Investigation of Ribonuclease T_1 Folding Intermediates by Hydrogen–Deuterium Amide Exchange–Two-Dimensional NMR Spectroscopy[†]

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ABSTRACT: The rate of hydrogen bond formation at individual amino acid residues in ribonuclease T1 (RNase T₁) has been investigated by the hydrogen-deuterium exchange-2D NMR (HDEx-2D NMR) technique (Udgaonkar & Baldwin, 1988; Rder et al., 1988) to gain insight into the mechanism and pathways of protein folding. The HDEx-2D NMR technique combines rapid mixing and 2D NMR methods to follow the protection of backbone amide deuterons from exchange with solvent protons as a function of folding time. The technique depends on the difference in the exchange rates of hydrogen-bonded and non-hydrogenbonded amide residues so that as the protein folds, the amide residues involved in hydrogen bonding are protected from exchange with solvent to give structural information about early folding events. The observed time course for deuterium protection was followed for 24 backbone amide residues that form stable hydrogen bonds in RNase T_1 . The time courses are biphasic with 60-80% of the protein molecules showing rapid hydrogen bond formation (12-119 s⁻¹) in the α -helix and the β -sheet. The remaining 20-40% of the molecules are protected in a slow phase with a rate constant that has a lower limit of 0.01 s⁻¹. If the rate constants in this first phase are arbitrarily subdivided into two classes, fast ($\geq 25 \text{ s}^{-1}$) and intermediate (<25 s^{-1}), then the amide residues that are found in the hydrophobic core are in the fast class while those located on the periphery of the three-dimensional structure are in the intermediate class. The HDEx-2D NMR results indicate that, in the early stages of folding, RNase T1 folds on at least two parallel pathways, each having at least one intermediate. We propose that these two intermediates resemble a native-like intermediate and a "nonstructured" molten globule.

There is intense interest in the mechanism of folding of proteins both in vivo and in vitro. On the basis of recent studies, the mechanism of protein folding in vivo has become more complicated as it has become clearer that a number of other proteins are involved, but the mechanism of protein folding in vitro has become less complicated as some of the more complex models have been eliminated. Experimental results supporting at least three descriptions of protein folding pathways in vitro have been presented (Baldwin, 1989, 1990). These depictions include molten globules (Kuwajima, 1989), the framework model (Kim & Baldwin, 1982), and the subdomain model (Oas & Kim, 1988). The molten globule model postulates a compact intermediate with considerable formation of secondary structure. In the framework model, the initial formation of the secondary structural elements precedes subsequent folding events. The subdomain model proposes the initial formation of small domains of secondary and tertiary structure that later dock to form the native protein structure. The three models are complementary in that each predicts a small number of sequential pathways with a finite number of transiently formed intermediates. The integration of one or more of these models into a unified description of protein folding requires a more detailed structural characterization of protein folding intermediates. This has, however, been difficult to achieve experimentally due to the transient nature of these intermediates. This limitation has been overcome, in part, by the development of the hydrogendeuterium exchange-2DNMR (HDEx-2DNMR) technique by Udgaonkar and Baldwin (1988) and Roder *et al.* (1988). The HDEx-2D NMR technique enables the structural elucidation of transiently formed folding intermediates and thus permits a more detailed structural characterization of protein folding pathways. This information can therefore provide valuable insight into the parameters governing secondary and tertiary structure formation during protein folding.

The HDEx-2D NMR technique exploits the difference in the chemical exchange rates of hydrogen-bonded and nonhydrogen-bonded amide residues. The protein is unfolded in D_2O to allow all amide protons to exchange with deuterium. At time t_0 the denaturant is rapidly diluted below the critical denaturant concentration and the protein allowed to refold. After various times, t_1 , the refolding protein is exposed to an elevated pH H₂O pulse for a time t_2 during which all nonhydrogen-bonded amide deuterons exchange for protons (Udgaonkar & Baldwin, 1988; Roder et al., 1988). The pH is then reduced to enable the protein to complete the folding process under conditions where all of the amide exchange reactions are quenched. The protein at this point has a mixture of hydrogen and deuterium labels whose occupancy can be determined by 2D J-correlated (COSY) NMR spectroscopy (Bax & Freeman, 1981) for the slowly exchanging amide sites of the fully folded protein. The folded and unfolded portions of the protein can therefore be differentiated as a function of the refolding time, t_1 , because only the portions of the protein which have folded during the period t_1 will contain the deuterium label.¹ The time course for deuterium protection thus provides a measure of the rate of hydrogen bond formation for each of these residues during the folding process. We have now investigated the rate of hydrogen bond formation at individual amino acid positions in ribonuclease

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Folding Pathway for RNase T_1

T_1 (RNase T_1) by the HDEx-2D NMR technique.

RNase T_1 is an excellent model for studies of the mechanism of protein folding. RNase T_1 is a small compact globular protein of 104 amino acids with a single hydrophobic core formed between a 4.5-turn α -helix and an antiparallel β -sheet. RNase T_1 has four prolines, two of which are in the *cis* conformation (Pro-39 and Pro-55) and two of which are in the trans conformation (Pro-60 and Pro-71). The native conformation of RNase T_1 has two disulfide bonds forming a large loop (Cys-6 to Cys-103) and a small loop (Cys-2 to Cys-10). The three-dimensional structure has been determined to 1.5-Å resolution by X-ray crystallography (Martinez-Oyanedel et al., 1991) and the complete NMR spectrum assigned for the resonances observed at 500 MHz (Hoffmann & Ruterjans, 1988). The energetics (Pace, 1990) and the kinetics (Kiefhaber et al., 1990-a-c, 1992a,b) of folding of wild-type RNase T_1 and a number of mutants have been studied. The overall folding of RNase T_1 is remarkably slow and quite temperature sensitive. Folding is slowest to reach equilibrium in the transition region; it requires minutes at 30 °C, hours at 25 °C, and days at 20 °C (Pace et al., 1989). Despite the slow overall folding, the formation of secondary structure in RNase T₁ appears to occur in milliseconds (Kiefhaber et al., 1990c, 1992b). The fluorescence emission maximum for Trp-59 that is characteristic of the folded protein is observed in less than 1 s, and enzymatic activity (50%) is restored within 20 s of refolding (Kiefhaber et al., 1992b). These results suggest that the secondary structure and at least parts of the hydrophobic core are formed very rapidly in the folding process.

EXPERIMENTAL PROCEDURES

Materials. RNase T_1 was purified as described by Shirley and Laurents (1990). The concentration of protein was determined using a molar extinction coefficient of 1.85×10^4 M^{-1} cm⁻¹ at 278 nm. All chemicals and solvents were obtained from Sigma Chemical Co.

Hydrogen-Deuterium Exchange Experiments. RNase T₁ (30 mg/mL) was denatured, and all exchangeable NH groups were deuterated by incubation in 5 M Gdm-DCl, 50 mM acetate-d₃, and 99.8% D₂O at pD 5.4 at 25 °C for 24 h. The protein was allowed to refold (6-7500 ms, 25 °C) by a 5-fold dilution into 0.1 M acetate-d₃ and H₂O, pH 5.0 (or D₂O, pD 5.4, for folding times >1000 ms), using a Bio-Logic QFM5 rapid-quench instrument. The refolding protein was diluted 5-fold (D₂O refold) of 2-fold (H₂O refold) into 0.3 M phosphate (H_2O) , pH 9.8, for a 50-ms labeling pulse. The pulse was quenched by a 2-fold dilution into 0.4 M acetate- d_3 , pH 4.5, and the sample then placed on ice. The final pH values of the three phases (refolding, labeling, and quench) were 5.0, 8.5, and 5.0, respectively. The samples were concentrated and exchanged into D₂O at 4 °C by repeated dilution and concentration in an Amicon 8000 series ultrafiltration cell using YM3 membranes. The samples were frozen (dry ice/acetone) and stored until analysis by 2D NMR spectroscopy. The concentration of the samples used for the NMR analysis was 50 mg/mL.

The reference NMR sample was prepared by unfolding RNase T_1 (30 mg/mL) in 5 M Gdm-DCl/HCl and 50 mM acetate, pH 5.0, in 90% H₂O at 25 °C for 24 h so that all the



FIGURE 1: Fingerprint region of a 400-MHz COSY NMR spectrum of RNase T_1 recorded at 20 °C in D₂O with 50 mM acetate- d_{3} , pD 5.4. The NH-C_aH cross-peaks [cross-peak assignments made by Hoffmann and Ruterjans (1988)] labeled are those used as structural probes in the HDEx-2D NMR experiment. Additional details are given in the text.

amide sites were about 90% protonated. The sample was then diluted 5-fold into 50 mM acetate, pH 5.0, in 90% H_2O to allow the protein to refold. The volume integrals determined from this sample were used as the intensity standards for proton occupancy.

NMR Spectroscopy. Magnitude COSY spectra were recorded at 20 °C on a Varian XL400 NMR spectrometer. One hundred twenty-eight transients of 1024 data points covering 5300 Hz were collected for each of $256 t_1$ increments. Each spectrum was processed using FELIX (Hare Research) on a Silicon Graphics 4D/25GT workstation. Before Fourier transformation, the data were multiplied by a sine-squared function phase-shifted by $\pi/12$ in the t_1 and the t_2 dimensions. Cross-peak intensities were measured by volume integration. Each spectrum was internally referenced to two nonexchangeable aromatic cross-peaks (W59 and F80).

RESULTS

All of the backbone amide protein resonances in RNase T_1 have been assigned in the two-dimensional COSY NMR spectrum (Hoffmann & Ruterjans, 1988). Under our experimental conditions, it was possible to unambiguously follow the kinetic protection for 24 separate amide residues involved in secondary and tertiary interactions. Figure 1 shows the fingerprint region of the COSY NMR spectrum for the amide groups used as structural probes in this study. Figure 2 shows the time course for the protection of these amide groups for a representative sample of the protons followed during the refolding of RNase T₁. All but four (A22, L26, I61, and F80) of the residues were found to obtain 100% protection within 10 min of refolding. The volumes of these four residues were too small to calculate accurately. The residues that were followed are listed in Table I along with their secondary structure location and hydrogen-bonding partner.

As shown in Figure 2, the curves are, at least, biphasic with rapid hydrogen bond formation $(12-119 \text{ s}^{-1})$ in the α -helix

¹ Wuthrich and co-workers have observed pockets of residual secondary structure even under highly denaturing conditions (Neri *et al.*, 1992). Existing structure in the "unfolded" protein could influence the folding pathway detected in the HDEx-2D NMR experiments.



FIGURE 2: Time courses for hydrogen incorporation into backbone amide groups. Represented are residues from the sheet (V101), helix (A19), turn (H92), and a tertiary interaction (Q85). Residues W59, V16, and D76 are in the fast class of rate constants ($\geq 25 \text{ s}^{-1}$). The intermediate class of rate constants ($\leq 25 \text{ s}^{-1}$) are represented by C6 and D76. The fraction of exchangeable deuterons is defiend as 1 at time 0. The data were fit to the following equation: $y = 1 + A[\exp(-kt) - 1]$. Additional details are given in text.

| residue | NH location | type and location of contact | phase 1 rate constant (s ⁻¹) | amplitude |
|---------|----------------|------------------------------------|--|-----------------|
| T5 | sheet | backbone (T104) | 26 ± 8 | 0.82 ± 0.07 |
| C6 | sheet | backbone (N9) | 13 ± 5 | 0.76 ± 0.08 |
| Y11 | sheet | backbone (Y4) | 15 ± 5 | 0.67 ± 0.06 |
| V16 | helix | backbone (S12) | 21 ± 4 | 0.62 ± 0.04 |
| A19 | helix | backbone (D15) | 55 ± 8 | 0.73 ± 0.03 |
| A21 | helix | backbone (S17) | 23 ± 11 | 0.58 ± 0.09 |
| A22 | helix | backbone (T18) | 23 ± 5 | 0.58 ± 0.04 |
| K25 | helix | backbone (A21) | 14 ± 6 | 0.73 ± 0.15 |
| L26 | helix | backbone (A22) | 53 ± 13 | 0.68 ± 0.04 |
| W59 | sheet | backbone (V78) | 25 ± 6 | 0.56 ± 0.04 |
| I61 | sheet | backbone (D76) | 31 ± 21 | 0.52 ± 0.10 |
| D76 | sheet | backbone (I61) | 12 ± 6 | 0.75 ± 0.12 |
| R77 | sheet | backbone (I90) | 119 ± 21 | 0.88 ± 0.04 |
| V79 | sheet | backbone (G88) | 28 ± 8 | 0.52 ± 0.04 |
| F80 | sheet | backbone (Y57) | 18 ± 6 | 0.64 ± 0.08 |
| N81 | turn | backbone (Q85) | 19 ± 6 | 0.72 ± 0.07 |
| N84 | turn | backbone (N81) | 19 ± 7 | 0.73 ± 0.11 |
| Q85 | sheet | tertiary (N81:OD1) | 34 ± 6 | 0.73 ± 0.04 |
| A87 | sheet | backbone (V79) | 59 ± 13 | 0.72 ± 0.05 |
| G88 | sheet | backbone (V79) | 16 ± 11 | 0.83 ± 0.11 |
| T91 | sheet | backbone (V101) | 37 ± 14 | 0.61 ± 0.06 |
| H92 | turn | backbone (A75) | 59 ± 9 | 0.82 ± 0.04 |
| F100 | sheet | tertiary (E46:OE2) | 51 ± 10 | 0.71 ± 0.04 |
| V101 | sheet | backbone (T91) | 89 ± 10 | 0.81 ± 0.03 |

and the β -sheet structure in 60–80% of the protein molecules. The rate constants and the amplitudes of the fast phase are given in Table I. If we arbitrarily subdivide the rate constants for protection of the amide NH groups shown in Table I into two classes, fast ($\geq 25 \text{ s}^{-1}$) and intermediate ($\leq 25 \text{ s}^{-1}$), then the amide positions protected in the fast class appear to be primarily associated with the hydrophobic core whereas those in the intermediate class are located on the periphery of the folded protein structure (Figure 3). The remaining 20-40% of the protein molecules are protected in a slow phase with a rate constant that has a lower limit of 0.01 s⁻¹. The solvent exchange time for unprotected amides is about 10 s under the quench conditions used (pH 5, 0-4 °C). Since the rate for the overall RNase T₁ folding is very slow (half-life at 10 °C = 4500 s) for these conditions, the amide protons not protected by secondary structure formation could exchange with the bulk solvent even under the experimental quench conditions.



FIGURE 3: Backbone amide groups protected from exchange during the time course of refolding. The fast ($\geq 25 \text{ s}^{-1}$) and intermediate phases ($\leq 25 \text{ s}^{-1}$) are represented as yellow and green CPK spheres, respectively. The RNase T₁ structure was made with Insight II from BIOSYM using the coordinates of the crystal structure determined by Martinez-Oyanedel et al. (1991).

It is unlikely, however, that the slow phase observed in these experiments is due to incomplete quenching because preliminary experiments utilizing a D₂O quench buffer yielded the same results as later experiments with a H₂O quench buffer (data not shown). Further support for this conclusion is the experimental observation of 100% protection for 20 of the 24 residues after 10 min.

The stability of the intermediate formed in the slow-folding phase of the HDEx-2D NMR experiment (see Figure 2) was probed by varying the H₂O pulse conditions. Intermediates with limited structural stability may partially unfold under the pulse conditions (50 ms at pH 8.5) used to allow deuterium/ hydrogen exchange to occur and thus mask the formation of transient intermediates. Four pulse conditions were tested on the 100-ms refolding time point by combining two pulse pH values (pH 7.8 and 8.5) and two pulse durations (50 and 150 ms). The two pulse pH conditions were chosen because the exchange rate for amide protons is reduced about 10-fold

Table II: Percent Proton Occupancy after 100 ms of Folding for Eight Residues of RNase T_1 Obtained from Variable Pulse Conditions

| | proton occupancy (%) | | | | | |
|-------------|--------------------------|--------|--------------------------|--------|--|--|
| | pulse duration at pH 8.5 | | pulse duration at pH 7.8 | | | |
| | 50 ms | 150 ms | 50 ms | 150 ms | | |
| T5 | 25 | 22 | 28 | 28 | | |
| C6 | 44 | 47 | 39 | 35 | | |
| Y 11 | 42 | 42 | 34 | 33 | | |
| A19 | 23 | 27 | 23 | 38 | | |
| N81 | 32 | 38 | 35 | 36 | | |
| Q85 | 34 | 37 | 33 | 38 | | |
| H92 | 21 | 26 | 19 | 24 | | |
| F100 | 23 | 16 | 17 | 20 | | |

with each unit pH reduction creating a weaker pulse. Because the pulse is weak, increasing the duration of the pulse increases the effectiveness (this is true only if the rate of folding is slower than the pulse duration such that little folding is occurring during the pulse). An increase in proton occupancy as the pulse duration is increased or, conversely, any decrease in proton occupancy as the pulse pH is decreased would be indicative of an unstable intermediate in the slow phase. The percent proton occupancy obtained under the variable pulse conditions is listed in Table II for 8 of the 24 residues followed. These changes in pulse conditions had no significant effect on the extent of proton incorporation (Table II). Therefore, any intermediate structure formed during the refolding time period, t_1 , does not appear to undergo destabilization that results in loss of structure under our pulse conditions.

DISCUSSION

The two major kinetic phases observed in the HDEx-2D NMR experiments with RNase T_1 indicate the existence of at least two parallel folding pathways. This observation of an initial heterogeneous population is also supported by the insensitivity of the folding molecules to the labeling pulse conditions, and thus the extent of labeling depends only on the fraction of the molecules forming the intermediate. The majority (70%) of the molecules fold on one pathway by forming an intermediate(s) in which hydrogen bonds are rapidly formed (<100 ms) to protect 24 amide NH groups from exchange. The remaining 30% of the protein molecules fold along a second pathway in which amide hydrogen protection is slow but complete within 10 min.

The complete folding of RNase T_1 is a complex process demonstrated to be primarily dependent upon the rate of cistrans isomerization of the two amide bonds to Pro-39 and Pro-55 (Kiefhaber et al., 1990a-c). The unfolded RNase T₁ has four possible conformations of Pro-39 and Pro-55: U(t39,t55), U(t39,c55), U(c39,t55), and U(c39,c55) [terminology of Kiefhaber et al. (1990a-c)]. Schmid and co-workers have proposed a detailed kinetic folding mechanism where approximately 4% of the molecules have the proper conformation for all of the proline residues and fold completely within milliseconds. The remaining protein molecules fold along a branched pathway with four intermediates: I(t39,t55), I(t39,c55), I(c39,t55), and I(c39,c55). The flux through the pathway depends upon which of the amide bonds to the two critical proline residues is the first to isomerize. Utilizing unfolding assays, Kiefhaber et al. (1990a, 1992b) demonstrated that the folding intermediates with only one trans conformation of either Pro-39, I(t39,c55), or Pro-55, I(c39,t55), form very rapidly and show native-like properties. In contrast, the intermediate with both trans Pro-39 and Pro-55,

I(t39,t55), appears loosely structured because both proline residues are accessible to catalysis by prolyl isomerase. The population of the three intermediates with incorrect prolines, I(t39,t55), I(t39,c55), and I(c39,t55), accounts for an amplitude of approximately 90% in the unfolding assays performed under the same conditions reported here for the HDEx-2D NMR experiments. An amplitude of approximately 50% is observed for the intermediate with two *trans* proline [I(t39, t55)] and 40% for the two intermediates with one *trans* proline [I(t39,c55) and I(c39,t55)].

The results from the HDEx-2D NMR experiments are slightly different from those obtained with the proline isomerization model. The fast-folding molecules observed in Schmid's experiments are probably included in our fast phase of folding but are present at a concentration too low to be distinguished from the rest of the protein. It is possible that the fast phase observed in our HDEx-2D NMR experiment might correspond to the formation of the intermediates observed in Schmid's unfolding assay which have one incorrect proline. However, the observed amplitude of 60-70% in the HDEx-2D NMR experiment is much greater than the observed amplitude (40%) in the unfolding experiments. Likewise, the intermediate with slow amide hydrogen protection might relate to the intermediate with two incorrect prolines. But again, the amplitudes determined from the two experiments do not match. This difference is difficult to explain only by proline isomerization. The two experiments are not necessarily contradictory, however, in that the HDEx-2D NMR experiments are observing hydrogen bond formation occuring early in protein folding whereas the proline isomerization experiments are detecting the rate-limiting steps of slow overall protein folding. The recent experiments of Kiefhaber et al. (1992b) utilizing stopped-flow CD in which they observed greater formation of apparent secondary structure than could be accounted for by the fast-folding phase (17%) for a mutant of RNase T_1 (S54G P55N) are in agreement with HDEx-2D NMR experiments presented in this paper. The effect of proline isomerization on the kinetics of individual hydrogen bond formation will be investigated in future HDEx-2D NMR experiments.

The HDEx-2D NMR technique has been previously applied to cytochrome c (Roder et al., 1988), ribonuclease A (Udganokar & Baldwin, 1988), ubiquitin (Briggs & Roder, 1992), barnase (Bycroft et al., 1990; Matouschek et al., 1992), and most recently T4 lysozyme (Lu & Dahlquist, 1992). With these proteins it was shown that specific secondary structural elements were formed faster than the overall rate of protein folding. Portions of the β -sheet structure in ribonuclease A were formed in the first major folding event. In cytochrome c, rapid (<10 ms) protection of the N- and C-terminal α -helices was observed, followed by slower protection throughout the structure. In contrast, barnase, ubiquitin, and T4 lysozyme showed rapid formation of both α -helical and β -sheet structures. Ubiquitin shows the most rapid formation of hydrogen bonds of all the proteins studied with the HDEx-2D NMR technique. Within 8 ms, protons in the α -helix and β -sheet and the interface between the two structures are 80% protected. T4 lysozyme is interesting in that the degree of protection for some residues varies with the pulse pH and is indicative of an intermediate with variable structural stability.

The half-lives for the slowest folding phases of these proteins are 0.5 s at 23 °C for T4 lysozyme (Lu & Dahlquist, 1992), 3 s at 10 °C for cytochrome c (Roder et al., 1988), 6 s at 25 °C for barnase (Bycroft et al., 1990; Matouschek et al., 1992), 7 s at 25 °C for ubiquitin (Briggs & Roder, 1992), 104 s at 10 °C for ribonuclease A (Udganokar & Baldwin, 1988), and 4500 s at 10 °C for RNase T_1 (Kiefhaber *et al.*, 1989a–c). Thus, RNase T_1 folds much more slowly than these other proteins, and this has been shown to be due to the rate of proline isomerization. Nevertheless, the formation of the secondary structure, as evidenced by hydrogen bond formation, occurs on a similar time scale for RNase T_1 and the faster folding proteins. It is evident, therefore, that the factors governing the rate of secondary structure formation in RNase T_1 are largely independent of proline isomerization.

RNase T_1 and barnase are in the same family of microbial ribonucleases and as such are the most closely related proteins of those studied by the HDEx-2D NMR technique. The similarity of the two proteins is emphasized by the superposition of their tertiary structure (Mauguen et al., 1982; Hill et al., 1983). Even though RNase T_1 contains two disulfide bonds, Hill et al. (1983) found six regions (all in the β -sheet structure) in which the α -carbons of barnase and RNase T₁ closely correspond. The HDEx-2D NMR experiments on barnase (Bycroft et al., 1990; Matouschek et al., 1992a) show rapid formation of all or part of the α -helices, some β -turns and the β -sheet structure. Complementary unfolding (Serrano *et al.*, 1992) and refolding (Matouschek et al., 1992b) experiments indicate the center of the main hydrophobic core is formed early in folding and is more stable than the edges. These data for barnase are in good agreement with the results presented here for RNase T_1 : rapid formation of the α -helix, β -sheet, and some turns as well as formation of the center hydrophobic core prior to the periphery of the core.

The main differences in the folding of barnase and RNase T_1 are in the kinetics of folding. The overall folding of barnase is significantly faster than for RNase T_1 . Both proteins show slow-folding kinetics, but barnase has only a 20% slow-folding phase compared to the 96% slow-folding phases of RNase T_1 under similar conditions. The difference in the proportion of the slow-folding phases results because the native structure of barnase contains no *cis* proline bonds. Fersht and co-workers (Bycroft *et al.*, 1990; Matouschek *et al.*, 1992a) observe biphasic curves in their HDEx-2D NMR experiments with barnase as a result of a long labeling pulse (5-15 s) which traps the protein molecules with incorrect prolines. As previously indicated, the biphasic curves obtained for RNase T_1 are not an artifact of the pulse-labeling conditions.

Two different molten globule species have been described by Baldwin (1991): the structured molten globule and the collapsed unfolded globule. The structured molten globule is a collapsed form containing blocks of protected amide protons. The collapsed unfolded globule contains chiral structure observed by circular dichroism but shows no observable protection of amide protons. The major RNase T_1 folding intermediate observed in our experiments could be described by the structured molten globule form of the type observed with cytochrome c (Jeng & Englander, 1991) and α -lactalbumin (Ewbank & Creighton, 1991) or more probably as a native-like intermediate. The intermediate in which the hydrogen bonds are formed more slowly may be a collapsed unfolded form of the type observed with thermally unfolded ribonuclease A (Robertson & Baldwin, 1991).

In summary, the HDEx-2D NMR technique is improving our understanding of the *invitro* mechanism of protein folding. Results from Schmid's group have shown that, in the first seconds of folding, RNase T_1 collapses to form an intermediate-(s) in which the secondary structure appears to be completely formed, Trp-59 is buried, and a substantial amount of enzyme activity has been regained. Our results indicate that at least two intermediates are formed during this early stage of folding: a major intermediate in which at least 24 of the amide protons are protected from exchange and a minor intermediate in which protection takes at least several minutes. We suggest that these two intermediates may resemble a native-like intermediate and a "nonstructred" molten globule.

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