114. In both cases, the slow rate of cleavage by an ISP at a neighboring bond was thought to reflect a cis proline in a flanking residue. It should be noted that RNase A had to be partially degraded by either pepsin or mercaptoethanol to expose the cleavage site to the ISP in these studies. It is possible that the slow step in the cleavage of RNase A by the ISP was not the cis to trans isomerization of the flanking proline bond, as the authors presume. Instead, the rate-limiting step may have been a conformational change at a second site. For example, the conformation of the disulfide bond between Cys-40 and Cys-95 may affect the rate of cleavage of RNase A by trypsin at Tyr-92, leading to artificially low estimates of the trans proline content at Pro-93. Alternatively, further degradation of RNase A by the ISP may have been needed to expose the intended site.

It is unlikely that the discrepancies between our results and those of Lin and Brandts (1983b, 1984, 1987a) reflect differences between the heat-denaturated and urea-denatured protein. Although no direct comparison was made, a variety of experiments indicate that the proportion of conformational isomers in unfolded RNase A is the same, no matter what technique is used to unfold the protein. Garel et al. (1976) demonstrated that the rate and proportion of the various phases are unchanged whether the protein is unfolded at a 47 °C, pH 2.0, 0.5 M Gdn·HCl (very close to our own conditions), or whether the protein is unfolded at 47 °C, pH 6.0, 5 M Gdn·HCl. The final folding conditions were 47 °C, pH 6.0, 0.5 M Gdn·HCl. Garel et al. (1976) and Mui et al. (1985) obtained identical results using either urea or Gdn·HCl as the denaturant. These experiments indicate that the exact conditions used to unfold the protein do not affect the ratio of conformational isomers.

Of course, there may be systematic errors in our own measurements. For example, if the conformation of the disulfide bond between Cys-40 and Cys-95 altered the chemical shift of Tyr-92 $C^{\alpha}H$, then our estimate of the amount of trans Pro-93 would be too low.

However, our measurements of the percentage of trans Pro-93 would have to be decreased by a factor of 2 in order to be consistent with the work of Lin and Brandts (1983b, 1987a). It is even more unlikely that our estimate of the amount of trans Pro-114 is in error by an order of magnitude. Thus, there is no simple explanation for the discrepancies between our results and those of Lin and Brandts.

The cis/trans isomerization of Pro-93 and Pro-114 has been studied in blocked tripeptides containing flanking amino acids (Stimson et al., 1982). Tyr-Pro-Asn, which corresponds to residues 92–94, forms a 25/75 mixture of cis and trans isomers. Asn-Pro-Tyr, residues 113–115, forms a 14/86 mixture of cis and trans. Compared to these peptides, heat-unfolded RNase A stabilizes the native cis conformation by a factor of 2–3. However, the nonnative trans isomer still predominates in heat-unfolded RNase A.

Models for Refolding of RNase A. As mentioned in the introduction, the percentages of the fast- and slow-folding phases, U_f, U_s^I, and U_s^{II}, are thought to be 20%, 30%, and 50%, respectively, based on changes in absorbance measured at 287 nm. A similar estimate of 22% for the fast phase was obtained by Baldwin and co-workers (Tsong et al., 1972; Garel & Baldwin, 1973; Garel et al., 1976) by studying the binding of cAMP, an inhibitor of native RNase A, during refolding.

On the basis of the data presented here and a statistical argument, assuming that there is no interaction between the prolines in the denatured protein, our results indicate that 15% of the unfolded protein has the native cis isomers of *both* Pro-93 and Pro-114. Another 38% of the protein has *both* nonnative trans isomers. Finally, 47% of the protein has a single trans isomer at either Pro-93 or Pro-114 (25% and 22%, respectively) but not at both. An argument could be made, on the basis of these estimates, that the isomer containing the cis conformation of *both* Pro-93 and Pro-114 corresponds to the fast phase, the double trans isomer is responsible for U_s^I, and the two isomers with a single trans proline at either Pro-93 or Pro-114 together make up U_s^{II}. Given the experimental errors in our results, 1σ = 7%, there is good agreement between the amount of the various cis and trans isomers with the observed percentages of the fast and slow phases.

However, the folding properties of U_s^{II} indicate that it is not a mixture of two isomers. At neutral pH and in the absence of denaturants, U_s^{II} refolds to an intermediate called I_N that has both native structure and enzymatic activity (Cook et al., 1979; Schmid & Blaschek, 1981; Mui et al., 1985; Schmid, 1986; Lin & Brandts, 1987a). The refolding takes place before all bonds isomerize to the native form. The rate of folding of U_s^{II} to I_N is 100-fold slower than in the fast phase, U_f to N. In addition, I_N is less stable to chemical denaturants than native. The refolding of U_s^{II} to I_N has been studied extensively, and it behaves like a single isomer, not a heterogeneous mixture (Cook et al., 1979; Schmid & Blaschek, 1981; Mui et al., 1985; Schmid, 1986; Lin & Brandts, 1983a, 1987a). It is unlikely that two distinct nonnative proline isomers, located at opposite ends of the native structure, would inhibit refolding to the same extent under a variety of conditions. Therefore, other models for refolding of RNase A should be examined.

Alternatively, there may be a third unidentified nonnative isomer. Let us hypothesize that 30% of the unfolded protein contains this isomer and that the protein cannot refold until this isomer adopts the native conformation. Furthermore, we will assume that one of the two trans isomers at Pro-93 and Pro-114 does *not* interfere with refolding. Thus, 27% of the protein refolds in the fast phase, 30% refolds as U_s^I, and 43% refolds as U_s^{II}. Once again, the relative percentages of the various phases agree with our result given the 1σ = 7% experimental error.

If this hypothesis is true, then one of the two trans isomers at Pro-93 or Pro-114 does not inhibit refolding at all. The proline which does not affect the rate of refolding can be referred to as the silent isomer. Since the trans isomer of the silent proline would be equally distributed in all phases, the fast phase would be a heterogeneous mixture of both the native and the nonnative forms, with the native isomer accounting for less than half the total of U_f.

This hypothesis is consistent with the theory of Schmid and co-workers (Schmid et al., 1986; Schmid, 1986) that trans Pro-93 is found in both U_s^I and U_s^{II} and that it does reduce the rate of refolding. Therefore, trans Pro-93 should account for roughly 70% of the unfolded species, close to our own estimates for the percentage of trans Pro-93. Trans Pro-114 would then correspond to the silent isomer. However, the theory of Schmid and co-workers is based on kinetic evidence gathered

from fluorescence spectroscopy, and the interpretation of the data is dependent on the model used for refolding (Schmid, 1986; Lin & Brandts, 1987b). Measurements reported in this paper were made under equilibrium conditions, and the role that each isomer plays during refolding is still unknown. Direct measurements of the percentages of isomers during refolding are needed to resolve the remaining questions.

Discussion of Silent Isomers. The next question is as follows: "How could half the protein refold in the rapid phase even if it contains a nonnative proline isomer(s)?" Two theories may be invoked. First, the silent proline may not participate in the rate-limiting step in the fast phase. Whatever compact, locally order structures form (or do not form) around the proline, they may have no relevance to the overall rate of folding. Alternatively, similar structures may form around both the cis and trans isomers of the proline, and each can participate in the subsequent steps in refolding.

The second theory receives support from the experimental and theoretical work of Scheraga and co-workers (Stimson et al., 1982, 1986; Montelione et al., 1984; Oka et al., 1984). Their results indicated that a β-bend still forms around trans Pro-114 in short fragments. In the crystalline state, terminally blocked Asn-Pro-Tyr, residues 113–115, is 100% trans and forms a type I β-bend (Montelione et al., 1984). Solution measurements, made by both NMR and Raman spectroscopy, gave results consistent with the pattern of intramolecular hydrogen bonds seen in the crystal, confirming that the β-bend surrounding Pro-114 is partially stable in solution (Montelione et al., 1984).

It is important to note that the β-bend is shifted by one residue further toward the N-terminus in the type I β-bend (trans Pro) compared to the native type VI β-bend (cis Pro) (Wlodawer et al., 1982; Borkakoti et al., 1982; Montelione et al., 1984). However, the β-sheet that extends away from Pro-114 toward the C-terminus contains two β-bulges (Richardson et al., 1978). Therefore, a shift in the β-bend may be accommodated by removing one of the β-bulges.

The blocked tripeptide Tyr-Pro-Asn, which corresponds to residues 92–94 in RNase A, is in the trans conformation in the crystalline form and predominantly in the trans conformation in solution (Montelione et al., 1984). In contrast to Asn-Pro-Tyr, the data indicate that the peptide forms an extended structure with little or no hydrogen bonding (Stimson et al., 1982, 1986; Montelione et al., 1984).

The evidence from small peptides indicates that trans Pro-114 may indeed be a silent isomer, whereas trans Pro-93 may significantly inhibit refolding.

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