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Involvement of Prolines-114 and -117 in the Slow Refolding Phase of Ribonuclease A As Determined by Isomer-Specific Proteolysis†

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ABSTRACT: Using the method of isomer-specific proteolysis (ISP), the cis–trans nature of the peptide bonds involving prolines-114 and -117 in ribonuclease (RNase) has been investigated. These studies involve the pretreatment of RNase first with either a short pepsin pulse or a short mercaptoethanol pulse to irreversibly unfold the protein and then with a short chymotrypsin pulse to quickly cleave the Tyr_{115}–Val_{116} bond so that the chain is suitably trimmed for the subsequent stereospecific cleavage either by aminopeptidase P, to investigate proline-117, or by a proline-specific endopeptidase, to investigate proline-114. The most reasonable interpretation of our results suggests that proline-117 is essentially 100% trans in both the native and unfolded states, so it apparently makes no direct contribution to the slow refolding kinetics of RNase. It is also determined that proline-114 is 100% cis in native RNase and ca. 95% cis in reversibly unfolded RNase so only 5% of the unfolded RNase can be rate limited by trans to cis isomerization of proline-114 during refolding. Careful spectroscopic studies of refolding show that the smallest and slowest of the refolding phases, the ct phase, has the proper amplitude (5%), relaxation time (400 s at 10 °C), and activation energy (17 kcal) for a phase that is rate limited by the trans to cis isomerization of proline-114. Measurements of the kinetics of binding of cytidine 2′-monophosphate during refolding further show that RNase does not become active until proline-114 has isomerized to the native cis configuration. It is concluded that none of the three prolines thus far examined (i.e., prolines-93, -114, and -117) by the ISP method is involved in the formation of a fully active, nativelike intermediate which has "incorrect" proline isomers. The specific structural process which is responsible for the largest of the three slow refolding phases, the XY phase, is still undetermined. Although ISP results on proline-42 are not yet available, it seems possible that this slow phase may be rate limited by a process other than proline isomerization. In unrelated studies, results from chymotrypsin hydrolyses of several short peptides containing the sequence -X-Y-Pro- show that cleavage of an active X–Y bond is very slow when it is immediately adjacent on the amino side of a proline peptide bond. Thus, chymotrypsin cleavage may not be generally useful as the analytical step in isomer-specific proteolysis.

The refolding of ribonuclease (RNase) occurs in both a fast (ca. 20% of the spectroscopic amplitude) and a slow (ca. 80%) kinetic phase, with the end product of each phase being a fully active form of RNase. It is now generally accepted, as proposed some time ago (Brandts et al., 1975), that the fast phase results from refolding of denatured molecules which have all proline residues in the correct native configuration while the slow phase involves refolding of molecules which have one or more incorrect proline isomers. Nevertheless, there are still many unanswered questions concerning the molecular details of the slow phase, since RNase has four proline residues and since certain features of the slow refolding kinetics may not be fully explained by proline isomerization alone. It has often been suggested (Schmid & Baldwin, 1978; Schmid, 1981; Schmid & Blaschek, 1981), for example, that unfolded RNase can be slowly refolded to a nativelike intermediate which has one or more prolines in an incorrect nonnative configuration.

The slow phase of refolding itself is known to be complex. When refolding into solutions containing a low concentration of denaturants, it was shown (Lin & Brandts, 1983c) to consist mainly of a faster phase (i.e., the XY phase) of ca. 50% of the spectroscopic amplitude and a slower phase (i.e., the CT phase) of ca. 25–30% of the amplitude. In addition, careful studies revealed (Lin & Brandts, 1983c) a third very slow phase of ca. 5% amplitude, which will be characterized in more detail in this paper and which will be referred to as the spectroscopic ct phase.

The best approach toward understanding the role of proline isomerization in each of the component processes of the complex slow phase of refolding is to directly measure the isomerization of each of the four prolines by using isomer-specific proteolysis (ISP). Two of these, prolines-93 and -114, are cis in the native protein while the other two, prolines-42 and -117, are trans. In previous work (Lin & Brandts, 1983b), which utilized trypsin as the stereospecific protease, we were able to show that the trans to cis isomerization of proline-93 is the rate-limiting step in the spectroscopic CT refolding phase and that RNase is unable to refold to an active form while proline-93 is in the incorrect trans state.

In a continuing effort to utilize the ISP method for studying the other prolines of RNase, particularly prolines-114 and -117 in the sequence -Gly_{112}-Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}-Pro_{117}-, proteases such as papain and chymotrypsin have been examined. We found that papain, which has low solubility in aqueous solution, hydrolyzes the Gly_{112}-Asn_{113} bond too slowly

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to be useful for investigating proline isomerization. However, it will be seen that chymotrypsin can completely cleave the Tyr_{115}–Val_{116} bond in ca. 1 min for oxidized, native, and equilibrium-unfolded RNase, if the latter two forms are first irreversibly unfolded by a short pulse (20 s) of pepsin activity in urea solutions or by a short (20 s) reduction pulse using mercaptoethanol. Once the Tyr_{115}–Val_{116} bond is cleaved by chymotrypsin, the carboxy-terminal Pro_{114}–Tyr_{115} bond is an excellent substrate for proline-specific endopeptidase if and only if the preceding Asn_{113}–Pro_{114} bond is trans, leading to the release of free Tyr_{115}. Thus, studies on RNase using pepsin (or mercaptoethanol reduction) and chymotrypsin and proline-specific endopeptidase in tandem should give direct information on the isomeric state of proline-114 in the intact chain, if the total hydrolytic sequence can be carried out rapidly relative to the rate of isomerization.

In addition, cleavage of the Tyr_{115}–Val_{116} bond by chymotrypsin also produces a fragment which has Val_{116}–Pro_{117} at the amino terminus. Aminopeptidase P is known (Lin & Brandts, 1979b, 1980) to have an absolute specificity for the trans form of amino-terminal X–Pro bonds, so that the use of chymotrypsin and aminopeptidase P in tandem, followed by analysis for free Val_{116}, should give definitive information on the isomeric state of the Val_{116}–Pro_{117} bond in RNase.

Utilizing the above ISP methodology, our results show that the trans to cis isomerization of proline-114 is, in all likelihood, the rate-limiting step in the small spectroscopic ct phase and, furthermore, that RNase will not refold into an active conformation unless proline-114 is in the correct cis configuration. In addition, the results strongly suggest that proline-117 makes no detectable contribution to the slow refolding phase since this proline is probably 100% trans in both the native and unfolded forms.

The studies leave unanswered the question as to the origin of the largest component process in the slow phase, i.e., the XY process. Although this could involve the isomerization of proline-42, which has not yet been examined by the ISP method, it seems possible that the XY process might be rate limited by a structural change other than proline isomerization.

Materials and Methods

Materials. Proline-specific endopeptidase (EC 3.4.21.26), purchased from Miles Laboratories (catalog no. 32-082-1; lot no. 13A, 13B, and 13C), was dialyzed against a large volume of 0.045 M sodium phosphate buffer (pH 7.0) to remove small-molecule contaminants before use. Chymotrypsin (EC 3.4.4.5, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone treated, catalog no. C3142; lot no. 92F-8045 and 70F-8000), lima bean trypsin inhibitor (catalog no. T-9378; lot no. 127C-8011), cytidine 2′-monophosphate [(2′CMP) catalog no. C-0388; lot no. 109C-7340], and sodium cacodylate (catalog no. C-0250; lot no. 37C-0121), obtained from Sigma Chemical Co., were directly used without further purification. Bradykinin (catalog no. 4130; lot no. G3473M) and [Tyr^5]bradykinin (catalog no. 21229; lot no. 04L1202) were obtained from Vega Biochemicals, while bradykinin-potentiating pentapeptide (catalog no. 52353) was purchased from Serva Fine Biochemicals. The sources, grades, or preparation methods for other enzymes, proteins, and chemicals used here have been described in earlier papers (Lin & Brandts, 1979a,b, 1983a–c).

Assay for the Rate of Chymotrypsin Hydrolysis of Short Peptides. In an attempt to establish the isomeric specificity of chymotrypsin toward the following X–Pro bond, several peptides with a -Tyr-(Phe or Trp)-X-Pro sequence were subjected to high chymotrypsin activity. The peptides were bradykinin (Arg_1-Pro_2-Pro_3-Gly_4-Phe_5-Ser_6-Pro_7-Phe_8-Arg_9), [Tyr^5]bradykinin, and bradykinin-potentiating pentapeptide (pGlu-Lys-Trp-Ala-Pro). The experimental procedures were very similar to those used in the trypsin hydrolysis of Gly-Gly-Lys-Phe-Pro and of the Lys_{91}–Tyr_{92} bond in oxidized RNase described previously (Lin & Brandts, 1983a). Bradykinin-potentiating pentapeptide has only one cleavage point for chymotrypsin activity. The rate of hydrolysis of the Trp–Ala bond was assayed by further hydrolyzing the resulting Ala–Pro dipeptide by prolidase to release free proline, which can be easily quantitated by the acid ninhydrin method (Lin & Brandts, 1979a,b). Bradykinin or [Tyr^5]bradykinin has two cleavage points for chymotrypsin. One is the carboxyl-terminal Phe_8–Arg_9 bond. The rate of hydrolysis of this bond was assayed by quantitating free arginine by high-performance liquid chromatography (HPLC), coupled with precolumn fluorescence derivatization with OPA reagent. The rate of hydrolysis at the other cleavage point, Phe_5–Ser_6 or Tyr_5–Ser_6, was assayed by further hydrolyzing the resulting N-terminal Ser_6–Pro_7 bond by aminopeptidase P to release free serine, which was also derivatized with OPA reagent and analyzed by HPLC.

Assay for the Rate of Chymotrypsin Hydrolysis of the Tyr_{115}–Val_{116} Bond of RNase. These experiments were performed for oxidized RNase (i.e., performic acid treated, devoid of disulfide bonds), native RNase, and reversibly unfolded RNase (i.e., disulfide bonds intact) as the initial states. Since the Tyr_{115}–Val_{116} bond in oxidized RNase is readily accessible for chymotrypsin hydrolysis, no pretreatment of substrate was necessary. However, both native RNase and reversibly unfolded RNase are very poor substrates for chymotrypsin in the native state buffer which must be used to maintain high chymotrypsin activity, so these must first be irreversibly unfolded. This was accomplished either by treating RNase with a short but intensive pepsin pulse (20 s) in a buffer (ca. 4.5 M urea, pH 2.0) which is a native-state buffer for pepsin but a denaturing buffer for RNase (Lin & Brandts, 1983b) or by reducing RNase with a short pulse (20 s) of mercaptoethanol in high urea concentration and at high pH immediately before chymotrypsin was added. The detailed experimental procedures are as follows:

(*1*) *Oxidized RNase.* For initiation of the reaction, 50 μL of substrate ($\sim 9 \times 10^{-4}$ M, in H_2O, pH 8.0, at 10 °C) was mixed with 400 μL of chymotrypsin solution ($\sim 1 \times 10^{-3}$ M, in 0.05 M veronal or phosphate buffer, pH 8.0, at 10 °C). At suitable time intervals, a 50-μL aliquot of solution was quickly pipetted into a centrifuge tube containing 1 mL of methanol to stop enzyme activity. The contents were mixed with a vibrator, heated in a water bath at 85–90 °C for 5 min, and then dried by nitrogen. To the residues was added 150 μL of deionized water. The amount of the Tyr_{115}–Val_{116} bond cleaved at various times was assayed by either further releasing free Tyr_{115} from the carboxyl terminus by a long hydrolysis with proline-specific endopeptidase or further releasing free Val_{116} from the N-terminus with a long hydrolysis by aminopeptidase P (APP). If Tyr_{115} was to be determined, the pH of the solution was first adjusted to 7 with a predetermined amount of 0.1 N HCl, and then 50 μL of proline-specific endopeptidase (0.05 units in 0.045 M phosphate buffer, pH 7.0) was added. The solutions were incubated at 35 °C for 60 min. Then 2.2 mL of methanol was added to stop enzyme activity and to precipitate out enzymes and large peptide fragments. If Val_{116} was to be assayed, the pH of the solution was adjusted to 8.5 with 0.1 M NaOH, and 50 μL of APP solution [1 unit, in 0.05 M veronal buffer with manganese(II) citrate reagent, pH 8.6] was added for 60 min at 35 °C. Then

2.2 mL of ethanol was added. The resulting precipitates for either assay method were centrifuged at 7500 rpm for 20 min. The supernatants were quantitatively transferred to test tubes and heated in a water bath at 85 °C for 3 min. The solution was then dried by nitrogen. The residues were dissolved by adding 250 μL of 0.4 M borate buffer, pH 9.5, and were filtered with the aid of a centrifugal filter (Rainin Instrument Co., nylon 66 membrane, 0.2 μm) to remove any particles. The samples were then ready for HPLC analysis (Lin & Brandts, 1983a,b). Control runs without oxidized RNase were also carried out in an identical manner, and the peak area obtained in the tyrosine or valine peak position was subsequently subtracted from that obtained for the sample. Tyrosine and valine standards were used to quantitate the amount of Tyr_{115} or Val_{116} released.

(*2*) *Native or Reversibly Unfolded RNase.* Before the experimental procedures similar to those described for oxidized RNase were performed, the substrates were irreversibly unfolded with a pulse of pepsin activity or a reductive pulse of mercaptoethanol. The detailed procedures are as follows:

(*a*) *Mercaptoethanol Reduction.* For initiation of the reaction, 10 μL of native RNase ($\sim 1 \times 10^{-2}$ M, in H_2O) or reversibly unfolded RNase (5.0 M urea, pH 2.0) at 10 °C was mixed with 100 μL of 0.2 M mercaptoethanol (8.5 M urea, pH 12.0, 10 °C) for 20 s to reduce the disulfide bonds. Then 20 μL of this solution was quickly transferred into 1.0 mL of chymotrypsin solution (2×10^{-4} M in 0.023 M sodium phosphate buffer, pH ~7.6, at 10 °C). At suitable time intervals, 100 μL of incubated solution was quickly pipetted into a centrifuge tube containing 1.0 mL of methanol to stop enzyme activity. The rest of the procedures are identical with those described for oxidized RNase. Only proline-specific endopeptidase was used to assay the rate of hydrolysis.

(*b*) *Pepsin Treatment.* For initiation of this reaction, 10 μL of native RNase ($\sim 1 \times 10^{-2}$ M in H_2O, at 10 °C) was reacted with 100 μL of pepsin solution (~20 mg/mL, in 5 M urea, pH 1.9, at 10 °C) for 20 s. Then 20 μL of the mixture was quickly added to 1.0 mL of chymotrypsin solution (2×10^{-4} M, in 0.023 M phosphate buffer, pH 8, at 10 °C). At suitable time intervals, 100 μL of incubated solution was quickly pipetted into a centrifuge tube containing 1 mL of methanol. The rest of the procedures are identical with those described for oxidized RNase. Only proline-specific endopeptidase was used to assay the rate of hydrolysis. This experiment was carried out for native RNase only.

Assay for the Isomeric States of Proline-114 or Proline-117 in RNase. The fast cleavage of the Tyr_{115}–Val_{116} bond of oxidized, native, or irreversibly denatured RNase (see Figure 2) by chymotrypsin then allows investigation of the isomeric states of both the Asn_{113}–Pro_{114} and the Val_{116}–Pro_{117} bonds in those various forms of RNase by using time-dependent pulses of either proline-specific endopeptidase (to probe the Asn_{113}–Pro_{114} bond) or aminopeptidase P (to probe the Val_{116}–Pro_{117} bond) subsequent to the chymotrypsin pulse (60–80 s). The rate of release of free Tyr_{115} or Val_{116} can be used to determine the isomeric states of proline-114 or proline-117, respectively, since proline-specific endopeptidase exhibits isomeric specificity toward the trans form of the preceding Asn_{113}–Pro_{114} bond while only the trans form of the Val_{116}–Pro_{117} bond can be cleaved by aminopeptidase P. Also, the isomeric states of proline-114 or -117 in the RNase fragments can be determined by these two enzymes by waiting for isomerization to reach equilibrium after the completion and inhibition of the chymotrypsin pulse. The detailed procedures are summarized below.

(*1*) *Oxidized RNase.* After substrate and chymotrypsin were mixed for 60 s to completely cleave the Tyr_{115}–Val_{116} bond under the conditions described in the preceding section for oxidized RNase, chymotrypsin activity was inhibited by adding 250 μL of lima bean trypsin inhibitor (120 mg/mL, in 0.05 M veronal buffer, pH 8.8, or in 0.045 M phosphate buffer, pH 6.0, depending on which enzyme was used). Either immediately or after waiting for 30 min, 100 μL of proline-specific endopeptidase (10 units, in 0.045 M phosphate buffer, pH 7.0) or 1.0 mL of aminopeptidase P solution [35 units, in 0.05 M veronal buffer containing manganese(II) citrate reagent, pH 8.8] was added to the solution, depending on whether assaying for proline-114 or proline-117. The final pH of the solution was ca. 7 for proline-specific endopeptidase and ca. 8.6 for aminopeptidase P. At suitable time intervals, an aliquot of the reaction mixture (100 μL for the proline-114 assay and 150 μL for the proline-117 assay) was quickly pipetted into a centrifuge tube containing 2.2 mL of methanol or absolute ethanol, and the contents were mixed with a vibrator to stop enzyme activity and precipitate out proteins and large peptide fragments. The resulting precipitate was centrifuged at 7500 rpm for 20 min. The supernatant was quantitatively transferred to a test tube and heated at 85 °C for 3 min. The solution was then dried by nitrogen. The residues were dissolved by adding 250 μL of 0.4 M borate buffer, pH 9.5, and were filtered with the aid of a centrifugal filter to remove any particles. The sample was then ready for HPLC analysis. Control runs without substrate were also carried out in an identical manner, and the peak areas obtained in the tyrosine or valine position were subsequently subtracted from that obtained for the sample.

(*2*) *Native or Reversibly Unfolded RNase.* (*a*) *Mercaptoethanol Reduction.* After native RNase or reversibly unfolded RNase was reduced with 0.2 M mercaptoethanol for 20 s or 30 min (for native RNase only) and then reacted with chymotrypsin for 60–80 s according to the procedures described in the preceding section, 250 μL of lima bean trypsin inhibitor was added. A high activity of proline-specific endopeptidase was immediately added in the same fashion as for oxidized RNase, to probe the isomeric states of proline-114. The experimental procedures were identical with those for oxidized RNase. For proline-117 isomerization, only reversibly unfolded RNase (initial state) was investigated. After reversibly unfolded RNase was reduced with 0.2 M mercaptoethanol for 20 s, and reacted with chymotrypsin for 80 s, 250 μL of lima bean trypsin inhibitor and 1.0 mL of aminopeptidase P (35 units) were immediately pipetted into the solution to assay the isomeric state of proline-117. The rest of the procedures are the same as those for assaying proline-117 in oxidized RNase.

(*b*) *Pepsin Treatment.* This experiment was carried out for proline-114 only. After native RNase or reversibly unfolded RNase was irreversibly unfolded by pepsin for 20 s and then reacted with chymotrypsin for 60–80 s (see preceding section for experimental conditions), the chymotrypsin activity was inhibited by 250 μL of lima bean trypsin inhibitor. Immediately or after waiting for 40 min (for native RNase only), a high activity of proline-specific endopeptidase was added to probe the isomeric states of proline-114. The rest of the procedures are the same as those for assaying proline-114 in oxidized RNase.

HPLC Analysis of Free Amino Acids. This was performed on a Varian 5020 gradient liquid chromatograph with a Fluorichrom fluorescence detector and a reverse-phase Servachrom HPLC precision column (C18, 5 μm, 4.6 mm × 25

cm; Serva Fine Biochemicals Inc.). A 100-μL sample loop was used with a Valco injector. The gradient run started at 40% MeOH–60% 0.09 M sodium phosphate buffer (pH 6.8) and increased to 70% MeOH in 35 min. The flow rate was 0.5 mL/min. Before a sample was injected, 50 μL of the sample solution was reacted with 150 μL of OPA reagent (Lin & Brandts, 1983a) for exactly 2 min. Amino acid standards, also derivatized with OPA reagent in the same fashion, were used to identify and quantitate the amount of free amino acids in each sample. The peak area was used to quantitate the degree of hydrolysis at each time interval, using a long incubation sample as the 100% reference.

RNase Refolding Monitored by Absorbance Change. The experimental procedures are very similar to those described in an earlier paper (Lin & Brandts, 1983c) except that a Cary 219, instead of a Cary 14, instrument was used. The experiment was carried out at either 10 or 16.5 °C. The unfolded RNase ($\sim 10^{-3}$ M, in 5 M urea, pH 2.0) was diluted 16 times in 0.05 M sodium cacodylate buffer (pH 5.5), and the absorbance change at 287 nm was continuously monitored until total equilibrium was achieved. Extrapolation to zero time was employed to obtain the total absorbance change of the slow refolding phases. The peel-off method was used to resolve the separate phases.

RNase Refolding Monitored by 2'CMP Binding. The experiment was also performed on a Cary 219 instrument, also at either 10 or 16.5 °C. The procedures are similar to those of Garel et al. (1976) and Cook et al. (1979). Unfolded RNase ($\sim 10^{-3}$ M, in 5 M urea, pH 2.0) was diluted 16 times into 0.05 M cacodylate buffer (pH 5.5) containing 1.5×10^{-4} M 2'CMP. The binding of 2'CMP to native RNase was continuously monitored at 250 nm until it leveled off. The data treatment was the same as that described for the tyrosine absorbance change.

Results

Short Peptides. One of our first objectives in this work was to try to establish, using short peptides, that chymotrypsin hydrolysis of an active X–Y bond in a sequence -X-Y-Pro- would only occur when the following Y–Pro bond was trans, in analogy with what has already been demonstrated to occur with trypsin and its substrates (Lin & Brandts, 1983a,b). Although we were not successful in achieving this objective, a brief summary of results is given below.

When bradykinin (Arg_1-Pro_2-Pro_3-Gly_4-Phe_5-Ser_6-Pro_7-Phe_8-Arg_9) and [Tyr^5]bradykinin are subjected to chymotrypsin hydrolysis, two bonds are cleaved. One is the Phe_5–Ser_6 (or Tyr_5–Ser_6) bond preceding the isomerizing proline bond, and the other is the Phe_8–Arg_9 bond at the carboxyl terminus. The rates of hydrolysis of these two bonds as a function of time are shown in Figure 1. Hydrolysis of bradykinin was carried out at 10 °C with chymotrypsin and substrate concentrations at 2×10^{-4} and 2.5×10^{-4} M, respectively, while hydrolysis of [Tyr^5]bradykinin was performed at 5 °C with enzyme and substrate concentrations at 8×10^{-4} and 6×10^{-4} M, respectively. Figure 1 shows that the cleavage of the Phe_8–Arg_9 bond is rather fast for both peptides (open triangles and open circles), with almost 100% cleavage in a couple of minutes. However, the cleavage of the Phe_5–Ser_6 bond (closed triangles) or the Tyr_5–Ser_6 bond (closed circles) in the peptides is surprisingly slow, considering the very high enzyme concentration. The half-time is about 38 min for Phe_5–Ser_6 cleavage and 24 min for Tyr_5–Ser_6 cleavage. These results are unexpected. Chymotrypsin is an endopeptidase and will normally hydrolyze peptide bonds inside a chain much faster than those at the carboxyl terminus.

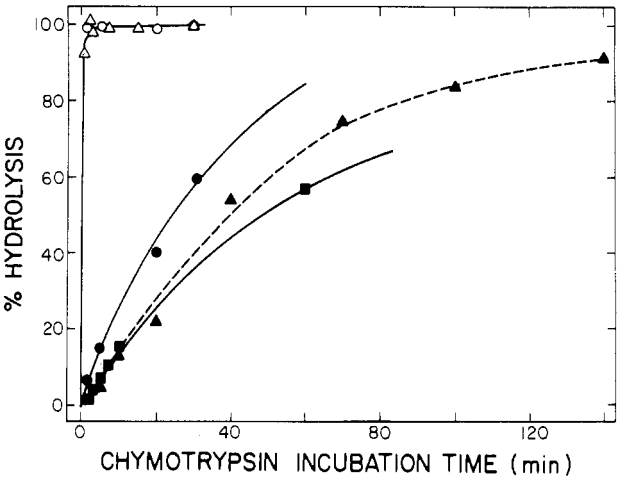


FIGURE 1: Hydrolyses of short peptides catalyzed by chymotrypsin. Open and closed triangles are the data for the Phe_8–Arg_9 bond and the Phe_5–Ser_6 bond, respectively, in bradykinin at 10 °C. Open and closed circles are the data for the Phe_8–Arg_9 bond and the Tyr_5–Ser_6 bond, respectively, in [Tyr^5]bradykinin at 5 °C. Closed squares are for the Trp–Ala bond in bradykinin-potentiating pentapeptide at 25 °C.

The results of chymotrypsin hydrolysis of bradykinin-potentiating pentapeptide (pGlu-Lys-Trp-Ala-Pro) are also plotted in Figure 1 (closed squares). Only one bond (Trp–Ala) was cleaved. The experiment was carried out at 25 °C with chymotrypsin and substrate concentrations at 4.4×10^{-4} and 6.8×10^{-4} M, respectively. The half-time of hydrolysis is also very long, about 48 min.

The purpose of carrying out these experiments was, as stated above, to establish the isomeric specificity of chymotrypsin toward the following X–Pro bond. Due to the slow cleavage of Phe_5–Ser_6, Tyr_5–Ser_6, and Trp–Ala bonds in these short peptides and because only one kinetic phase is seen in these hydrolyses, we are unable to establish the isomeric specificity of chymotrypsin, as we previously did for trypsin (Lin & Brandts, 1983a). NMR has shown that bradykinin in aqueous solution has about 10% cis content for the Ser_6–Pro_7 bond (London et al., 1978) although the relaxation time for isomerization has not been obtained. We would expect it to be several minutes at 10 °C, in analogy with similar peptides. It seems then that the rate of cleavage is about 10 times slower than that of isomerization. Bradykinin-potentiating pentapeptide, which has proline at the carboxyl end, should also have a substantial amount of the cis form and a relatively slow rate of isomerization. However, the rate of cleavage of the Trp–Ala bond is also too slow to detect the isomerization. Other data not shown in Figure 1 show that the cleavage of the Trp–Ala bond cannot be significantly accelerated by tripling the enzyme to substrate ratio.

In addition to these three peptides, three other peptides (Gly-Gly-Tyr-Phe-Pro, Gly-Gly-Tyr-Gly-Pro, and the oxidized insulin B chain, which has a -Tyr-Thr-Pro- sequence near the carboxyl end) were also subjected to high chymotrypsin activity. All of these show very slow cleavage and one kinetic phase, with the half-time ranging from 20 to 100 min (unpublished results) at 10 °C.

Rate of Chymotrypsin Hydrolysis of the Tyr_{115}–Val_{116} Bond in the RNase Sequence -Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}-Pro_{117}-. Even though it seems likely that chymotrypsin, like trypsin, does have an absolute specificity for a trans peptide bond following the active bond, this could not be demonstrated experimentally with short peptides since the location of a proline residue adjacent to the active bond slowed down the rate of cleavage to a point where it was much slower than

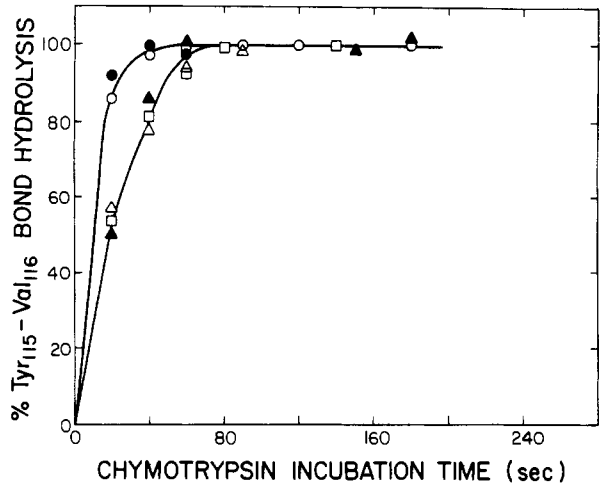


FIGURE 2: Chymotrypsin hydrolysis (10 °C) of the Tyr_{115}–Val_{116} bond in the RNase sequence -Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}-Pro_{117}-. Circles are for oxidized RNase; closed circles represent the data assayed by the aminopeptidase P release of free Val_{116} from the N-terminus; open circles represent the data assayed by the proline-specific endopeptidase release of free Tyr_{115} from the carboxy terminus. The chymotrypsin and oxidized RNase concentrations are 10^{-3} and 10^{-4} M, respectively. Open triangles are the data for native RNase (initial state) which was first subjected to 20-s mercaptoethanol reduction immediately before the addition of chymotrypsin. Closed triangles are for equilibrated reversibly unfolded RNase (in 5 M urea, pH 2, as the initial state) which was first subjected to 20-s mercaptoethanol reduction immediately before the addition of chymotrypsin. Squares are for RNase which was initially in the native state and was first subjected to 20-s pepsin treatment immediately before the addition of chymotrypsin. The chymotrypsin and substrate concentrations for these two experiments were 2×10^{-4} and 2×10^{-5} M, respectively.

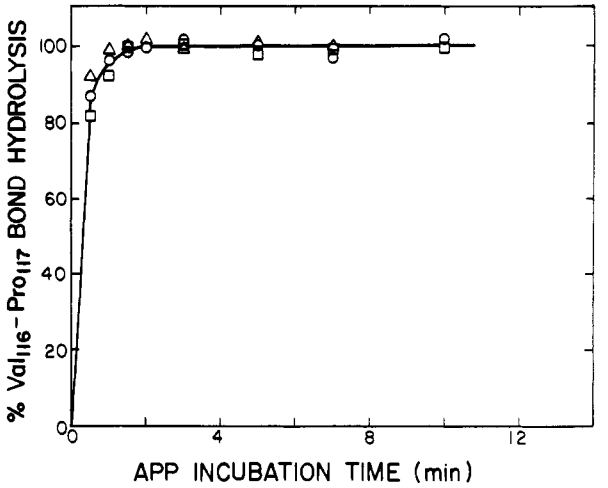


FIGURE 3: Rates of hydrolysis of the Val_{116}–Pro_{117} bond in RNase, catalyzed by aminopeptidase P at 10 °C. Triangles are for oxidized RNase which was first subjected to 60-s chymotrypsin activity immediately before aminopeptidase P (35 units) was added. Squares are for oxidized RNase which was first subjected to 60-s chymotrypsin activity, and aminopeptidase P (35 units) was added after waiting for 30 min following inhibition of chymotrypsin activity. Circles are for equilibrated, reversibly unfolded RNase (initial state) which was first reduced by mercaptoethanol for 20 s and reacted with chymotrypsin for 80 s, and aminopeptidase P (35 units) was added immediately after the chymotrypsin activity was inhibited. The chymotrypsin and substrate concentrations are 1×10^{-3} and 1×10^{-4} M, respectively, for oxidized RNase, and are 2×10^{-4} and 2×10^{-5} M, respectively, for equilibrated, reversibly unfolded RNase.

isomerization. Therefore, in the studies which follow, we will make no assumptions about the isomeric specificity of chymotrypsin toward the following bond.

In contrast to the short peptides examined above, the Tyr_{115}–Val_{116} bond in oxidized RNase can be hydrolyzed rather quickly by chymotrypsin even though proline-117 is located immediately adjacent to the active bond. The rates of hydrolysis (10 °C) for different initial states of RNase are plotted in Figure 2. The data show that oxidized RNase (circles) was completely hydrolyzed in less than 1 min. The ratio of enzyme to substrate concentration is about 10 (10^{-3} and 10^{-4} M, respectively). Hydrolysis was assayed either by long-time exposure to aminopeptidase P (closed circles; to further release free Val_{116} from the N-terminus) or by long-time exposure to proline-specific endopeptidase (open circles; to further release free Tyr_{115} from the carboxy terminus), and the results are identical within the experimental errors. The amount of Tyr_{115} or Val_{116} released from the samples with long chymotrypsin incubation time (10 or 20 min), which was always very close to 1 mol/mol of RNase, served as the 100% reference.

Since native or reversibly unfolded RNase is not a good substrate for chymotrypsin in native-state hydrolysis buffers, these forms of RNase must first be quickly and irreversibly unfolded before chymotrypsin can be added. This was carried out with a short pulse (20 s) of either pepsin activity or mercaptoethanol reduction. The rates of hydrolysis of the Tyr_{115}–Val_{116} bond catalyzed by chymotrypsin for both pepsin-unfolded and mercaptoethanol-reduced RNase are plotted in Figure 2. For mercaptoethanol reduction, the initial state was either native RNase (open triangles) or equilibrated, reversibly unfolded RNase in 5 M urea, pH 2.0 (closed triangles), while for pepsin treatment, only native RNase was investigated (squares). These data show that the Tyr_{115}–Val_{116} bond can be completely hydrolyzed in less than 1.5 min regardless of the initial state. The ratios of chymotrypsin to substrate concentration were also ca. 10, but the solutions were more dilute (2×10^{-4} to 2×10^{-5} M) than for oxidized RNase, since it was necessary to dilute out the high urea concentration employed in pepsin cleavage and mercaptoethanol reduction. The slightly slower cleavage of Tyr_{115}–Val_{116} in pepsin-treated or mercaptoethanol-reduced RNase could be due to this dilution or could be due to the presence of urea (0.10–0.17 M), pepsin, or mercaptoethanol in the solution.

Isomeric States of Proline-117. Data in Figure 2 show that the Tyr_{115}–Val_{116} bond in oxidized RNase can be completely hydrolyzed in 40–60 s, while that for native or equilibrium-unfolded RNase can be completely cleaved in 60–90 s after first being treated with mercaptoethanol or pepsin for 20 s. Since we are unable to establish the isomeric specificity of chymotrypsin, the fast 100% cleavage of the Tyr_{115}–Val_{116} bond by chymotrypsin might mean the following: (1) Chymotrypsin exhibits no isomeric specificity toward the following Val_{116}–Pro_{117} bond [*or* to the preceding (two bonds away) Asn_{113}–Pro_{114} bond]. (2) Chymotrypsin exhibits isomeric specificity toward the following Val_{116}–Pro_{117} bond, but this bond has little (<5%) or no cis content. (3) Chymotrypsin exhibits isomeric specificity toward the following Val_{116}–Pro_{117} bond, but the rate of isomerization is too fast compared to the Tyr_{115}–Val_{116} cleavage.

There is not much we can do, using the ISP method, to distinguish between possibilities (2) and (3). However, if (1) is true, we should be able to verify it by adding a high activity of aminopeptidase P immediately after the Tyr_{115}–Val_{116} bond is completely cleaved by the short chymotrypsin pulse, since the isomeric specificity of aminopeptidase P toward the trans form of an X–Pro (here Val_{116}–Pro_{117}) bond is well established (Lin & Brandts, 1979b, 1980, 1983a). Figure 3 shows the results of such a study, in which the rate of hydrolysis of Val_{116}–Pro_{117} in RNase structures at 10 °C is plotted as a function of aminopeptidase P incubation time. Two experi-

ments were performed for oxidized RNase. One was designed to probe the isomeric state of proline-117 in oxidized RNase at the end of the 60-s chymotrypsin pulse, and in this case, a high activity of aminopeptidase P (~35 units) was added immediately after chymotrypsin activity was inhibited (triangles). The other was designed to examine proline-117 in the equilibrated oxidized RNase fragment containing the Val_{116}–Pro_{117} bond, so high activity of aminopeptidase P (35 units) was added after waiting for 30 min following inhibition of chymotrypsin activity (squares). Finally, Figure 3 also shows the rate of hydrolysis of the Val_{116}–Pro_{117} bond for equilibrated, unfolded RNase (initial state) which was first reduced by mercaptoethanol for 20 s and reacted with chymotrypsin for 80 s (circles). Aminopeptidase P (35 units) was added immediately after the chymotrypsin activity was inhibited. The chymotrypsin and substrate concentrations are 2×10^{-4} and 2×10^{-5} M, respectively, for all three experiments.

Data in Figure 3 show that in all cases the hydrolyses are fast and only one kinetic phase, with a half-time of ca. 20 s at 10 °C, is observed. These results show that the Val_{116}–Pro_{117} bond in oxidized RNase or unfolded RNase has no or little cis content or that the rate of isomerization of the Val_{116}–Pro_{117} bond in these RNase structures is too fast to be studied by the ISP method.

Isomeric State of Proline-114. After the Tyr_{115}–Val_{116} bond in RNase is completely hydrolyzed with a short chymotrypsin pulse, high activity of proline-specific endopeptidase can be added to probe the isomeric state of proline-114 which exists at the instant when proline-specific endopeptidase is added. Since this enzyme has an absolute requirement for a trans peptide bond at the position immediately preceding the active bond, only those Pro_{114}–Tyr_{115} bonds with a neighboring trans Asn_{113}–Pro_{114} bond can be hydrolyzed fast while those Pro_{114}–Tyr_{115} bonds with a cis Asn_{113}–Pro_{114} neighboring bond must first isomerize to trans before they can be cleaved. The study of the release of free Tyr_{115}, which was assayed by HPLC, as a function of incubation time with proline-specific endopeptidase can then be used to obtain the cis/trans ratio of proline-114 in various RNase structures. This method has been described in detail previously (Lin & Brandts, 1979a,b, 1980, 1983a,b,e).

(*1*) *Oxidized RNase.* Figure 4 shows the semilog plot of the rate of hydrolysis of the Pro_{114}–Tyr_{115} bond (10 °C) catalyzed by high activity of proline-specific endopeptidase after oxidized RNase was first subjected to a 60-s pulse of chymotrypsin activity. In one experiment (crosses), proline-specific endopeptidase was added immediately after chymotrypsin activity was inhibited with lima bean trypsin inhibitor. In the other experiment (circles), proline-specific endopeptidase was added after waiting for 30 min following chymotrypsin inhibition. The amount of Tyr_{115} released from the sample with long incubation time (30 min), which is close to 1 mol/mol of RNase, served as the 100% reference. Data in Figure 4 implicitly indicate two kinetic phases. The slow phase for both experiments accounts for ca. 17% of the total amplitude and has a relaxation time of ca. 5.5 min at 10 °C. The fast phase accounts for ca. 83% of the total amplitude, and the rate is too fast to be evaluated. These kinetic patterns are very similar to those reported for *N*-Cbz-Gly-Pro-MAC and *N*-Cbz-Gly-Pro-Leu-Gly when hydrolyzed by proline-specific endopeptidase (Lin & Brandts, 1983e), and a similar interpretation seems to apply in the present instance. This means that proline-114 in oxidized RNase or in the equilibrated chymotrypsin-produced fragment has ca. 17% of the cis form

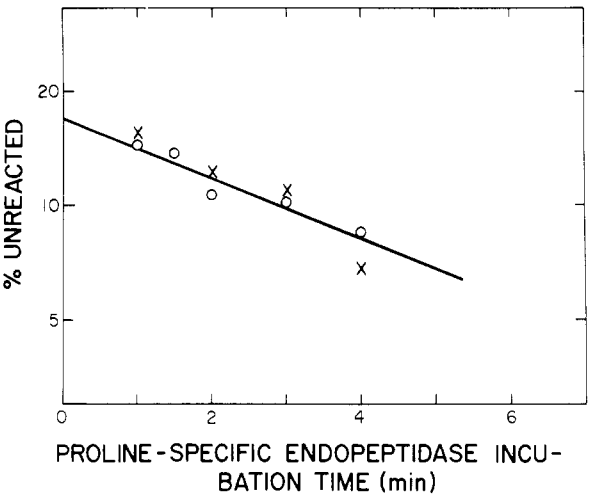


FIGURE 4: Semilog plot of the rates of hydrolysis of the Pro_{114}–Tyr_{115} bond in oxidized RNase catalyzed by proline-specific endopeptidase at 10 °C. Crosses are for oxidized RNase which was first subjected to 60-s chymotrypsin activity, and proline-specific endopeptidase (10 units) was added immediately after the chymotrypsin activity was inhibited. Circles are the data for oxidized RNase which was first subjected to 60-s chymotrypsin activity, and proline-specific endopeptidase (10 units) was added after waiting for 30 min following chymotrypsin inhibition. The chymotrypsin and substrate concentrations are 1×10^{-3} and 1×10^{-4} M, respectively.

and 83% of the trans form in solution. It should be noted that the relaxation time derived from data in Figure 4 is not for the whole oxidized RNase chain, but for the fragment containing Asn_{113}-Pro_{114}-Tyr_{115} at the carboxy terminus.

(*2*) *Native RNase and Equilibrated, Unfolded RNase.* As mentioned above, native and reversibly unfolded RNases, unlike oxidized RNase, are not good substrates for chymotrypsin and must therefore be irreversibly unfolded before chymotrypsin is added. This was carried out in two ways: with a short pulse of either mercaptoethanol reduction or pepsin digestion.

(*a*) *Mercaptoethanol Reduction.* Semilog plots of the rate of cleavage of the Pro_{114}–Tyr_{115} bond as a function of incubation time with the proline-specific endopeptidase (10 °C) are shown in Figure 5. The following three different experiments, designed to detect the isomeric states of proline-114 in native RNase, in equilibrated, unfolded RNase, and in reduced RNase, were carried out: (1) *native RNase in H_2O* was subjected to 20-s mercaptoethanol reduction and then 60 s of chymotrypsin activity before proline-specific endopeptidase was added (circles); (2) *equilibrated, unfolded RNase* (in 5 M urea, pH 2.0) was subjected to 20-s mercaptoethanol reduction and then 80-s chymotrypsin activity before proline-specific endopeptidase was added (triangles); (3) *native RNase* was subjected to 30-min mercaptoethanol reduction and then 80-s chymotrypsin activity before proline-specific endopeptidase was added (crosses). In all these experiments, there is a 60–80-s lag between the time when chymotrypsin begins its cleavage and the time of addition of the proline-specific endopeptidase to probe proline-114. As noted in a previous paper (Lin & Brandts, 1983b), isomerization will begin to occur as soon as RNase is unfolded and the peptide bond is cleaved by chymotrypsin unless the equilibrium of the proline bond is not affected by peptide bond cleavage and protein unfolding. Data in Figure 5 show ca. 92% cis for proline-114 when the initial state is native RNase (circles). It is well-known that proline-114 in native RNase is 100% cis. It is likely that the 80-s lag in adding proline-specific endopeptidase has resulted in proline-114 isomerizing from 100% to 92%. Figure 5 shows that the relaxation time of cis to trans isom-

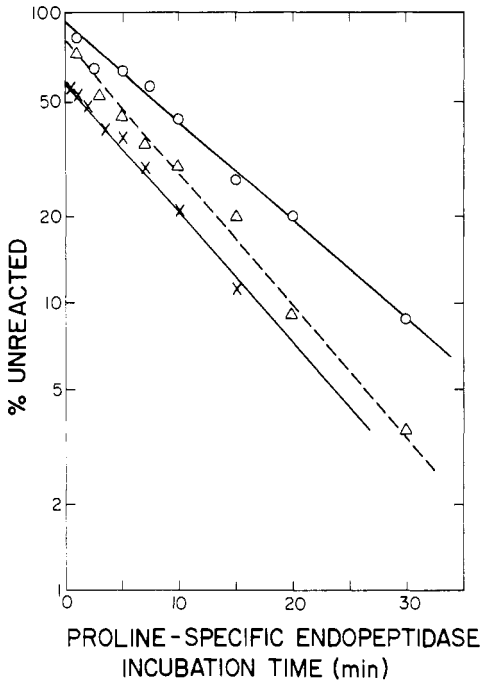


FIGURE 5: Semilog plot of the rates of hydrolysis of the Pro_{114}–Tyr_{115} bond in RNase catalyzed by proline-specific endopeptidase at 10 °C. Circles are for native RNase (initial state) which was first reduced by mercaptoethanol for 20 s and reacted with chymotrypsin for 60 s, and proline-specific endopeptidase (10 units) was added immediately after the chymotrypsin activity was inhibited. Triangles are for equilibrated, reversibly unfolded RNase (initial state) which was first reduced by mercaptoethanol for 20 s and reacted with chymotrypsin for 80 s, and proline-specific endopeptidase (10 units) was added immediately after the chymotrypsin activity was inhibited. Crosses are for native RNase which was first reduced by mercaptoethanol for 30 min and reacted with chymotrypsin for 80 s, and proline-specific endopeptidase (10 units) was added immediately after the chymotrypsin activity was inhibited. The chymotrypsin and substrate concentrations are 2×10^{-4} and 2×10^{-5} M, respectively.

erization is ca. 11.5 ± 1.5 min at 10 °C. If we assume that the cis/trans ratio (58/42) obtained from the sample with 30-min mercaptoethanol reduction (crosses) can be used as the final equilibrium of proline-114 in the reduced RNase as well as in the proline-114 fragment, then the two-way relaxation time for proline-114 isomerization is a chymotrypsin fragment is ca. 4.9 ± 0.6 min. Using these estimates, it can be shown by calculation that the 92% estimate obtained at the time of addition of the proline-specific endopeptidase would be corrected to ca. 101% at the time the native state was initially unfolded.

The same correction can be applied to the data when the initial state of RNase is the equilibrated, unfolded form. Data in Figure 5 (triangles) show ca. 82% cis proline-114 at the time of addition of the proline-specific endopeptidase. Correction for the 100-sec lag leads to an estimate of 92% cis for equilibrated, unfolded RNase in 5 M urea, pH 2.0.

(*b*) *Pepsin Treatment.* These experiments are very similar to those using mercaptoethanol reduction except that a short pulse of pepsin digestion was used to irreversibly unfold RNase. The semilog plots of the rate of Pro_{114}–Tyr_{115} hydrolysis catalyzed by proline-specific endopeptidase at 10 °C are shown in Figure 6. The following three different experiments were also carried out: (1) *native RNase* was subjected to 20-s pepsin and 60-s chymotrypsin digestion before proline-specific endopeptidase was added (circles); (2) *equilibrated, unfolded RNase* was subjected to 20-s pepsin and 80-s chymotrypsin digestion before proline-specific endopeptidase was added (triangles); (3) *native RNase* was subjected to 20-s pepsin and

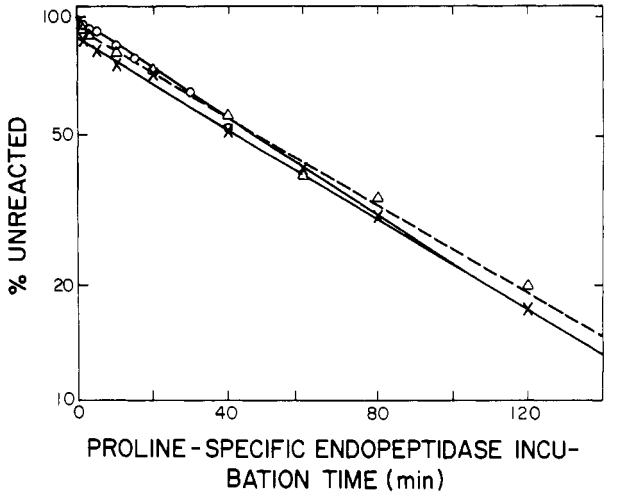


FIGURE 6: Semilog plots of the rates of hydrolysis of the Pro_{114}–Tyr_{115} bond in RNase catalyzed by proline-specific endopeptidase at 10 °C. Circles are for native RNase (initial state) which was first subjected to 20-s pepsin and 60-s chymotrypsin digestion, and proline-specific endopeptidase (10 units) was added immediately after the chymotrypsin activity was inhibited. Triangles are for equilibrated, reversibly unfolded RNase (initial state) which was first subjected to 20-s pepsin and 80-s chymotrypsin digestion, and proline-specific endopeptidase (10 units) was added immediately after the chymotrypsin activity was inhibited. Crosses are for native RNase which was first subjected to 20-s pepsin and 80-s chymotrypsin digestion, and proline-specific endopeptidase (10 units) was added after waiting for 40 min following chymotrypsin inhibition. The chymotrypsin and substrate concentrations are 2×10^{-4} and 2×10^{-5} M, respectively.

80-s chymotrypsin digestion, and chymotrypsin activity was immediately inhibited by lima bean trypsin inhibitor. The solution was then incubated at 10 °C for 40 min for equilibrium to occur before proline-specific endopeptidase was added (crosses).

These experiments also have an 80–100-s lag in assaying the isomeric state of the initial RNase structure. However, the relaxation time for these three disulfide-intact forms of RNase (i.e., 73 ± 5 min for all three) is about 10 times slower than that for the corresponding reduced forms (Figure 5) so that the correction for the lag time need not be made in this case, particularly in view of the fact that all three forms have nearly the same cis content (i.e., slow phase amplitude) so that the potential shift in equilibrium following unfolding is very small. The data in Figure 6 then lead to an estimate of 98% cis (circles) for the native form (compared to the corrected estimate of 101% cis from the independent data in Figure 5), 94% cis (triangles) for the equilibrated, unfolded form (compared to 92% from Figure 5), and 90% cis (crosses) for the equilibrated RNase fragment containing proline-114. Note that this latter pepsin fragment has substantially more equilibrium cis content (90% vs. 58%) than the fully reduced, intact RNase chain. This is similar to what was found earlier for proline-93, where the cis content was nearly twice as high for those unfolded chains with disulfide bonds intact relative to the oxidized form.

Although the one-way, cis to trans relaxation times are much longer for the disulfide-intact forms (Figure 6) than for the reduced forms (Figure 5), the calculated two-way relaxation times are very similar. For example, the two-way relaxation time for the equilibrated pepsin–chymotrypsin fragment with disulfides intact is ca. 7.3 min, compared to 4.9 min for the mercaptoethanol-reduced form.

RNase Refolding Monitored by Absorbance Change and 2'CMP Binding. In a previous paper (Lin & Brandts, 1983c) which reported on the refolding of RNase in low urea con-

Table I: Kinetic Parameters for the Three Slow Refolding Phases of RNase, Monitored by the Absorbance Change (287 nm) and by Binding of 2′CMP

		XY phase		CT phase		ct phase[a]	
temp (°C)	method	α (%)	τ (s)	α (%)	τ (s)	α (%)	τ (s)
10	absorbance change	51	23	24	70	5	390
10	2′CMP binding	53	24	23	70	4	400
16.5	absorbance change	49	17	27	36	4	200
16.5	2′CMP binding	49	18	26	37	5	210

	activation energy (kcal/mol)		
	XY phase	CT phase	ct phase[a]
absorbance change	8	16	17
2′CMP binding	7	16	16

[a] Because of the small amplitude of the ct phase, a large uncertainty is inherent in these kinetic parameters. The expected errors of these values are of the order of 20–30%.

centration, two slow kinetic phases (designated as the XY and CT phases) were obtained when 96% of the total absorbance change was used to resolve the folding kinetics. In that paper, we indicated that the last ca. 5% of the refolding reaction is complicated by a small and very slow kinetic phase which was also apparent in earlier data from other laboratories (Cook et al., 1979; Schmid, 1981). No serious effort was made to characterize this small phase. Here, in order to correlate the ISP data on proline-114 and -117 isomerization with the kinetics of RNase refolding, we have reexamined the refolding of RNase monitored by the absorbance change, using a more sensitive spectrophotometer (i.e., the Cary 219), and have analyzed the entire slow absorbance change, instead of only 96% which was used to resolve the XY and CT kinetic phases in the earlier work. In addition, 2′CMP binding studies have been carried out to see if the recovery of RNase activity is consistent with refolding kinetics monitored by the absorbance change. Figure 7 shows some results from these two types of studies at 10 °C. The final urea concentration for both experiments was 0.31 M and 0.048 M cacodylate buffer, pH 5.5. Data in Figure 7 show the excellent agreement between the refolding of RNase monitored by the absorbance change (triangles) and the recovery of RNase activity monitored by 2′CMP binding (circles), suggesting that all of the amplitude changes resulting from tyrosine perturbations are due to the refolding of RNase with full binding activity. Three kinetic phases, instead of two, can be resolved from Figure 7 by the peel-off method. These kinetic data are listed in Table I, along with those obtained at 16.5 °C.

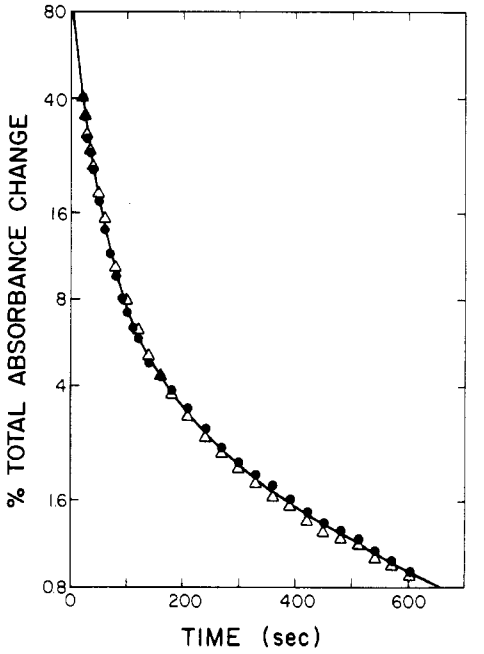


FIGURE 7: Refolding kinetics of RNase monitored by absorbance changes (triangles) at 287 nm and by 2′CMP binding (circles) at 10 °C. Unfolded RNase ($\sim 10^{-3}$ M, in 5 M urea, pH 2.0) was diluted 16 times into 0.05 M sodium cacodylate buffer (pH 5.5) or 0.05 M sodium cacodylate buffer (pH 5.5) containing 1.5×10^{-4} M 2′CMP. The final urea concentration is 0.31 M. The experiments were carried out on a Cary 219 spectrophotometer by using hand mixing. The fast phase of 20% amplitude was not seen in these experiments but has been included in the ordinate intercept.

In analogy with our previous data (Lin & Brandts, 1983c) under only slightly different conditions (0.31 M urea and acetate buffer, pH 5.0), the major phase accounting for ca. 50% of the total amplitude is assigned here as the XY phase since the amplitude, relaxation time, and activation energy are virtually the same. However, the rest of the slow amplitude was resolved here into two phases. One of these has 23–27% of the total refolding amplitude, a relaxation time of ca. 70 s at 10 °C, and an activation energy of ca. 16 kcal/mol. These compare favorably to earlier estimates for the spectroscopic CT phase (i.e., amplitude of 27–30%, relaxation time of 85 s at 10 °C, and activation energy of 17 kcal/mol), which was shown to be due to trans to cis isomerization of proline-93 since the parameters of the CT phase correlated well with direct measurements of proline-93 isomerization by the ISP method.

Finally, the slowest refolding phase seen in the data of Figure 7 has only 4–5% of the total amplitude change, a relaxation time of ca. 400 s at 10 °C, and an activation energy of ca. 17 kcal/mol. We will refer to this as the spectroscopic ct phase. There seems to be a strong likelihood that the ct phase is due to the trans to cis isomerization of proline-114 during refolding, since the ISP results discussed earlier show that reversibly unfolded RNase has only ca. 5% of proline-114 in the trans form and that all of this changes to cis in the native form. The activation energy of the ct phase is also consistent with the involvement of isomerization as the rate-limiting step. Because of the small trans content, we were not able to directly measure, by the ISP method, the relaxation time for proline-114 isomerization during RNase refolding. However, the calculated trans to cis relaxation time for proline-114 in the pepsin–chymotrypsin-treated RNase fragment with disulfide bonds intact is ca. 450 s, on the basis of the ISP results, which agrees well with the value of 400 s for the ct phase. Thus, the amplitude, activation energy, and relaxation time of the ct spectroscopic phase all suggest that this involves the trans to cis isomerization of proline-114 during refolding of RNase. It is also clear from the results of Figure 7 that recovery of enzymatic activity (i.e., the ability to bind 2′CMP) occurs only after the proline-114 has isomerized to the cis form, so it seems certain that proline-114 is not involved in the formation of a

nativelike, fully active intermediate which has one (or more) proline in an incorrect isomeric state.

Discussion

Since the cleavage of the active X–Y bonds in six small substrates containing the sequence -X-Y-Pro- was very slow, the isomeric specificity of chymotrypsin for the trans form of the following Y–Pro bond could not be established, as had earlier been done for trypsin (Lin & Brandts, 1983a). It is likely that the presence of the proline residue on the carboxyl side of the active bond is responsible for the slow cleavage, since otherwise similar substrates which lack the proline residue are cleaved much faster (Yoshida et al., 1968; Baumann et al., 1973). This may limit the usefulness of chymotrypsin for ISP analyses.

The very fast chymotrypsin cleavage of the carboxy-terminal Phe_8–Arg_9 bond in bradykinin and [Tyr^5]bradykinin was unexpected, and we feel the presence of the Pro_7 residue on the amino side of the active bond may accelerate this normally slow cleavage at the chain terminus. There are other data in the literature to support this idea. For example, Baumann et al. (1973) examined five chymotrypsin substrates of the type Ac-M-Tyr-Gly-NH_2 where M was either Gly, Ala, Val, or Pro, or where M was absent altogether. The largest value for k_{cat} was obtained for the proline derivative, which was nearly an order of magnitude larger than that for the glycine derivative, which had the smallest k_{cat}. Thus, the incomplete results on small peptides are consistent with the notion that a proline residue on the carboxyl side of the active bond may slow down chymotrypsin cleavage while a proline residue on the amino side of the active bond may cause an acceleration in cleavage. A recent paper (Pace & Barrett, 1984) shows the similar effect of proline residues on trypsin activity. These effects are probably steric in nature and have little to do with the ability of proline peptide bonds to isomerize.

These speculations could have some relevance to the chymotrypsin cleavage of the Tyr_{115}–Val_{116} bond in RNase, since this bond has a proline residue on both its amino side (i.e., Pro_{114}) and its carboxyl side (i.e., Pro_{117}). At any rate, the Tyr_{115}–Val_{116} bond can be 100% cleaved by chymotrypsin in ca. 1 min, even for modified native and reversibly unfolded forms, which is sufficiently fast to permit ISP analysis of both proline-114 and proline-117.

The subsequent aminopeptidase P cleavage of the amino-terminal Val_{116}–Pro_{117} bond, produced by the chymotrypsin cleavage, shows only a fast phase with a relaxation time of ca. 20 s. This suggests *either* that the Val_{116}–Pro_{117} bond in intact RNase chains (i.e., oxidized, native, and reversibly unfolded RNases) is present almost exclusively (i.e., >97%) in the trans form *or* that isomerization is fast relative to the aminopeptidase P hydrolysis. We strongly prefer the first interpretation since a cis–trans relaxation time of 20 s or faster would be highly unusual for any proline peptide bond and particularly for proline-117, which has bulky valyl side chains as the two nearest neighbors. Thus, it seems likely that proline-117 does not make any measurable contribution to the slow refolding phases of RNase, because it exists almost exclusively as the trans isomer in both the native and unfolded forms.

On the other hand, the cleavage of the carboxy-terminal Pro_{114}–Tyr_{115} bond, produced by chymotrypsin cleavage at the Tyr_{115}–Val_{116} bond, by subsequent addition of the proline-specific endopeptidase does occur in two well-separated kinetic phases, which then allows an estimation of the isomeric state of the Asn_{113}–Pro_{114} bond in intact RNase chains. The results show 17% cis content for oxidized RNase and 58% for reduced RNase in 8.5 M urea, pH 12 (see Table II for a summary of the amount of cis isomer found for various forms of RNase by the ISP method). These can be compared to an earlier estimate of 12% cis for oxidized RNase fragment 105–124 (Stimson et al., 1982) obtained from NMR spectra of marginal signal/noise ratio. The ISP results also show essentially 100% cis form for native RNase, in agreement with the X-ray structure of RNase S (Wyckoff et al., 1970). For the reversibly unfolded form (5 M urea, pH 2.0), the ISP estimate is 92–95% cis for proline-114, which is unexpectedly high in view of the 17% cis found in oxidized RNase and 58% in reduced RNase. This implies a strong stabilization of the cis form of proline-114 by disulfide bonding (disulfide bond 110–58 is the only one which is close to proline-114 in the primary sequence) to an extent similar to that previously found for the stabilization of cis proline-93 (which has disulfide bonds 95–40 and 84–20 on either side) when disulfides are formed.

Table II: Cis Content for Three Prolines in RNase Determined by the ISP Method

	Pro_{93} (%)	Pro_{114} (%)	Pro_{117} (%)
oxidized RNase	35	17	0
reduced RNase		58	
reversibly unfolded RNase	70	95	0
native RNase	100	100	0

On the basis of the results found (cf. Table II) for both proline-93 and proline-114, it seems possible that other proline residues which show a strong propensity for the cis configuration in chains lacking disulfide bonds may also be found to exhibit an even stronger cis character in disulfide-linked chains. Although local interactions specific to particular sequences must also be important, there is a general statistical effect which should shift the equilibrium to the cis form when disulfide bonds are formed. The presence of a cis peptide bond in a completely random polypeptide chain will tend to partially fold the chain back on itself, relative to the trans form, and this will act to decrease the average hydrodynamic dimensions and correspondingly lower the configurational entropy of the entire chain. This effect will be much smaller in a disulfide-linked chain, since the very extended configurations are already disallowed because of the disulfide bonds and the presence of a cis peptide bond may introduce only minor additional restrictions on chain dynamics.

Although we are still unable to draw any conclusions about the stereospecificity of the chymotrypsin cleavage of the Tyr_{115}–Val_{116} bond relative to the cis–trans nature of the Val_{116}–Pro_{117} bond (since this bond is probably 100% trans), the absence of any slow phase in the chymotrypsin cleavage does imply that the Tyr_{115}–Val_{116} bond can be cleaved fast whether or not the Asn_{113}–Pro_{114} bond is cis or trans.

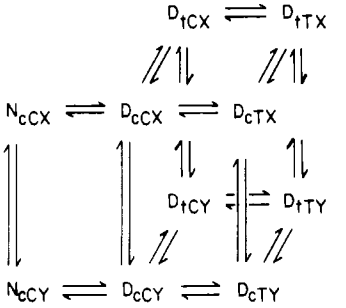
The ISP results then suggest that proline-114 is ca. 95% cis in equilibrium-unfolded RNase and 100% cis in native RNase and that it isomerizes very slowly, i.e., substantially slower than previously found for proline-93. It might be expected then that there would be a very slow, low-amplitude relaxation in RNase refolding kinetics which is rate limited by isomerization of proline-114. The spectroscopic ct phase, seen in tyrosine absorbance changes, was shown here to have the amplitude (4–5%), activation energy (17 kcal/mol), and relaxation time (ca. 400 s at 10 °C) which would be expected for the trans to cis isomerization of proline-114 during refolding, so it seems likely that this is the rate-limiting step in the ct phase. The fact that the ct phase is also seen when refolding is monitored by 2′CMP binding shows that the enzymatic activity of RNase is not recovered until and unless proline-114 is in the "correct" cis state.

It was suggested by Wyckoff [see Kim & Baldwin (1982)] that proline-114, being located near the outer surface of the molecule, is a "nonessential" proline residue and that RNase may refold into an active, nativelike structure whether or not proline-114 is in the correct isomeric state. Although nonessentiality seems to be a reasonable expectation for some proline residues in some proteins, as was first suggested in the original publication on proline isomerization (Brandts et al., 1975), it seems not to be the case for proline-114 nor is it the case for proline-93 (Lin & Brandts, 1983b) or, apparently, for proline-117 in RNase. No direct ISP results are available on the remaining proline in RNase, proline-42, which is known to be trans in the native state and located close to the active site.

The ISP results appear to be leading toward a rather surprising conclusion to the RNase refolding story. Three slow spectroscopic refolding phases have been found, and two of these (i.e., the 25–30% CT phase and the 5% ct phase) can be attributed to the isomerization of prolines-93 and -114, respectively. The largest of the slow refolding phases, i.e., the XY phase of 50% amplitude, is still unassigned. It apparently is not associated with prolines-93 or -114, or with proline-117. The XY phase must then be due either to the isomerization of proline-42 or to some process other than proline isomerization. Although the precise way in which proline-42 is involved in refolding will not be known for certain until ISP studies are completed, it seems unlikely a priori that it would be involved in a slow phase of 50% amplitude since proline-42 is trans in native RNase. Because of this, we must allow for the possibility that the XY phase may not involve isomerization of any of the four prolines in RNase.

Although indirectly related to the present results, we would like to comment on conclusions reached in previous work from this lab. In analyzing the very pronounced effect of ammonium sulfate, urea and guanidine hydrochloride (Gdn·HCl) on the relaxation time for the XY refolding phase, our interpretation emphasized the simplest model (i.e., fewest number of states) which would satisfactorily explain the kinetic results which existed at the time. Thus, the strong effect of additives on the XY kinetics was felt to be consistent with a simple $X \rightleftharpoons Y$ process occurring only in the unfolded form, which exhibited rate constants that were sensitive to the presence of these additives. However, a recent study by Schmid (1983) which utilized a new unfolding assay to follow spectroscopically invisible structural changes that occur during refolding shows rather clearly that slow structural changes continue to occur in refolded RNase after the point where nearly full activity has been regained from completion of the spectroscopic XY phase. This conclusion is also consistent with results by Schmid et al. (F. X. Schmid, M. H. Bunocore, and R. L. Baldwin, unpublished results) and earlier suggestions by Cook et al. (1979). It was suggested that these kinetic complexities are caused by formation of an active, nativelike intermediate which has one or more incorrect proline isomers. We feel the new evidence argues strongly in favor of the existence of one additional active form of RNase other than the true native form and therefore necessitates modification of the minimal mechanism of Lin & Brandts (1983c). It does seem possible to us, in view of the above discussion, that this additional active form might not have an incorrect proline isomer as proposed by Schmid. This point should be resolved when ISP results are available on proline-42.

Taking cognizance of these new results from Schmid's laboratory and our new results on proline-114 and the spectroscopic ct phase, the minimal mechanism which now seems consistent with all existing data is



The N_{cCX} state is the true native form, while the N_{cCY} state is the active, nativelike form implied by the data of Schmid (1983) which becomes populated during refolding into low urea or Gdn·HCl. Although prolines-114 and -93 are in the correct cis configuration (designated as c and C, respectively), the N_{cCY} state is nonnative since it has a Y or modified Y configuration rather than the native X configuration. The remaining six possible nativelike states omitted from the above mechanism (i.e., states N_{tCX}, N_{tTX}, N_{tTY}, N_{tCY}, N_{cTX}, and N_{cTY}), which have either an incorrect trans (t) isomer for proline-114 or an incorrect trans (T) isomer for proline-93, or both, have been shown specifically not to become populated during refolding by our ISP results, so the N_{cCY} state seems to be the only possibility among the states currently defined that might exist as a "fully active, nativelike intermediate". It is also clear from the results of Schmid (1983) that this additional active state is involved with the $X \rightleftharpoons Y$ refolding path, and not with the $C \rightleftharpoons T$ or $c \rightleftharpoons t$ paths, which further substantiates its assignment as N_{cCY}.

Although not anticipated before the ISP studies began, it has been found that different proline residues in unfolded RNase may have vastly different amounts of the cis form (see Table II), ranging all the way from ca. 0% for proline-117 to ca. 95% for proline-114. The conventional assumption, first suggested by Brandts et al. (1975) and later used by other investigators, that proline residues in unfolded proteins will be ca. 10–20% cis, seems incorrect and should be discarded since it may lead to unwarranted conclusions. For example, Krebs et al. (1983) have found that RNases from different species containing a different number of proline residues (ranging from four to six) have the same refolding kinetics in terms of the distribution of amplitude between slow and fast phases. This was interpreted as meaning that some prolines are nonessential for refolding; i.e., some prolines have nonnative configurations in unfolded RNase but can still rapidly refold in spite of this. An equally likely interpretation which might explain these results is that some prolines are virtually 100% in the trans configuration in both the native and unfolded forms and therefore have no effect on the slow phase amplitude.

Added in Proof

Since acceptance of the manuscript, we have become aware of new results from the laboratory of G. Fischer. This group has purified (Fischer et al., 1984) a new enzyme, peptidylprolyl *cis,trans*-isomerase, which catalyzes proline isomerization in peptides and proteins. They found (G. Fischer and H. Bang, unpublished results; G. Fischer, personal communication) that both the CT and ct refolding phases of RNase A are catalyzed by this enzyme. The XY phase is not catalyzed even during refolding in solutions containing moderate amounts of urea, where the XY phase becomes slower than the CT phase. They propose that their results do not support the involvement of proline isomerization in the XY phase but do support its involvement in the CT and ct phases. This is in agreement with

our above interpretations based solely on ISP results. In other studies from the same laboratory (Fischer et al., 1985), results on peptide nitroanilide substrates suggest that chymotrypsin is unable to cleave the cis form of substrates containing the sequence -Ala-Pro-Phe-NHNp, in contrast to our finding that chymotrypsin rapidly cleaves 100% of the active bond in the RNase sequence -Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}-. We do not know whether this is due to a difference in specificity toward amide and peptide substrates or to some other factor.

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Registry No. RNase, 9001-99-4; 2′-CMP, 85-94-9; L-proline, 147-85-3.

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