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Early folding intermediate of ribonuclease A

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ABSTRACT **Pulsed hydrogen exchange (^2H–^1H) is used to characterize the folding process of ribonuclease A (disulfide bonds intact). The results show one principal early folding intermediate (I_1), which is formed rapidly after the start of folding and whose proton-exchange properties change with the time of folding. All probes that are hydrogen bonded within the β-sheet of native ribonuclease A are protected in I_1. Thus, the results suggest that the β-sheet is formed rapidly and cooperatively. The initial protection factors of probes in the β-sheet are between 10 and 100, but they increase with time of folding and exceed 1000 at 400 msec from the start of folding. Thus, the β-sheet is only moderately stable when it is first formed, but subsequent events stabilize it, possibly through interactions involving hydrophobic side chains. The large protection factors of the β-sheet probes in an early folding intermediate are unexpected and remarkable. Probes in the three α-helices are fewer in number and give less accurate data than the β-strand probes. The folding kinetics expected for a simple sequential model of folding are outlined. An important difference between the observed and predicted behavior is that the early folding intermediate is not fully populated when it is first formed.**

A recently developed method (1, 2) of studying kinetic folding intermediates uses ^2H–^1H exchange to pulse label accessible peptide amide protons in folding intermediates. A preliminary study of the folding of RNase A, disulfide bonds intact, has been reported (1). Since then, the complete amide proton assignments of RNase A have been reported (3, 4), and the results, which have been extended, can now be interpreted in structural terms. Twenty-seven amide protons are used as probes of structure in this work: their locations are shown in Fig. 1. All but two are hydrogen bonded and all are located in α-helices or β-strands; more than half are in the three-stranded antiparallel β-sheet.

Our report is focused on early events in the folding process. In kinetic experiments using pulse labeling to measure protection factors, the measurable range is 1–1000. Larger protection factors cannot be quantitated, but ones measured for native proteins are as large as 10^9 (7). The protection factor is defined as the ratio of two exchange rate constants: k(solvent exposed)/k(observed), where the solvent-exposed rate is measured on a peptide with the same sequence or is calculated from data on model peptides (8). Pulsed hydrogen exchange shows which protons are protected in a folding intermediate and can give their protection factors, but it does not show directly whether protection occurs by hydrogen bonding and it does not reveal hydrogen-bond acceptor groups. The first step in structural interpretation is to compare the pattern of protection results with the structure of native RNase A.

Significant changes in methodology have been made since our previous report (1). In that work, introduction of pulse labeling was found to be an important advance over the competition method (9, 10) because pulse labeling allows the straightforward measurement of folding kinetics. In our previous work, exchange was initiated by dilution from ^2H_2O to ^1H_2O, and a very long pulse of exchange (10 sec) was used. If the exchange pulse is longer than the folding steps, it is not possible to measure the protection factors of the folding intermediates. In this work, we adopt the suggestion of Roder *et al.* (2) to initiate folding at pH 4.0 and 10°C, where exchange is slow compared to steps in folding. The dilution of the denaturant, which initiates folding, can be made directly into ^1H_2O, and a second large dilution to initiate exchange is avoided. Then a short (37-msec) pulse of exchange can be triggered by raising the pH to 9 or 10 and quenched by dropping the pH to 3. Since exchange is base catalyzed, its rate is increased 10^5-fold by raising the pH from 4 to 9.

A second important change in methodology is to measure the pH profile of exchange in the pulse once the folding intermediate of interest is populated. Measurement of the pH profile of the pulse exchange data makes it possible to calculate the protection factors of the probes and to find out whether or not different probes show the same folding kinetics.

The predicted behavior for a simple sequential mechanism of folding* is as follows. If folding follows a simple sequential pathway (5, 12), as in

$$U \rightleftharpoons I_1 \rightleftharpoons I_2 \rightleftharpoons \cdots I_n \rightleftharpoons N, \qquad [1]$$

then the kinetics of each step should be the same when monitored by two different proton probes, provided the step is detected by both probes. Because the extent of pulse labeling measures directly the fraction of protein molecules labeled in a pulse, comparison of the amplitudes measured by two different probes is easy. Although the individual steps are written as reversible, because the unfolding and refolding reactions are known to be reversible, the equilibrium constant for each step is likely to be large enough that the back reaction is barely detectable.

When a probe becomes protected in a given step, its protection factor is usually large enough that the probe becomes completely exchange resistant at the end of the step. This depends, of course, also on the pH and duration of the pulse. On the other hand, the conditions of the pulse are chosen so that all unprotected protons exchange completely in the pulse. Thus, the typical behavior for each probe in a simple sequential model of folding is that it changes from being freely exchangeable (and shows complete exchange in a pulse) before a particular step in folding to being completely protected (and shows no exchange in a pulse) after that step. If, by chance, the protection factor is marginal so that partial exchange still occurs after the folding step, this can be found out by varying the pH of the pulse (see below). The kinetic folding data should then be a collection of single-exponential reactions, the number of which depends on the number of folding intermediates, and each probe should become com-

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Abbreviation: COSY, spin-correlated spectroscopy.

*Compare with the recent discussion of the folding pathway of barnase by Fersht and co-workers (11).

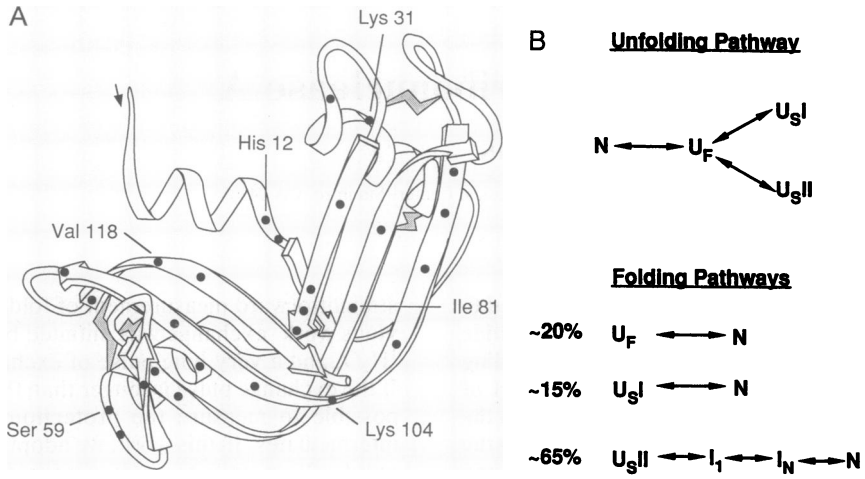

FIG. 1. (*A*) Location of amide protons used as structural probes to investigate the folding pathway of RNase A. Some have been labeled on this ribbon diagram of the protein (diagram courtesy of Jane Richardson), so that others may be identified from the complete list in Table 1. Helix 1 encompasses residues 3–13; helix 2, residues 25–34; and helix 3, residues 50–60. Approximately 40 amide protons are stable to exchange with ^2H_2O in native RNase A, under the conditions of recording of a correlated spectroscopy (COSY) NMR spectrum. Of the 40 amide protons, only 27 could be used as probes for folding; the NMR resonances of the others were either too weak or showed overlap under our spectrum recording conditions. (*B*) Pathways of unfolding and folding of RNase A. Unfolded RNase A (U) is kinetically heterogenous: it consists of a mixture of a fast-folding species, U_F (20%), and at least two slow-folding species, U_SI (15%) and U_SII (65%) (5), each of which folds to native RNase A (N) by an independent pathway. A native-like intermediate, I_N, had previously been identified on the folding pathway of U_SII. I_N differs from N by the same isomerization that differentiates U_SII from U_F, probably (6) a trans isomer of Pro-93 in I_N versus a cis isomer in N. I_1 is a hydrogen-bonded intermediate formed on the folding pathway of U_SII within 100 msec (see text and Fig. 2), which is likely to be on the direct pathway of folding. I_N is not formed on the unfolding pathway of N. The scheme for the refolding of U_SII that is shown is the simplest possible minimal model that can account for the data.

pletely protected in some one step of this collection. Its protection factor may change, however, at later steps in folding.

Exchange in proteins usually follows an *EX*2 mechanism, which is base catalyzed (7). Thus, if a probe undergoes only 50% exchange in a pulse at pH 9 and if folding follows the behavior outlined above, then the probe is expected to show complete exchange at pH 10 and negligible exchange at pH 8.

The experiments presented here were designed to test this simple model of sequential folding. Key ingredients of the experimental design are the use of a short exchange pulse and measurement of the pH profile of exchange once a folding intermediate is populated. Some of the results were not foreseen and are difficult to explain. Use of the sequential model helps to pinpoint these unexpected results. We consider possible explanations for them, but further work is needed to find the actual explanations. Some of the results do not agree with the simple sequential model, but the disagreement is marginal and further work is needed to find out if it represents experimental error or real disagreement. We list these questionable results as inconsistencies.

MATERIALS AND METHODS

Bovine pancreatic ribonuclease A (grade XII A, Sigma), purified chromatographically (13), was deuterated as described (1). All experiments were done at 10°C.

Exchange Experiments. A rapid (millisecond) mixing, pulsed quench-flow machine that has been described in detail (14) was used. The deuterated RNase A was unfolding in an unfolding buffer (2.65 M guanidine hydrochloride/40 mM glycine, in ^2H_2O at a final pH of 2). Refolding of the unfolded RNase A solution (60–70 mg of RNase A per ml) at pH 4 was initiated by diluting 10.5-fold into a refolding buffer (0.442 M sodium sulfate/0.055 M sodium formate, in H_2O at pH 4.25). At different times after beginning refolding, the exchange pulse was initiated by diluting 1.5-fold into an exchange buffer [0.4 M sodium sulfate/0.25 M guanidine hydrochloride/0.1 M (final) glycine, in H_2O] so that the final pH was 9 or 10. The 37-msec exchange pulse was terminated by diluting 1.33-fold into a quench buffer (0.4 M sodium sulfate/0.25 M guanidine hydrochloride/0.1 M sodium formate), so that the final pH was 2.9. The refolding reaction was then allowed to go to completion (10 min) at this pH. The mixing dead time was 5 msec for each of the three mixing events described above. For the zero time point, the exchange pulse was applied directly to the unfolded protein solution. The NMR sample of the fully folded RNase A was then prepared (1), and the pH was adjusted to 3.5. A two-dimensional homonuclear spin-correlated spectroscopy (COSY) spectrum (15) of the sample from each time point was recorded at 30°C on a General Electric GN-500 spectrometer. Data acquisition and processing were as described (1), except that 350 values for t_1 were used in lieu of 450. The intensities of the C_αH-NH crosspeaks (the proton occupancies) in each spectrum were determined by calculating the volume integrals of the crosspeaks after first setting the baseline of the spectrum to zero. Spectra were normalized to one another as described previously (1). In the COSY spectrum, the Val-63 and Ile-81 crosspeaks are much more intense than the His-12 and Met-13 crosspeaks; consequently, the estimated error in determination of the proton occupancies for Val-63 and Ile-81 is around ±10%, whereas it is around ±20% for His-12 and Met-13.

Optical Experiments. The modified Gibson-Durrum stopped-flow instrument that was used has been described in detail (16, 17). As with the exchange experiments described above, refolding at pH 4 was initiated by a 10.5-fold dilution of the unfolded protein solution into refolding buffer. The unfolding and refolding buffers were the same as those described above; the protein concentration, however, was 5-fold lower.

RESULTS

Optical Stopped-Flow Measurements of Refolding. The unfolding and refolding pathways of RNase A found in earlier studies, based on optical probes (5, 18), are shown in Fig. 1*B*. Note that the unfolding and refolding pathways are not the

same, because the conditions are different: the refolding intermediates are not stable in unfolding conditions. We study here the refolding pathway of the major unfolded species U_SII. It is separated by slow isomerization reactions, probably proline isomerization, from the fast-folding species U_F (20%) and from the minor slow-folding species U_SI (15%). U_SII is thought to differ from U_F (which has native proline isomers) by a nonnative trans isomer of Pro-93 (6). The native-like intermediate I_N has been well studied (5, 6, 19). Although I_N resembles native RNase A (N) in having RNase catalytic activity (20), I_N is readily distinguished from N by special assays devised for this purpose (5, 19). Note that U_F forms N directly, whereas U_SII forms I_N in the time range of our experiments (0–4 sec), and only later does I_N form N. The intermediate I_1 was detected in early studies of RNase A folding intermediates using ^3H–^1H exchange (9, 21), but I_1 has not been characterized. We found earlier (1) that I_1 is formed rapidly and contains several strongly protected amide protons.

A "baseline" for our pulse labeling results is provided by optical stopped-flow experiments, which monitor the formation of tertiary structure (in species I_N or N) by the absorbance change that accompanies burial of tyrosine side chains. N is formed from U_F, and I_N is formed from U_SII. Tyrosine absorbance, which does not distinguish I_N from N, gives the sum of both species. If no other folding intermediates are populated, the pulse labeling curves are predicted to follow the kinetic curve for the formation of I_N + N. This curve is shown as a dashed line in Fig. 2. The fast-folding species U_F (20%) folds rapidly (τ = 40 msec), and the minor slow-folding species U_SI (15%) folds quite slowly ($\tau \approx 50$ sec). Thus, the dashed line for the sum of I_N + N (Fig. 2) falls to 80% near t = 0 as U_F folds and has the value of 15% at 4 sec, after U_SII has folded, because U_SI has only begun to fold.

Kinetics of Folding Measured by Amide Proton Pulse Labeling. Fig. 2*A* shows the kinetics of folding measured by pulse labeling for four probes. Two probes in the N-terminal helix (His-12 and Met-13) show curves that are superimposable on the baseline (dashed line). Consequently, these probes show no evidence for a folding intermediate preceding I_N, and the N-terminal helix appears to be stabilized only when I_N is formed. On the other hand, two probes in the β-sheet of RNase A (Val-63 and Ile-81) show substantially greater protection than that predicted by the dashed line, indicating that a folding intermediate (I_1) is populated. The pulse labeling curves of most other probes in the β-sheet resemble those of Val-63 and Ile-81.

The kinetic curves are different at early times for pulse labeling at pH 9 versus pH 10 (Fig. 2*B*). When I_1 is first formed, almost all protected probes show only weak protection, but the degree of protection increases with time. In Fig. 2*B*, Val-63 and Ile-81 initially are protected against exchange at pH 9 but are only partly protected at pH 10. This is shown by the divergence between the pulse labeling curves at pH 9 and pH 10 when I_1 is first formed (Fig. 2*B*). At later times there is increasing convergence of the pH 9 and pH 10 results, and convergence is essentially complete at 0.4 sec. Resistance to exchange in a 37-msec pulse means >10-fold protection at pH 9 and >100-fold protection at pH 10, since the time constant for exchange of a fully solvent-exposed amide proton (Ala-Ala) is estimated (7, 8) to be about 2 msec at pH 9 and 10°C.

Measurement of Protection Factors in I_1. The pH profile of pulse labeling at 0.4 sec has been used to estimate the degree of protection of each probe in I_1. If the only protected species are I_N and N (dashed line, Fig. 2), then the expected extent of labeling at 0.4 sec is 60%. Probes such as Val-63 and Ile-81, which are protected in I_1, show 40% labeling at 0.4 sec at pH 9. In the pH profile, the increase in the extent of labeling from 40% to 60% corresponds to the labeling of I_1. The pH profiles of the different probes can be examined to see if they all fit into this simple scheme: 40% labeling at pH 9, if the probe is protected in I_1, or 60%, if the probe is not protected. Three classes of probes are found by this test: class A, strongly protected (>1000-fold; I_1 not labeled below pH 11, extent of labeling = 40% above pH 9); class B, moderately protected (≈100-fold; I_1 labeled between pH 9 and pH 11, extent of labeling = 40% at pH 9 and 60% at pH 11); and class C, weakly protected or not at all (I_1 labeled below pH 9, extent of labeling = 60% above pH 9). If any probe is protected <1000-fold in I_N, its extent of labeling will rise above 60% near pH 11. Sample pH profiles are shown in Fig. 3, and all probes are listed by class in Table 1 together with the hydrogen-bond acceptor (if any) found in native RNase A. Three probes are found to be unprotected in I_1: His-12 and

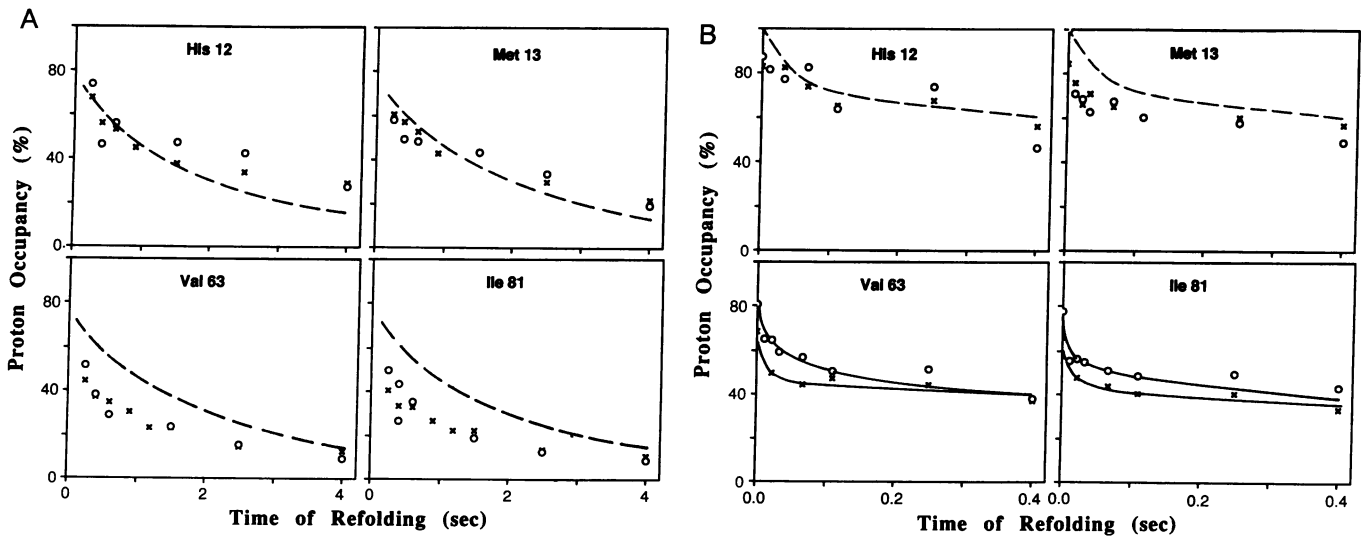

FIG. 2. Acquisition of protection of backbone amide deuterons from exchange with solvent protons. The proton occupancy of a backbone amide hydrogen site, in a COSY NMR spectrum, is a measure of the extent of labeling by exchange (proton incorporation) at that site and is plotted versus time after initiation of refolding for four different amide protons and on two different time scales: 0–4 sec (*A*) and 0–400 msec (*B*). The pH during the exchange pulse was 9 (×) or 10 (o). The dashed lines indicate the progress of the refolding reaction monitored by measuring the accompanying change in tyrosine absorbance at 287 nm and correspond to the formation of I_N from U_S II (Fig. 1*B*). The other lines through the data points were drawn by inspection only.

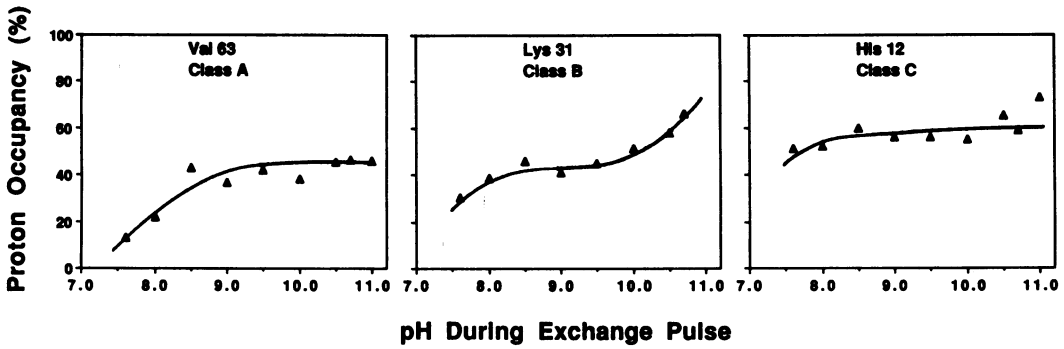

FIG. 3. pH profile for labeling by a 37-msec pulse applied 400 msec after initiation of refolding. The pH profiles are presented for three amide protons, each representative of one of the three major groups of amide protons, which are classified according to both the pH profile and the kinetics of acquisition of protection from exchange (see text and Table 1). The time constant for exchange of a free peptide amide proton is 2 msec at pH 9 and 10°C, but the inductive effect of side chains and neighboring residues may make exchange more than 10-fold faster (8). A decrease in labeling from a plateau level is seen at pH values below 9. This is the result of exchange being base catalyzed: as the pH during exchange is decreased, the duration of the pulse (37 msec) starts to limit complete labeling of an unprotected amide hydrogen site. The pH of the exchange pulse was varied between pH 7.6 and 11 by changing the composition and pH of the exchange buffer. Mops was used as the buffer below pH 8.5, and glycine was used for the higher pH values.

Met-13 in helix 1 and Glu-49, which is not hydrogen bonded in native RNase A.

Table 1. Classification of amide protons on the basis of degree of protection in I_1

Residue	Secondary structure	Hydrogen bond acceptor (native)
Class A: Strong protection		
Val-47	β-Sheet	His-12 O
His-48	β-Sheet	Ser-80 O
Val-54	Helix 3	Ser-50 O
Val-63	β-Sheet	Cys-72 O
Cys-72	β-Sheet	Val-63 O
Tyr-73	β-Sheet	Val-108 O
Ile-81	β-Sheet	Ala-102 O
Cys-84	β-Sheet	Asn-44 O
Lys-98	β-Sheet	Arg-85 O
Ile-106	β-Sheet	Ser-75 O^{γ}
Val-108	β-Sheet	Tyr-73 O
Val-116	β-Sheet	Glu-111 O
Val-118	β-Sheet	Ala-109 O
His-119	β-Sheet	Ala-109 O
Class A-1: Ill-defined protection		
Thr-100	β-Sheet	Asp-83 O
Ala-102	β-Sheet	Ile-81 O
Lys-104	β-Sheet	Met-79 O
Glu-111	β-Sheet	Val-116 O
Class B: Moderate protection		
Lys-31	Helix 2	Asn-27 O
Asn-34	Helix 2	Lys-31 O
Val-43	β-Sheet	None
Ser-59	Helix 3	Ala-56 O
Gln-60	Helix 3	Val-57 O
Tyr-97	β-Sheet	Asn-27 O^{δ}
Class C: Weak protection		
His-12	Helix 1	Phe-8 O
Met-13	Helix 1	Glu-9 O
Glu-49	β-Sheet	None

The location in the secondary structure and the type of hydrogen-bond acceptor group that is present in native RNase A are also shown (22). Two protons, Val-43 and Glu-49, are not hydrogen bonded in native RNase A. Three amide protons make tertiary hydrogen bonds: Val-47 is hydrogen bonded to the peptide C=O of His-12 in helix 1, Tyr-97 is hydrogen bonded to the side chain of Asn-27 in helix 2, and Ile-106 is hydrogen bonded to the side chain of Ser-75. Four protons in the β-sheet show ambiguous exchange behavior and have not yet been assigned to class A or B; they are listed separately in class A-1.

Four probes that do not fit the classification scheme used in Table 1 are shown as class A-1 (unassigned). There are also five probes whose folding kinetics (of the type shown in Fig. 2) are typical (Asn-34, Val-43, His-48, Ser-59, and His-119) and which need further work to be sure of their classification.

DISCUSSION

Comparison of the Results with a Simple Sequential Model of Folding. The basic test of the sequential model is that each probe should become protected in only a single step of folding, once allowance has been made for the different unfolded species present and the possibility has been tested (by varying the pH of the pulse) that a given probe has only a marginal protection factor in these pulse conditions. The interesting alternative model is a parallel pathway model in which folding occurs simultaneously on parallel pathways. In this case each probe will become protected in more than one folding step, one step for each folding pathway. The amplitude of each step (i.e., the change in extent of labeling) represents the fraction of molecules folding on that pathway. Returning to the simple sequential model, if only a few intermediates are populated, then only a few steps in folding will be observed and every probe should become protected in one of these few steps. Our results show that all but three probes become protected in a single step of folding and these three probes (His-12, Met-13, and Glu-49) become protected much later, as I_N is formed.

The major disagreement with the predictions of the sequential model is that I_1 is not fully populated in the $U_S II \rightarrow I_1$ step. As discussed in the Introduction, each folding step is expected to go essentially to completion. Consequently, the extent of labeling is expected to drop to 15% [the estimated amount of $U_S I$ (5)] as soon as I_1 is fully formed. Instead, the extent of labeling reaches a plateau level of 40%. We consider possible explanations for this effect below. It is not a case of simultaneous folding on two parallel pathways, A and B, because then two early-folding intermediates, $I_1(A)$ and $I_1(B)$, should be formed, whereas we see only a single intermediate.

The second basic difference from the predictions of the simple sequential model is that the proton-exchange properties of I_1 are not constant. Instead, the protection factors of all protons in I_1 increase with time up to 400 msec, except for the three probes that become protected later, as I_N is formed. This indicates that the sequential model is an oversimplification of the folding process. I_1 actually contains a broad distribution of species, as regards the protection factors of the

probes in I_1, and this distribution changes gradually with time.

There seem to be two possible explanations for the failure to populate I_1 fully when it is first formed. The first explanation is that optical methods of monitoring folding (5) overestimate U_SII (65%; to fit our data U_SII would have to be 40%) and underestimate U_SI (15%; this figure would have to be raised to 40%). This explanation seems unlikely. At 4 sec, when U_SI has barely begun to fold, the amount of pulse-labeled RNase A has dropped to the value expected for U_SI (about 15%).

The second explanation is that some kind of transient barrier exists to the folding of part of the U_SII molecules. What might this transient barrier be? An obvious possibility is the transient presence of one or more cis peptide bonds per unfolded RNase A molecule. Although the probability of any one peptide bond being cis is low (say 1%), nevertheless, there are 123 peptide bonds in RNase A and the probability of any unfolded molecule having at least one cis bond could be quite high (23). Once the cis bond isomerizes to trans, it would be trapped in the trans conformation by folding, and so the kinetic barrier presented by a cis peptide bond would be transient.

Data on the kinetics of cis–trans isomerization of peptide bonds are difficult to obtain because the cis isomer is rarely populated to a measurable extent. The cis isomer of N-methyl peptides is, however, populated (24), and the lifetime of the cis isomer was determined to be 1.7 sec at 21°C and can be calculated to be 5.4 sec at 10°C, which is comparable to the time constant of the $I_1 \rightarrow I_N$ step. Thus, the cis–trans isomerization of N-methyl peptide bonds is substantially faster than that of proline peptide bonds, and the cis–trans isomerization of ordinary peptide bonds may be still faster. If cis peptide bonds really are a significant factor in NMR-detected folding kinetics, then the folding reactions of other proteins should show similar phenomena to the one we report here for RNase A.

Properties of the Early-Folding Intermediate I_1. The most striking property of I_1 is the large protection factors of probes in the hydrogen-bonded β-sheet of native RNase A (Table 1). I_1 is evidently a highly stable folding intermediate. Since I_1 is formed before the rate-limiting step in folding ($I_1 \rightarrow I_N$), the rate-limiting step must occur late in the folding of RNase A. A second striking property of I_1 is its high content of secondary structure. The entire β-sheet of RNase A is probably present in I_1, and the data suggest that helices 2 and 3 are also present. Only helix 1 appears to be stabilized later, in the rate-limiting $I_1 \rightarrow I_N$ step. The third striking property of I_1 is the change with time of its amide proton exchange behavior. Since all of the β-sheet protons of native RNase A become protected in the initial reaction, as I_1 is formed, it seems likely that the β-sheet is formed rapidly and cooperatively but is only marginally stable when it is first formed. Subsequent side-chain interactions presumably then stabilize it.

Table 1 contains several interesting pieces of information in addition to the large protection factors of the probes in the hydrogen-bonded β-sheet of native RNase A. We consider, however, that at this stage in the work conclusions based on single probes, or even on two probes in one helix, are preliminary, for several reasons—particularly because of the complexity of unfolded RNase A and the failure to populate I_1 fully when it is first formed.

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