An Early Intermediate in the Folding of Ribonuclease A Is Protected against Cleavage by Pepsin†

Franz Schmid* and H. Blaschek

ABSTRACT: Folding of bovine pancreatic ribonuclease A (RNase A) is a sequential process which involves the formation of well-populated structural intermediates under suitable conditions. Two intermediates have been detected on the major slow-refolding pathway of RNase A: a late intermediate (I_N) which already resembles the native protein in a number of properties and a rapidly formed early intermediate (I_1) which shows extensive hydrogen-bonded secondary structure. Here competition experiments between refolding and proteolytic cleavage of the peptide chain are described which yield information about the decrease in accessibility of particular proteolytic cleavage sites during the folding process. Results obtained with pepsin as a proteolytic probe of folding indicate that the primary cleavage site for pepsin, Phe-120–Asp-121, becomes inaccessible early in the course of refolding, if folding is carried out under conditions which effectively stabilize the native state. Under marginally stable conditions, folding is very slow, and protection against peptic cleavage is not detectable prior to the final formation of native protein. The comparison with amide proton exchange experiments suggests that the protection against peptic cleavage occurs during the formation and/or stabilization of hydrogen-bonded secondary structure in the early intermediate (I_1). We conclude that the carboxy-terminal region of the RNase peptide chain, which is known to be important for the stability of the folded protein, may also be relevant for early steps of refolding.

The native folded structure of a protein and also the molecular mechanism of protein folding are determined ultimately by the amino acid sequence. The aim of theoretical and experimental approaches to the folding problem is to resolve the individual steps of folding and to find out how the amino acid sequence defines the pathway of folding. Experimental approaches involve (a) detection of kinetic folding intermediates, (b) placement of these intermediates into the right sequence, (c) characterization of their structures, and (d) elucidation of the nature of the rate-limiting steps between these intermediates.

The model of sequential folding [for a review, see Kim & Baldwin (1982)] suggests that protein folding proceeds on an ordered pathway via a definite sequence of structural folding intermediates. The relative rates of the individual steps may vary, depending on the folding conditions, and intermediates may or may not be well populated. Alternative pathways of folding may exist under different conditions. Structural intermediates have been found in the folding reactions of several small protein molecules (Ko et al., 1977; Crisanti & Matthews, 1981; Desmadril & Yon, 1981; Kato et al., 1981; McPhie, 1982; Goto & Hamaguchi, 1982; Nall, 1983).

Multiple unfolded forms coexist in unfolded ribonuclease A (RNase A)[1] (Garel & Baldwin, 1973; Cook et al., 1979; Schmid & Blaschek, 1981; Lin & Brandts, 1983b); this multiplicity is caused by proline peptide bond isomerizations in the unfolded chain (Brandts et al., 1975; Schmid & Baldwin, 1978; Lin & Brandts, 1983b). Of all unfolded RNase A molecules, 60–70% refold on the slow $U_S^{II} \rightarrow N$ refolding pathway (Schmid & Blaschek, 1981). This major refolding reaction starts from an unfolded state which contains at least one incorrect proline isomer (U_S^{II} is a slow-folding species), and therefore, the $U_S^{II} \rightarrow N$ refolding pathway involves both folding and proline isomerization steps.

Two folding intermediates have been detected on this major $U_S^{II} \rightarrow N$ pathway, an early hydrogen-bonded intermediate (I_1) which probably is formed rapidly (Nall et al., 1978; Schmid & Baldwin, 1979; Kim & Baldwin, 1980) and a late intermediate (I_N). I_N already shows some properties of the native state, but it still contains at least one incorrect proline isomer (Cook et al., 1979; Schmid & Blaschek, 1981; Schmid, 1982), and it is less stable than native RNase A (Schmid, 1983). Both intermediates are well populated only under strongly native folding conditions (Schmid & Baldwin, 1979; Kim & Baldwin, 1980; Schmid, 1983). The minimal kinetic mechanism for the folding of U_S^{II} is therefore

$$U_S^{II} \rightarrow I_1 \rightarrow I_N \rightarrow N \qquad (1)$$

It is not known whether I_1 is a single species or a population of related intermediates and whether rapid equilibria are involved in early steps of folding (Kim & Baldwin, 1982).

The intermediate I_1 probably has a hydrogen-bonded structure. Competition experiments between refolding and amide proton exchange of ^{3}H-labeled unfolded RNase A showed that about 20 amide protons are protected from exchange out at an early stage of folding, when folding was carried out under strongly native folding conditions (Schmid & Baldwin, 1979). Complementary experiments using a pulse-labeling method and unlabeled RNase A in the same folding conditions indicated that intermediates are formed rapidly, which provide protection against ^{3}H labeling (Kim & Baldwin, 1980). The structure of I_1 is still largely accessible to solvent, as the major changes in tyrosine absorbance occur at a later stage of folding.

The competition experiments between folding and amide proton exchange as well as the spectroscopic measurements in general detect only overall changes in the properties of the protein during folding. Proteolytic techniques have been used to search for equilibrium intermediates in the temperature-

† From the Institut für Biophysik und Physikalische Biochemie, Fakultät für Biologie und Vorklinische Medizin, Universität Regensburg, D-8400 Regensburg, West Germany. *Received September 22, 1983.* This research was supported by a grant from the Deutsche Forschungsgemeinschaft (Schm 444/2-1).

[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A (EC 3.1.27.5) with disulfide bonds intact; U_S^I, U_S^{II}, and U_F, slow- and fast-folding species of unfolded RNase A; I_1 and I_N, folding intermediates; 2′,3′-CMP, cytidine 2′,3′-phosphate; Gdn·HCl, guanidine hydrochloride; CM-cellulose, carboxymethylcellulose; $NaDodSO_4$, sodium dodecyl sulfate; PIR, pepsin-inactivated ribonuclease; STI, soybean trypsin inhibitor; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-2128$01.50/0

induced unfolding transition of RNase A [see Burgess & Scheraga (1975) and references cited therein] and to examine different steps on the denaturation and renaturation pathways of lactate dehydrogenase (Girg et al., 1981; Zettlmeissl et al., 1983).

Here we introduce a method which involves protease action during the folding of RNase A to obtain information about the accessibility of particular parts of the polypeptide chain during refolding. The basic outline is (i) to follow the kinetics of the loss of cleavage sites for specific proteases during the folding process, (ii) to localize in the amino acid sequence the cleavage sites that are blocked by folding, and (iii) to compare the results with the sequence of known intermediates in folding. The search for equilibrium or kinetic folding intermediates by using proteases is often hampered by the difficulty that binding of the protease may influence the structure of the intermediates and thereby shift equilibrium populations of species or direct the folding pathway. Therefore, in the present approach, we carry out folding *in the absence* of the protease and use a protease pulse to determine the *resistance to proteolytic cleavage* after various times of folding. First experiments, carried out with pepsin as a proteolytic probe of refolding, indicate that the carboxy-terminal end of the RNase chain is involved in a structure which is formed early in refolding.

Materials and Methods

Materials

RNase A (type XII A) and 2′,3′-CMP from Sigma (St. Louis, MO), pepsin (from porcine gastric mucosa) from Boehringer Mannheim, Sephadex G-10 from Pharmacia (Uppsala, Sweden), and Gdn·HCl (ultrapure) from Schwarz/Mann (Orangeburg, NY) were used. CM-cellulose, acrylamide, bis(acrylamide), ammonium persulfate, NaDodSO$_4$, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, sodium cacodylate, and Serva blue R-250 were from Serva (Heidelberg, FRG). All other reagents were from Merck (Darmstadt, FRG). RNase A concentrations were determined spectrophotometrically by using a molar absorbance at 278 nm of 9800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957).

Methods

Competition between Refolding and Peptic Cleavage. At time $t = 0$, 0.05 mL of unfolded RNase A (5 mg/mL in 4 M Gdn·HCl, pH 1, 10 °C) was pipetted into 0.95 mL of a solution of 0.1 mg/mL pepsin in 0.05 M sodium formate, pH 3.5, to initiate the competition reaction of refolding and peptic cleavage. The pepsin solution was kept at 10 °C by a circulating water bath and was vigorously agitated by a magnetic stirring bar to ensure rapid mixing. After variable times (t_i), pepsin action was stopped by addition of 0.2 mL of 0.5 M sodium cacodylate, pH 8.5, which raised the pH to 7. Afterwards, the samples were kept at 10 °C, pH 7, for 30 min to allow the refolding of unmodified RNase to go to completion. The amount of uncleaved RNase A was determined by measuring the activity toward 2′,3′-CMP (Crook et al., 1960) and by NaDodSO$_4$–polyacrylamide gel electrophoresis. Control experiments under identical final conditions starting with native RNase A confirmed that folded RNase A is not attacked by pepsin. The zero time point (100% activity) was derived from an experiment in which pepsin was replaced by buffer.

Pepsin Pulse Experiments. At time $t = 0$, 0.1 mL of unfolded RNase A (5 mg/mL in 4 M Gdn·HCl, pH 1, 10 °C) was pipetted into 1.7 mL of vigorously stirred 0.05 M sodium formate buffer, pH 3.5, 10 °C, to initiate refolding. After various times of folding, t_i, 0.2 mL of pepsin solution (1 mg/mL in 0.2 M Gdn·HCl–0.05 M sodium formate, pH 3.5) was added. Pepsin action was stopped after 10 min by a jump to pH 7 produced by addition of 0.5 mL of 0.5 M sodium cacodylate, pH 8.5. Uncleaved RNase A was detected by activity assays using 2′,3′-CMP (Crook et al., 1960) and by gel electrophoresis. The zero time point was derived from an experiment where pepsin was already present in the refolding buffer. The values for 100% activity and gel band intensity were obtained from an experiment where the pepsin solution was replaced by buffer.

Preparation of Pepsin-Inactivated RNase (PIR). A mixture of PIR and of native RNase A was produced by limited proteolysis by pepsin for 10 min at 37 °C (following the procedure given by Anfinsen (1956) and Puett (1972).

Analytical Ion-Exchange Chromatography. The mixture of PIR and native RNase A and also the products of the competition experiments were resolved by ion-exchange chromatography by a method similar to that of Puett (1972). The samples were first adjusted to 0.13 M phosphate buffer, pH 6.45, by passage over a Sephadex G10 column (0.6 × 19 cm) equilibrated with this buffer. Then they were applied to a 0.8 × 47 cm CM-cellulose column which was equilibrated with 0.13 M phosphate buffer, pH 6.45. The samples were eluted with the same buffer at room temperature with a flow rate of 10 mL/h. Individual fractions of 2 mL were collected and assayed for absorbance at 277 nm and for RNase activity.

Gel Electrophoresis. Before being processed, soybean trypsin inhibitor (STI) was added to the samples as an internal concentration standard. A dialysis step was included prior to electrophoresis to remove residual Gdn·HCl (Gdn·H^+ ions precipitate NaDodSO$_4$). Twenty-microliter samples were applied on top of a discontinuous NaDodSO$_4$–polyacrylamide slab gel consisting of a 5% stacking gel and a 20% running gel (running gel buffer, 0.38 M Tris–0.1% NaDodSO$_4$, pH 8.8; stacking gel and electrophoresis buffer, 0.19 M glycine, 0.025 M Tris, and 0.1% NaDodSO$_4$, pH 8.3).

Results

Competition Experiments between Refolding and Cleavage by Pepsin. To determine the accessibility of folding intermediates to peptic cleavage, competition experiments between refolding and cleavage by pepsin were carried out at 10 °C, pH 3.5, in 0.2 M Gdn·HCl and concentrations of pepsin ranging from 0.1 to 0.5 mg/mL. Under these conditions, pepsin still shows high activity. Native RNase A is not sensitive to peptic cleavage, and both absorbance- and fluorescence-detected refolding kinetics of the U_S species, measured at pH 3.5, are identical with the respective refolding kinetics observed at neutral pH. This suggests that the refolding pathway at pH 3.5, 10 °C, is very similar to the refolding pathway at pH 6.0, where the intermediates I_1 and I_N have been detected previously (Schmid & Baldwin, 1979; Cook et al., 1979; Schmid & Blaschek, 1981). A decrease in pH below 3.5 at 10 °C results in a progressive deceleration of the slow-refolding reaction [see also the 25 °C results of Nall et al. (1978)].

Figure 1 shows the result of a competition experiment between refolding and inactivation by pepsin. Refolding of RNase A (unfolded by 4 M Gdn·HCl, pH 1) was initiated by a 20-fold dilution into 0.5 M formate, pH 3.5, containing 0.1 mg/mL pepsin at 10 °C. Samples were withdrawn after different times of folding and cleavage, and pepsin action was stopped by a jump to pH 7. Residual folding was then allowed to go to completion at pH 7, and the remaining fraction of the sample which showed catalytic activity was determined.

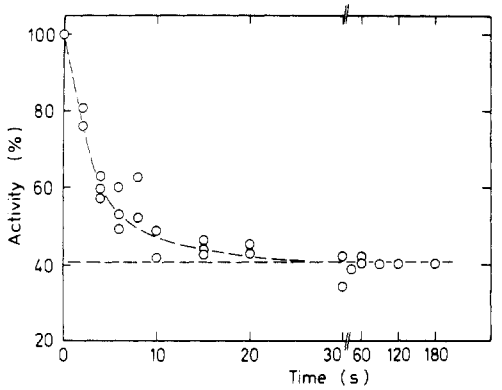

FIGURE 1: Competition experiments between refolding and inactivation by pepsin at 10 °C, pH 3.5. The experiments were started at time zero by a 20-fold dilution of unfolded RNase A (5 mg/mL in 4 M Gdn·HCl, pH 1) with the refolding buffer (0.05 M sodium formate, pH 3.5) which contained 0.1 mg/mL pepsin. After the indicated time intervals, pepsin action was stopped by a jump to pH 7. The residual RNase activity found in the samples after complete refolding of the unmodified protein molecules at pH 7 is shown as a function of the time, when pepsin action was stopped. The zero time point was determined in experiments where pepsin was omitted.

Figure 1 indicates that deactivation by pepsin during folding occurs very rapidly ($\tau_{1/2}$ = 2.5 s) compared to absorbance-detected refolding ($\tau_{1/2}$ = 20 s). However, although cleavage by pepsin appears to be much faster than the absorbance-detected formation of the nativelike intermediate I_N, only about three-fourths of all U_S molecules are inactivated. At the end of the competition experiment, 41% of active RNase A remains, 21% of which is formed during the $U_S \rightarrow N$ refolding reaction. Cleavage by pepsin cannot compete with the fast $U_F \rightarrow N$ refolding reaction [$\tau_{1/2} < 50$ ms; cf. Lin & Brandts (1983a)], which results in a basis of 20% active RNase A generated by this fast-refolding reaction. The results of Figure 1 suggest that the sensitivity to pepsin is lost rapidly during $U_S \rightarrow N$ refolding. A quantitative analysis of the data, e.g., in terms of a simple kinetic competition model between folding and cleavage by pepsin, is, however, difficult as U_S is composed of at least two subspecies, U_S^I and U_S^{II}, which refold with different rates. They probably differ also in sensitivity to peptic cleavage during refolding. In these experiments, pepsin was present during the refolding process. An interaction of the protease with partially structured intermediates and hence an influence on the refolding pathway cannot be excluded definitely.

A Pepsin-Resistant Intermediate Is Formed More Rapidly Than the Intermediate I_N. The decrease in sensitivity toward inactivation by pepsin during refolding was detected by protease pulse experiments. The refolding experiments were carried out under the same final conditions as in Figure 1 (0.2 M Gdn·HCl, pH 3.5, 10 °C); however, refolding was started at time t = 0 *without pepsin*. Then, after different times of refolding, t_i, in the absence of protease, pepsin was added to give a final concentration of 0.1 mg/mL. Pepsin action was stopped after 10 min by a jump to pH 7. This was sufficient time for the competing reactions of folding and cleavage to go to completion (cf. Figure 1). The residual RNase activity found in the different samples is shown in Figure 2 as a function of the time (t_i) when pepsin was added to the refolding protein. The zero time point was determined in an experiment where pepsin was already in the refolding buffer at the beginning of folding (it is equivalent to the final value in Figure 1). The sensitivity against inactivation by pepsin decreases rapidly with increasing time of prefolding prior to the addition of the protease. When pepsin is present at the beginning of

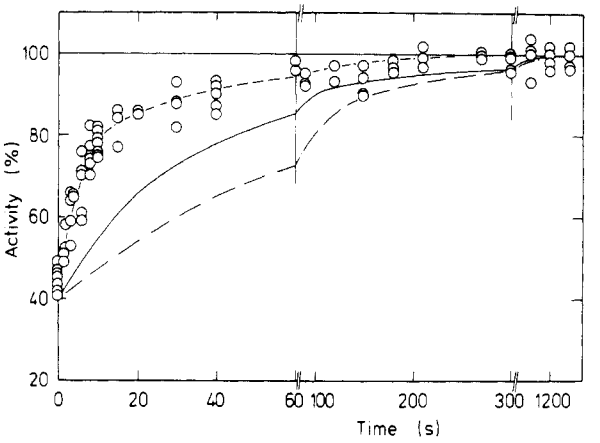

FIGURE 2: Decrease of sensitivity toward peptic cleavage during the slow-refolding reaction of RNase A at 10 °C in 0.05 M sodium formate, pH 3.5, and 0.1 mg/mL pepsin containing 0.2 M Gdn·HCl. At time zero, refolding was initiated by a 20-fold dilution of unfolded RNase A (5 mg/mL in 4 M Gdn·HCl, pH 1). After the indicated time intervals, pepsin was added, and competition between folding and cleavage was allowed to proceed for 10 min. The residual RNase activity of the samples is shown as a function of the time when pepsin was added. The kinetics of the loss of sensitivity toward pepsin-induced inactivation are compared with absorbance (—) and fluorescence detected (–·–) slow-refolding kinetics. The kinetic progress curves are matched so that initial and final values coincide.

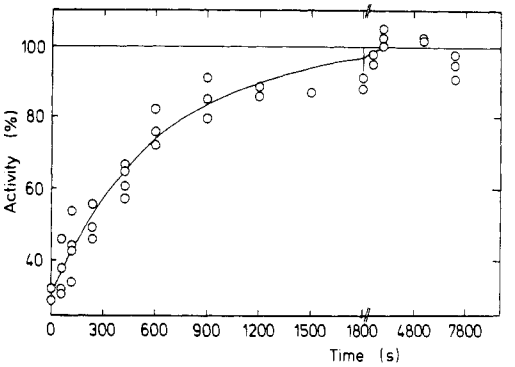

FIGURE 3: Decrease of pepsin sensitivity during the slow-refolding reaction at 10 °C in 0.05 M glycine, pH 2.5, and 0.1 mg/mL pepsin in the presence of 1 M Gdn·HCl. Refolding was initiated at time zero by a 20-fold dilution of unfolded RNase A. Pepsin was added after the indicated time intervals, and the competition between refolding and cleavage was allowed to proceed for 60 min. The residual RNase activity of the samples is shown as a function of the time when pepsin was added. Absorbance-detected refolding kinetics are given for comparison (—). The data are plotted in such a way that initial and final values of the kinetics coincide.

refolding, only about one-fourth of all refolding U_S molecules escape inactivation by pepsin. A 10-s prefolding step leads to an increase of this fraction to about three-fourths of all U_S species. The kinetics of the formation of pepsin-resistant molecules during folding are compared in Figure 2 to the kinetics of the fluorescence-detected formation of fully native protein and to the absorbance-detected formation of the nativelike intermediate I_N. If the I_N molecules were the first ones protected from proteolytic cleavage during refolding, then the appearance of pepsin-resistant molecules should parallel the absorbance-detected refolding kinetics. Figure 2 shows that the sensitivity toward pepsin is lost much more rapidly during refolding than the formation of I_N occurs. This indicates that early intermediates are formed which are already protected against proteolysis by pepsin. Because of the complex nature of the slow refolding of RNase A, the data of Figure 2 do not allow a quantitative analysis in terms of rate constants or the degree of protection against peptic cleavage.

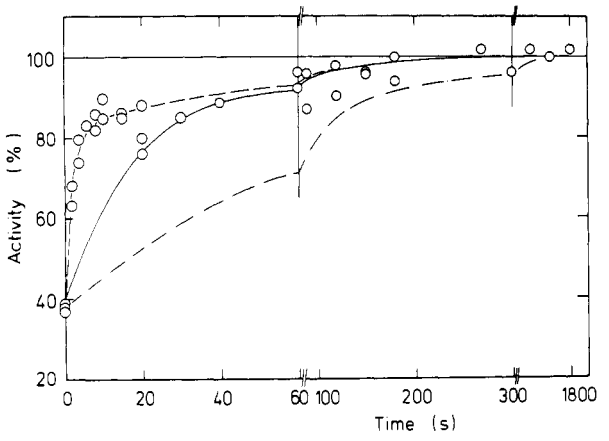

FIGURE 4: Decrease of pepsin sensitivity during the slow-refolding reaction at 10 °C in 0.05 M sodium formate, pH 3.5, 0.5 mg/mL pepsin, and 0.2 M Gdn·HCl in the presence of 0.2 M $(NH_4)_2SO_4$. Refolding was initiated at time zero by a 20-fold dilution of unfolded RNase A. Pepsin was added after the indicated time intervals, and the competition between refolding and cleavage was allowed to proceed for 10 min. The residual RNase activity of the samples is shown as a function of the time when pepsin was added. Absorbance (—) and fluorescence-detected (–·–) folding kinetics are included for comparison. The kinetic progress curves are matched so that initial and final values coincide.

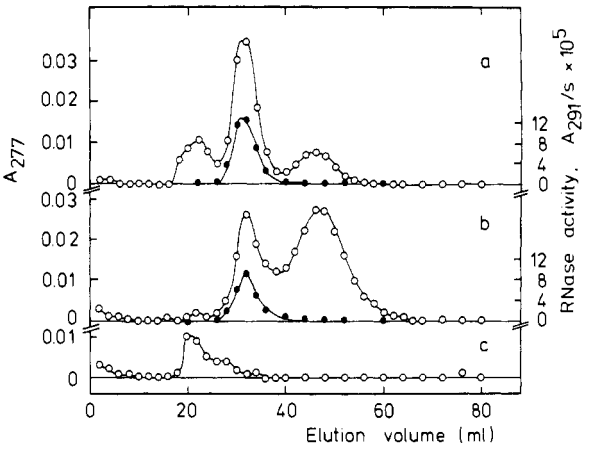

FIGURE 5: Characterization of the products of the competition experiment between refolding and cleavage by pepsin. Chromatography was on a 0.8 × 47 cm CM-cellulose column equilibrated with 0.13 M sodium phosphate, pH 6.45 at room temperature. (○) Absorbance at 277 nm; (●) RNase activity. (a) Chromatography of the products of a competition experiment carried out at 10 °C, pH 3.5, in 0.2 M Gdn·HCl and 0.1 mg/mL pepsin. Pepsin action was stopped after 10 s. (b) Elution pattern of a mixture of native and pepsin-inactivated RNase prepared according to Puett (1972). (c) Pepsin control. Experimental setup as in (a) but without RNase.

At pH 2.5 and in the presence of 1 M Gdn·HCl, native RNase A is only marginally stable. Under these conditions, the $U_S \rightarrow N$ refolding reaction is very slow (cf. Figure 3) and no longer complex. Fluorescence-detected and absorbance-detected folding kinetics coincide; both monitor the formation of native RNase A. There is no kinetic evidence for folding intermediates. Under these marginally stable folding conditions, the loss of sensitivity toward pepsin parallels the absorbance-detected refolding (Figure 3); i.e., protection against peptic cleavage does not occur prior to the rate-limiting step of refolding of U_S.

Ammonium sulfate in general stabilizes folded protein structures and enhances the rates of protein folding reactions (von Hippel & Wong, 1965; Cook et al., 1979; Schmid, 1981). Addition of ammonium sulfate to the refolding buffer strongly accelerates the formation of pepsin-resistant intermediates during the slow-refolding reaction of RNase A. Figure 4 shows the kinetics of the loss of sensitivity toward peptic cleavage at pH 3.5, 10 °C, in the presence of 0.2 M ammonium sulfate. Note that 0.5 mg/mL pepsin has been used rather than 0.1 mg/mL as in the experiments without ammonium sulfate (Figure 2). This was necessary because even when pepsin was present already at the beginning of folding, inactivation by 0.1 mg/mL pepsin could hardly compete with the rapid formation of pepsin-resistant species during $U_S \rightarrow N$ refolding.[2]

The results of the pepsin pulse experiments suggest that under strongly native folding conditions the rapid formation of an intermediate can be detected on the slow-refolding pathway of RNase A. This intermediate has lost its sensitivity toward pepsin (or is much less sensitive than the unfolded protein); it is stabilized by ammonium sulfate, and it is not detectable under marginally stable folding conditions, where the native protein is at the edge of its stability.

[2] The pepsin concentrations in the different pepsin-pulse experiments (Figures 2–4) were selected such that about 40 ± 5% residual RNase activity was constantly observed in the experiments, where pepsin was already present at the beginning of refolding (the zero time points). The activity of pepsin and the rate of refolding of RNase A both depend on pH and on the concentration of Gdn·HCl; therefore, matching of the zero time points Figures 2–4 improved the comparability of the individual results.

Characterization of the Peptic Cleavage Site Which Is Protected Early in Folding. Limited proteolysis of acid-unfolded RNase (at 37 °C, pH 1.8, for 10 min) by pepsin leads to primary cleavage of the Phe-120–Asp-121 peptide bond; the C-terminal tetrapeptide (121–124) is split off. The resulting des-(121–124) RNase, also known as PIR, is almost completely inactive (Anfinsen, 1955, 1956; Fujioka & Scheraga, 1965; Taniuchi, 1970; Puett, 1972). Extended exposure of RNase to pepsin at pH 1.8 leads to further degradation of the primary product of cleavage, PIR. Here we ask whether the cleavage site, which is lost early during refolding, is identical with the site which is first cleaved in acid-unfolded RNase A. Native RNase A and PIR differ in charge (Asp-121 is lost in PIR); therefore, separation of PIR from intact RNase A has routinely been achieved by using cation-exchange chromatography [cf., e.g., Puett (1972)]. A competition experiment between refolding and cleavage by pepsin was carried out at pH 3.5 and 10 °C in 0.2 M Gdn·HCl and 0.1 mg/mL pepsin as shown in Figure 1, and pepsin action was stopped after 10 s. After removal of the Gdn·HCl on a Sephadex G-10 column, the reaction mixture was applied to a CM-cellulose column and eluted with 0.13 M sodium phosphate, pH 6.45 (Puett, 1972). The elution profile is shown in Figure 5a. The column was calibrated with a mixture of native and pepsin-inactivated RNase (Figure 5b) and pepsin (Figure 5c). The primary product of the competition experiment between folding and cleavage by pepsin (Figure 5a) elutes at the same position as PIR (Figure 5b). When the competition is allowed to go on for 10 min, rather than 10 s, the last peak in the elution profile (Figure 5a) disappears. In contrast to intact RNase A, which is not attacked by pepsin under the given conditions, the product of the cleavage by pepsin is further degraded by the protease. This also explains why the amount of inactive material found under the conditions of Figure 5a is less than that expected from the 10-s time point in Figure 1.

The products of the competition experiment were also characterized by $NaDodSO_4$–polyacrylamide gel electrophoresis. A competition experiment between refolding and peptic cleavage was carried out under the conditions of Figure 1. Pepsin action was stopped after various times, and the samples

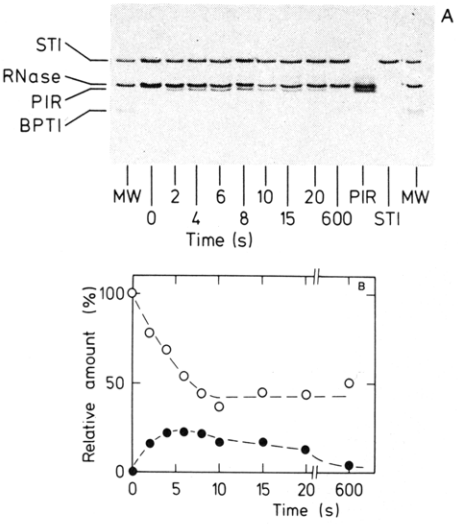

FIGURE 6: (A) $NaDodSO_4$–polyacrylamide gel electrophoresis of the products of competition between refolding and cleavage by pepsin. Experiments were carried out as described in Figure 1. Pepsin action was stopped after the indicated time intervals. The zero time point was obtained from an experiment without pepsin. STI was used as an internal concentration standard. A mixture of native and pepsin-inactivated RNase A (lane PIR), a sample without RNase A (lane STI), and a mixture of STI, RNase A, and BPTI (lane MW) were used as references. (B) Scan of the gel shown in (A). (○) Intact RNase A; (●) band comigrating with PIR. The areas of these peaks were determined relative to the area of the STI peak. The relative intensity of the RNase A band in the zero lane was set as 100%.

were applied to a $NaDodSO_4$–polyacrylamide gel. Gdn·HCl ions precipitate $NaDodSO_4$; therefore, the individual samples were dialyzed against water prior to electrophoresis. Soybean trypsin inhibitor was added as an internal concentration standard before processing of the samples for electrophoresis to correct for concentration changes during the dialysis step. Intact RNase A, pepsin, and a mixture of intact RNase A and PIR [prepared according to Puett (1972)] served as markers for the identification of the individual bands. Two closely spaced bands, unmodified RNase A and a band which comigrates with PIR, are observed after various times of the competition experiment (Figure 6A). The band equivalent to native RNase A decreases rapidly (Figure 6B) in the same manner as the enzymatic activity (cf. Figure 1), and concomitantly, the band which is equivalent to PIR increases with the time of the competition experiment. This molecule, however, is not stable toward pepsin: it is hydrolyzed at a considerable rate to smaller peptides which are not detected on the gel. After 10 min of pepsin action, this band has almost completely disappeared.

The results of both CM-cellulose chromatography and gel electrophoresis experiments suggest that the cleavage site for pepsin, which is protected early during the $U_S \rightarrow N$ refolding reaction, is identical with the PIR site, i.e., the Phe-120–Asp-121 peptide bond.

Discussion

The Carboxy-Terminal Region Is Structured in Early Folding Intermediates. On the slow $U_S \rightarrow N$ refolding pathway of RNase A, the sensitivity toward peptic cleavage is lost at an early stage of folding, prior to the formation of the nativelike intermediate I_N. The slow refolding is known to be a complex set of parallel reactions which originate from different unfolded states (Cook et al., 1979; Schmid & Blaschek, 1981; Lin & Brandts, 1983a). Therefore, it cannot be decided whether the early pepsin-resistant intermediates are formed exclusively on the major $U_S^{II} \rightarrow N$ pathway or whether protection against pepsin is generated in a single step. These uncertainties preclude an analysis of the data of Figure 2 in terms of stabilities and rates of formation of specific intermediates. Pepsin-resistant intermediates are not detected under conditions where the native protein is only marginally stable, such as 1 M Gdn·HCl, pH 2.5 (Figure 3). Here refolding is very slow, and there is no evidence for any folding intermediates under such conditions (Schmid, 1981; Schmid & Blaschek, 1981). In the presence of ammonium sulfate, which stabilizes folded protein structures, the rate of formation and/or the stability of pepsin-resistant intermediates is increased.

The cleavage site, which is protected early during refolding, is the Phe-120–Asp-121 peptide bond. It is identical with the site which is split most rapidly in unfolded RNase at acid pH (Anfinsen, 1955, 1956). This suggests that the carboxy-terminal region of the polypeptide chain is a part of early structural intermediates of folding. Other potential cleavage sites for pepsin may be protected still more rapidly: however, only sites which are cleaved very fast in the unfolded chain can be monitored by the assay employed.

Relation to Hydrogen-Exchange Experiments. By using a hydrogen-exchange pulse-labeling method, Kim & Baldwin (1980) found that part of the peptide NH of the refolding polypeptide chain is rapidly excluded from hydrogen exchange with ^3H_2O. After 3–4 s of refolding in the presence of 0.2 M ammonium sulfate, about 40% of those peptide NH's, which are highly protected in native RNase A, have already become inaccessible to exchange with the solvent. The time course of the decrease of tritium label incorporation into refolding RNase A (Kim & Baldwin, 1980) is very similar to the time course of the loss of the 120–121 peptic cleavage site under similar conditions (cf. Figure 4). This suggests that the formation or stabilization of a structure around Phe-120–Asp-121, which is inaccessible to pepsin, occurs parallel to the formation or stabilization of extended hydrogen-bonded structures during the refolding process. It appears unlikely that the formation of local structures solely in the carboxy-terminal region of an otherwise still unstructured molecule is the cause for the decreased accessibility for pepsin. The hydrogen-exchange experiments (Schmid & Baldwin, 1979; Kim & Baldwin, 1980) have provided evidence that the early intermediates consist of a significant amount of hydrogen-bonded structure; the present results with pepsin strongly suggest that a specific part of the polypeptide chain, i.e., the C-terminal region, belongs to this early structure.

The C-Terminal Region Is Important for the Pathway of Folding and for the Stability of the Native Protein. The removal of the C-terminal tetrapeptide from RNase A leads to a significant loss of conformational stability. The midpoint of the thermal transition at pH 6 of des-(121–124) RNase is decreased to about 45 °C (for intact RNase A, T_M = 62 °C) (Lin et al., 1972), and Gdn·HCl-induced unfolding at 25 °C shows a midpoint at 1.5 M Gdn·HCl, compared to 2.9 M for RNase A. This is equivalent to a reduction in the conformational free energy of about 30% (Puett, 1972). Removal of additional residues from the C-terminus (in particular, elimination of Phe-120) leads to a further destabilization of the protein (Lin et al., 1972). Reduced des-(121–124) RNase has lost the ability of re-forming the native disulfide bonds upon reoxidation by air (Taniuchi, 1970). The enzymatic activity of des-(121–124) RNase is almost zero (Anfinsen, 1956; Lin, 1970). All of these results demonstrate that the carboxy-terminal region is very important for the native conformation of RNase A.

In this work, we have shown that the region around the C-terminus becomes structured already at an early stage of refolding. Hence, the C-terminal sequence not only is crucial for the stability and activity of the native state but also is part of an early folding intermediate which may be important for determining the pathway of folding of the polypeptide chain. The importance of the C-terminus is reflected in the very high conservation of the amino acid sequence of this region during evolution (Blackburn & Moore, 1982). It remains to be worked out whether those parts of the polypeptide chain which are relevant for the stability of the folded state are also important for the stability of folding intermediates, i.e., for the pathway of folding. If the native structure is gained step by step in the course of refolding, then similar interactions may govern the stability both of folding intermediates and of the native protein.

Registry No. RNase, 9001-99-4.

References

Anfinsen, C. B. (1955) *Biochim. Biophys. Acta 17*, 593–596.

Anfinsen, C. B. (1956) *J. Biol. Chem. 221*, 405–412.

Blackburn, P., & Moore, S. (1982) *Enzymes, 3rd Ed. 15*, 317–433.

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Burgess, A. W., & Scheraga, H. A. (1975) *J. Theor. Biol. 53*, 403–420.

Cook, K. H., Schmid, F. X., & Baldwin, R. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6157–6161.

Crisanti, M. M., & Matthews, C. R. (1981) *Biochemistry 20*, 2700–2706.

Crook, E. M., Mathias, A. P., & Rabin, B. R. (1960) *Biochem. J. 74*, 234–238.

Desmadril, M., & Yon, J. M. (1981) *Biochem. Biophys. Res. Commun. 101*, 563–569.

Fujioka, H., & Scheraga, H. A. (1965) *Biochemistry 4*, 2197–2205.

Garel, J. R., & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3347–3351.

Girg, R., Rudolph, R., & Jaenicke, R. (1981) *Eur. J. Biochem. 119*, 301–305.

Goto, Y., & Hamaguchi, K. (1982) *J. Mol. Biol. 156*, 891–910.

Kato, S., Okamura, M., Shimamoto, N., & Utiyama, H. (1981) *Biochemistry 20*, 1080–1085.

Kim, P. S., & Baldwin, R. L. (1980) *Biochemistry 19*, 6124–6129.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Ko, B. P. N., Yazgan, A., Yeagle, P. L. Lottich, S. C., & Henkens, R. W. (1977) *Biochemistry 16*, 1720–1725.

Lin, L.-N., & Brandts, J. F. (1983a) *Biochemistry 22*, 564–573.

Lin, L.-N., & Brandts, J. F. (1983b) *Biochemistry 22*, 573–580.

Lin, M. C. (1970) *J. Biol. Chem. 245*, 6726–6731.

Lin, M. C., Gutte, B., Caldi, D. G., Moore, S., & Merrifield, R. B. (1972) *J. Biol. Chem. 247*, 4768–4774.

McPhie, P. (1982) *J. Biol. Chem. 257*, 689–693.

Nall, B. T. (1983) *Biochemistry 22*, 1423–1429.

Nall, B. T., Garel, J.-R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.

Puett, D. (1972) *Biochemistry 11*, 1980–1989.

Schmid, F. X. (1981) *Eur. J. Biochem. 114*, 105–109.

Schmid, F. X. (1982) *Eur. J. Biochem. 128*, 77–80.

Schmid, F. X. (1983) *Biochemistry 22*, 4690–4695.

Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764–4768.

Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 135*, 199–215.

Schmid, F. X., & Blaschek, H. (1981) *Eur. J. Biochem. 114*, 111–117.

Sela, M., & Anfinsen, C. B. (1957) *Biochim. Biophys. Acta 24*, 229–235.

Taniuchi, H. (1970) *J. Biol. Chem. 245*, 5459–5468.

von Hippel, P. H., & Wong, K.-Y. (1965) *J. Biol. Chem. 240*, 3909–3923.

Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1983) *Arch. Biochem. Biophys. 224*, 161–168.