Determination of Cis–Trans Proline Isomerization by Trypsin Proteolysis. Application to a Model Pentapeptide and to Oxidized Ribonuclease A†

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ABSTRACT: It is shown, by examination of a model pentapeptide, that trypsin will only cleave substrate bonds in a polypeptide chain when the peptide bond following the active bond is in the trans isomeric state. The cis form must isomerize to trans before it can be cleaved. Taking advantage of this isomeric specificity, the sequence -Lys_{91}-Tyr_{92}-Pro_{93}- is examined in oxidized RNase A. It is shown that the Tyr–Pro bond exists 33% in the cis form at equilibrium and that the cis-to-trans relaxation time for isomerization is 5.0 min at 10 °C. The fragment 92–98 has about the same cis content (35%) as does oxidized RNase A but has a much slower relaxation time (11 min). This suggests that overall chain dynamics may exert some effect on the kinetics of isomerization.

Proline isomerization is unique among the structural changes which are accessible to the typical protein, because of the double bond character of the peptide linkage. All other known conformational changes occur by rotation about single bonds, which are much faster. A concerted structural change involving many thousands of internal rotations can then be rate limited by a single isomerization process, so the importance of proline peptide bonds may far exceed their number. This has been recognized for protein denaturation reactions, where it now seems likely that proline isomerization can control the overall rate of unfolding and refolding (Brandts et al., 1975; Lin & Brandts, 1978; Schmid & Baldwin, 1978). There are also suggestions that more subtle conformational changes, occurring between two nativelike forms, can be rate limited by peptide isomerization (Brown et al., 1977; Marsh et al., 1979).

Up to the present time, only indirect evidence has implicated proline isomerization in structural processes of proteins. Although NMR is an excellent way of observing isomerization in small peptides, it has not been able to provide useful data on proteins. Our lab has been working for several years on the development of isomer-specific proteolysis (ISP) as a technique for recognition of proline isomerization. Previous studies (Lin & Brandts, 1979a,b, 1980) have demonstrated that proline-specific exopeptidases, such as prolidase and aminopeptidase P (APP), exhibit absolute specificity toward the trans form of an amino-terminal X–Pro bond (where X is any amino acid residue), while the cis form must first isomerize to the trans form before it can be cleaved. Although these exopeptidases have proven to be very useful in elucidating the structure of poly(L-proline) and of short peptides with X–Pro at the amino terminus, they cannot be used by themselves to study prolyl residues which are situated internally in a long polypeptide chain. Unfortunately, there are no endopeptidases known which will cleave a peptide bond on the N-terminal side of prolyl residues, so one is forced to use a more complicated protocol for looking at internal prolyl residues.

In a continuing effort to utilize the ISP method for probing the structure of proline-containing proteins, we recently attempted (Lin & Brandts, 1983) to employ trypsin in tandem with aminopeptidase P, to study the isomerization of the Tyr–Pro bond in the sequence -Lys_{91}-Tyr_{92}-Pro_{93}- of bovine RNase A. Much to our surprise, we found that trypsin itself exhibited isomeric specificity toward the following Tyr–Pro bond, i.e., trypsin can cleave the Lys–Tyr bond of RNase A only if the following Tyr–Pro bond is in the trans form. This was unexpected since we had earlier shown that leucine aminopeptidase can cleave the Leu–Phe bond in the tripeptide Leu-Phe-Pro, irrespective of whether the Phe–Pro bond is cis or trans (Lin & Brandts, 1979b). It seems possible that the difference between trypsin and leucine aminopeptidase, in terms of their specificity toward the following bond, may reflect a basic difference between the number of residues which normally "fit" into the active site of endopeptidases and that of aminopeptidases.

This paper presents results which confirm the above suggestions. The activity of trypsin toward a model pentapeptide, glycylglycyl-L-lysyl-L-phenylalanyl-L-proline, has been carefully examined to determine the isomeric specificity. It is known from NMR studies (Grathwohl & Wüthrich, 1976) of similar peptides which contain the Phe-Pro sequence that they exist in solution as a mixture of nearly equimolar amounts of the cis and the trans form. If trypsin does have isomeric specificity toward the following bond, then two kinetic phases should be seen during the time course of hydrolysis at high trypsin activity. First, there will be a fast phase corresponding to the direct attack on the Lys–Phe bond for those pentapeptide molecules having the Phe–Pro bond in the trans form. Second, there will be a slow phase corresponding to the cis-to-trans isomerization, and subsequent hydrolysis, for those pentapeptide molecules which initially have the Phe–Pro bond in the cis form. It will be shown that these two phases do exist and that the slow phase has all of the characteristics expected for isomerization.

For confirmation of trypsin's isomeric specificity, results from a study of the isomerization of the Pro_{93} residue are presented, in which both oxidized RNase and a fragment of RNase containing residues 92–98 are examined. This not only gives further evidence on the specificity of trypsin but also demonstrates the effect of the length of the polypeptide chain on the kinetics and thermodynamics of proline isomerization.

Materials and Methods

Materials. Trypsin (from porcine pancreas), soybean trypsin inhibitor, prolidase, and oxidized RNase A were purchased from Sigma Chemical Co. The oxidized RNase A (catalog no. R5625, lot no. 114C-8380) was subjected to

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elemental analysis and found to contain no detectable chlorine within the sensitivity of the analysis (±0.03%), indicating the absence of chlorotyrosine. Aminopeptidase P (APP) was purified from *Escherichia coli* B as described previously (Yaron & Berger, 1970; Lin & Brandts, 1979b). All of the chemicals used for hydrolysis studies were reagent grade. *o*-Phthalaldehyde, which was used for precolumn fluorescence derivatization of amino acids and peptides, was purchased from Pierce Chemical Co. Methanol (HPLC grade) was obtained from MCB, Inc. Phosphate buffer was passed through a fine fritted disk filter before use as an HPLC gradient component. Also, *N*-(carbobenzyloxy)glycylglycine, N^{ϵ}-(carbobenzyloxy)-L-lysine methyl ester hydrochloride, *N*-(carbobenzyloxy)-L-phenylalanine, and L-proline benzyl ester hydrochloride, which were used to synthesize the pentapeptide, were obtained from Sigma Chemical Co.

Synthesis of Glycylglycyl-L-lysyl-L-phenylalanyl-L-proline. The classic solution method was employed to synthesize the pentapeptide (Greenstein & Winitz, 1961; Yamamoto & Izumiya, 1967). This involved the following steps: (1) The compound *N*-(carbobenzyloxy)glycylglycine (in tetrahydrofuran) was coupled in the cold with N^{ϵ}-(carbobenzyloxy)-L-lysine methyl ester hydrochloride (in chloroform) in the presence of isobutyl chloroformate and triethylamine to form *N*-(carbobenzyloxy)glycylglycyl-L-N^{ϵ}-(carbobenzyloxy)lysine methyl ester. (2) *N*-(Carbobenzyloxy)-L-phenylalanine (in chloroform) was also coupled in the cold with L-proline benzyl ester hydrochloride (in chloroform) in the presence of isobutyl chloroformate and triethylamine. The resulting *N*-(carbobenzyloxy)-L-phenylalanyl-L-proline benzyl ester was treated with hydrobromic acid in glacial acetic acid solution to remove the N-protecting group. (3) The product of step 1, *N*-(carbobenzyloxy)glycylglycyl-L-N^{ϵ}-(carbobenzyloxy)lysine methyl ester, was then coupled with L-phenylalanyl-L-proline benzyl ester, obtained from step 2, via the azide method. (4) The resulting *N*-(carbobenzyloxy)glycylglycyl-L-N^{ϵ}-(carbobenzyloxy)lysyl-L-phenylalanylproline benzyl ester was hydrogenated in the presence of palladium black in HCl–acetic acid solution to remove all the protecting groups.

The overall yield of the preparation was found to be about 20%. Amino acid analysis showed that the molar ratios of glycine, lysine, phenylalanine, and proline in the pentapeptide are 1.99/0.98/0.96/1.04, respectively. An R_f value of 0.21 was obtained for the pentapeptide from TLC analysis (1-butanol/acetic acid/water, 4/1/1 by volume), compared to 0.39 for glycine and 0.70 for phenylalanine under the same experimental conditions. The melting point was estimated to be 180 °C (decomposition). Anal. Calcd for $C_{24}H_{36}N_6O_6 \cdot HCl$: C, 53.28; H, 6.89; N, 15.53; Cl, 6.55. Found: C, 53.46; H, 6.67; N, 15.50; Cl, 6.88. The conservation of optical asymmetry of lysine, phenylalanine, and proline residues during the synthesis was confirmed by the fact that phenylalanine and proline could be quantitatively released (>95%) from the pentapeptide when hydrolyzed by the combined activities of trypsin and prolidase.

Assay for the Rate of Trypsin Hydrolysis of Glycylglycyl-L-lysyl-L-phenylalanyl-L-proline. When the pentapeptide is subjected to trypsin hydrolysis, only the Lys–Phe bond is cleaved. For a convenient assay of trypsin activity, however, the resulting Phe–Pro fragment can be further hydrolyzed by prolidase to release free proline, which can be easily quantitated by the acid–ninhydrin method (Lin & Brandts, 1979a). The intact pentapeptide is completely resistant to prolidase activity. The experimental procedures are as follows: For initiation of the hydrolysis, 300 μL of ~0.02 M pentapeptide (in 0.05 M Tris buffer, pH 7.4), incubated at a controlled temperature, was quickly pipetted into 300 μL of trypsin solution (30 mg/mL, in 0.16 M Tris buffer, pH 8.5), thermostated at the same temperature, and mixed thoroughly with a stirring bar. At suitable time intervals, 50-μL aliquots of incubation solution were quickly pipetted into a test tube containing 250 μL of soybean trypsin inhibitor (5 mg/mL, in 0.1 M Tris buffer, pH 8.1) and mixed thoroughly with a vibrator to stop trypsin activity. Then, 20 units of prolidase was added to each tube, and the tubes were incubated at 35 °C for 60 min to release all proline from the Phe–Pro fragment. The acid–ninhydrin colorimetric method was then used to determine the amount of free proline in each tube (Lin & Brandts, 1979a, 1979b). A tube containing the pentapeptide subjected to only prolidase activity, in the presence of the same amount of trypsin and trypsin inhibitor, was assayed in the identical way as a control. The same hydrolysis study was also carried out for pentapeptide which was preequilibrated at pH 2.0, rather than at pH 7.4, before adding trypsin.

Assay for the Rate of Hydrolysis of Tyr_{92}–Pro_{93} Bond of Oxidized Ribonuclease A. Two experiments designed to detect the isomeric specificity of trypsin, as well as to study the effect of the length of the polypeptide chain on the kinetics and thermodynamics of proline isomerization, were carried out at 10 °C under high trypsin activity. In the first experiment, oxidized ribonuclease A was first subjected to a 30-s pulse of trypsin activity, and the resulting fragment 92–98 was further hydrolyzed by high APP activity to release Tyr_{92}, either immediately after the trypsin pulse or after waiting for 60 min following completion of the trypsin pulse. In the second experiment, the release of fragment 92–98 from oxidized ribonuclease A was studied as a function of the time exposed to high trypsin activity. Fragment 92–98 was then assayed by cleavage with APP to release Tyr_{92}. HPLC chromatography, coupled with precolumn fluorescence derivatization of amino acids, was used to analyze the resulting free tyrosine. The details are as follows.

(*1*) *First Experiment.* So that the reaction could be initiated, 100 μL of oxidized ribonuclease A (16 mg/mL, in 0.05 M veronal buffer, pH 8.6) at 10 °C was mixed with 100 μL of trypsin solution (35 mg/mL, in 0.05 M veronal buffer, pH 8.6) and incubated at 10 °C for 30 s, and then the trypsin was inactivated by adding 100 μL of soybean trypsin inhibitor (50 mg/mL, in 0.05 M veronal buffer, pH 8.6, at 10 °C). Either immediately or after waiting for 60 min after the trypsin activity was stopped, 600 μL of aminopeptidase P (~70 units, in 0.05 M veronal buffer with Mn^{2+}–citrate reagent, pH 8.6) at 10 °C was pipetted into the incubation solution. At suitable time intervals, 100-μL aliquots of reaction mixture were quickly pipetted into a centrifuge tube containing 1 mL of absolute ethanol, and the contents were mixed with a vibrator to stop APP activity and to precipitate out proteins and large peptide fragments. The resulting precipitate was centrifuged at 7500 rpm for 30 min. The supernatants were quantitatively transferred to test tubes and heated in the water bath at 85 °C for 3 min to inactivate any residual enzyme activity. The solution was dried by nitrogen. Then, the residues were dissolved by adding 250 μL of 0.4 M boric acid buffer, pH 9.5, and were centrifuged again, if necessary. The samples were then ready for HPLC analysis. A control sample, incubated with trypsin for 60 min instead of 30 s, was treated in an identical way, and the amount of tyrosine released from this sample was found to be 1.0 mol of tyrosine/mol of oxidized RNase. The oxidized RNase concentration was determined by absorbance measurement at 275 nm, using an extinction

coefficient of 9390,[1] while the tyrosine concentration was determined from HPLC analysis using a standard tyrosine solution for comparison. Another control solution of oxidized RNase, which was treated identically except the APP pulse was omitted, was run, and the small area obtained in the tyrosine peak position was subsequently subtracted from that obtained for samples.

In order to check for mass conservation, we carried out a control run in which a standard tyrosine solution was subjected to the same procedures as used for oxidized RNase. The recovery of tyrosine in the final solution was found to be more than 95%, as determined from HPLC analysis.

(*2*) *Second Experiment.* At zero time, 100 μL of oxidized ribonuclease A at 10 °C was mixed with 150 μL of trypsin solution at 10 °C. At suitable time intervals, 20-μL aliquots of this incubation mixture were quickly pipetted into a centrifuge tube containing 80 μL of soybean trypsin inhibitor (14 mg/mL, in 0.05 M veronal buffer, pH 8.6), and mixed with a vibrator. Then 50 μL of APP solution (2 units, in 0.05 M veronal buffer with Mn^{2+}–citrate reagent, pH 8.6) was added into each tube and incubated at 35 °C for 60 min to release all Tyr_{92} from the fragment 92–98. Then 1 mL of absolute ethanol was added. The rest of the procedures are the same as in the first experiment. A sample incubated with trypsin for 60 min showed a release of 1.0 mol of tyrosine/mol of RNase, and this was the 100% reference used to quantitate the percent of tyrosine released in the hydrolysis samples.

Assay for the Free Tyr_{92} Using HPLC. A Varian 5020 gradient liquid chromatograph with a Fluorichrom fluorescence detector and a reverse-phase Varian Micropak MCH 10 column (10 μm, 4 mm × 30 cm) was used. A 100-μL sample loop was used with a Valco injector. The gradient run starts at 40% methanol–60% 0.1 M sodium phosphate buffer (pH 6.8) and increases to 70% methanol in 35 min. After the methanol content is kept at 70% for 5 min, the initial conditions are restored by running a reverse methanol gradient at 2%/min. The flow rate was 0.5 mL/min. The sample turnover time was 60 min.

Before a sample was injected, 30 μL of the sample solution was reacted with 150 μL of *o*-phthalaldehyde reagent [prepared according to Lindroth & Mopper (1979)] for exactly 2 min. The tyrosine peak, eluted at 17.5 min, can be identified and quantitated by running a tyrosine standard. The degree of hydrolysis at each time interval can then be calculated from the peak area.

Results

Glycylglycyl-L-lysyl-L-phenylalanyl-L-proline. As in previous work (Lin & Brandts, 1979a,b, 1980), the hydrolysis of pentapeptide catalyzed by trypsin was carried out at a high ratio of trypsin activity to substrate concentration to ensure that the rate of hydrolysis of the Lys–Phe bond is fast if the structure of the peptide is "correct". The time dependence of hydrolysis for peptide preequilibrated at pH 7.4 and at 25 °C is shown in Figure 1A for two different trypsin activities. In spite of the high trypsin activity, two well-separated kinetic phases are seen. While the rate of hydrolysis for the fast phase is strongly dependent on trypsin activity, the semilog plot of substrate concentration vs. time (Figure 1B) indicates that the rate and amplitude for the slow phase are virtually independent of enzyme activity, suggesting that this phase is rate limited by a structural equilibrium. The relaxation time and amplitude can be estimated to be about 18 min and 54%, respectively.

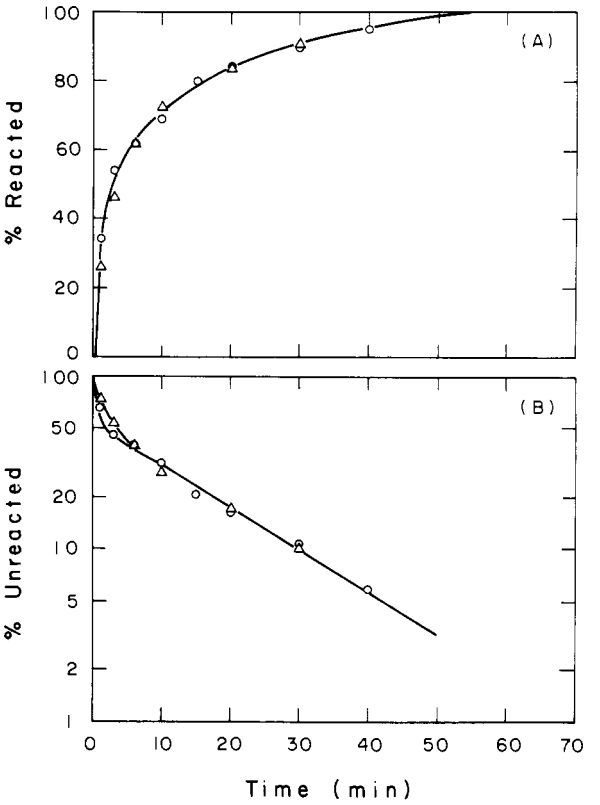


FIGURE 1: Rate of glycylglycyl-L-lysyl-L-phenylalanyl-L-proline hydrolysis catalyzed by trypsin at 25 °C: (A) the degree of hydrolysis vs. time; (B) semilog plot of substrate concentration vs. time. The ratio of trypsin activity to substrate concentration was 20000 (○) and 10000 BAEE units/μmol (△), respectively. The concentration of pentapeptide was 10 mM in 0.6 mL of 0.08 M Tris solution at pH 8.2.

The rates of hydrolysis were also measured at 32 °C for the pentapeptide preequilibrated as the zwitterionic form (at pH 7.4) and as the cationic form (at pH 2.0), but at the same enzyme activity to substrate concentration (20000 BAEE units/μmol) ratio. The results are plotted in Figure 2. They show that the relaxation times for the slow phase for both sets of preequilibrium conditions are identical within the experimental errors (7.3 ± 0.2 min), but that the amplitude depends significantly on the ionic state. The slow phase amplitude is 59% for the zwitterionic form and 45% for the cationic form. These kinetic patterns of hydrolysis are very similar to those reported earlier for L-Phe-L-Pro, Gly-L-Pro, Leu-Phe-Pro, and Gly-L-Pro-L-Ala when hydrolyzed by APP or prolidase (Lin & Brandts, 1979b). The slow phase observed in those studies has been attributed to the existence of a cis configuration at the X–Pro bond which is being hydrolyzed. Since the cis form of the active Lys–Phe bond of the pentapeptide in solution is probably present in amounts less than 1% (Brandts et al., 1975), the large slow phase in the hydrolysis of the pentapeptide can only be due to the isomerization of the following Phe–Pro bond, which is very likely to exist as a nearly 50:50 mixture of cis and trans forms as has been shown for similar peptides.

[1] The extinction coefficient used here for oxidized RNase corresponds with the value determined experimentally for both reduced RNase and reduced carboxymethylated RNase, by dry weight analysis (White, 1961). There is a difficulty in determining directly the extinction coefficient of performic acid oxidized RNase, due to the fact that formic acid readily attaches to some lysyl and arginyl groups, leading to an unknown change in molecular weight estimated to be ca. 5% (Hirs et al., 1956). If this factor is neglected, then the extinction coefficient obtained by dry weight analysis will be too low by a similar amount. Therefore, the values measured directly for the reduced protein in water are the more reliable, since its molecular weight is well-defined.

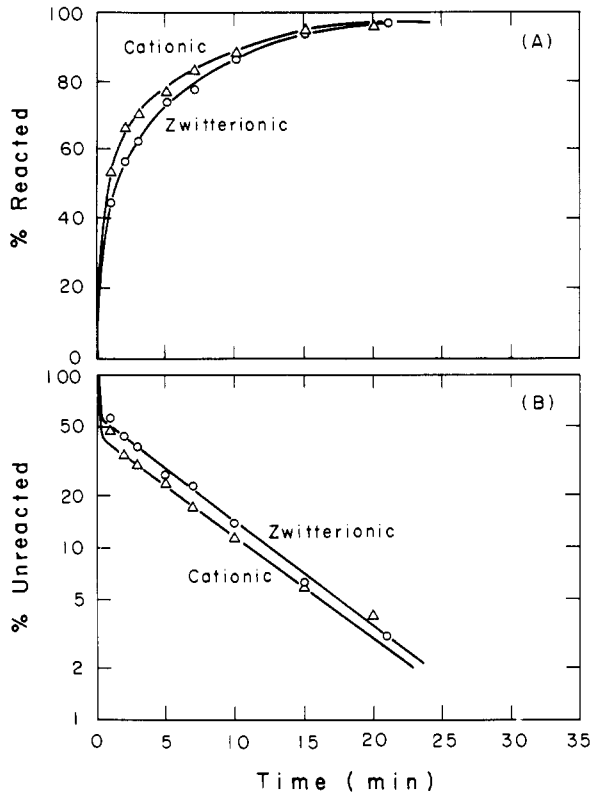


FIGURE 2: The rate of glycylglycyl-L-lysyl-L-phenylalanyl-L-proline hydrolysis catalyzed by trypsin at 32 °C: (A) the degree of hydrolysis vs. time; (B) semilog plot of substrate concentration vs. time. The ratio of trypsin activity to substrate concentration was 20 000 BAEE units/μmol. The concentration of pentapeptide was the same as that of Figure 1. Triangles and circles represent the cationic (pH 2.0) and zwitterionic (pH 7.4) forms of substrate, respectively, which were present when hydrolysis was initiated.

Table I: Relaxation Time and Amplitude of Slow Phase Seen in Hydrolysis of Glycylglycyl-L-lysyl-L-phenylalanyl-L-proline by Trypsin

temp (°C)	initial pH at 7.4			initial pH at 2.0		
	α (%)	τ (min)	k (min^{-1})	α (%)	τ (min)	k (min^{-1})
17.3	49	33.0	0.0303			
25	54	17.7	0.0565			
32	58	7.2	0.139	45	7.5	0.133
40	67	4.4	0.227			

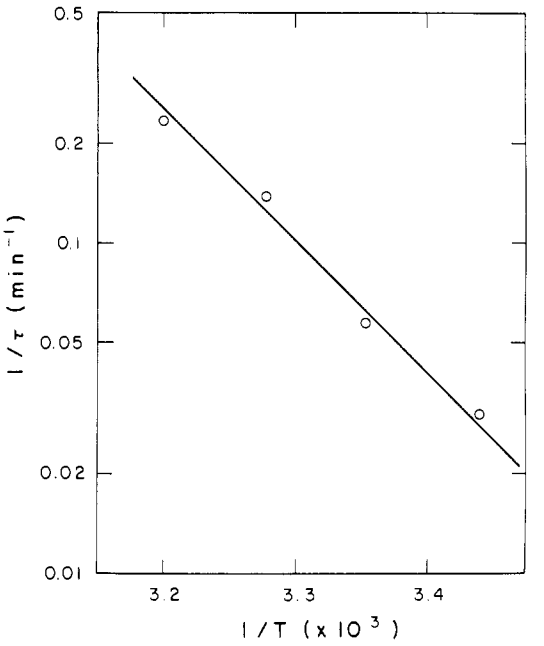


FIGURE 3: Arrhenius plot for the slow phase of hydrolysis of the zwitterionic form of the pentapeptide, catalyzed by trypsin. The experimental conditions were the same as those of Figures 1 and 2.

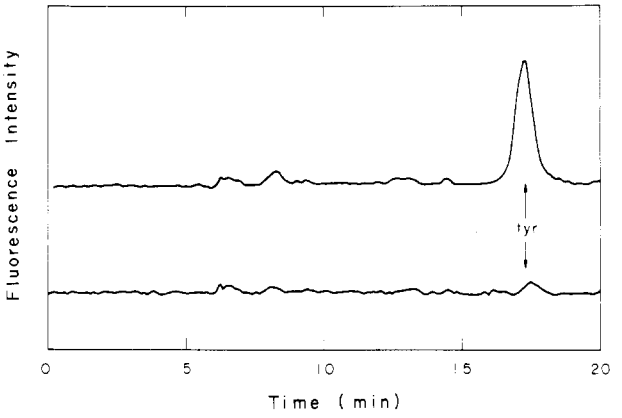


FIGURE 4: Chromatography of free tyrosine released from oxidized RNase by trypsin and aminopeptidase P activities in tandem. The lower profile is a control run; oxidized RNase was subjected to trypsin activity only. The upper profile is a sample run; oxidized RNase was first subjected to trypsin activity for 1 min and then subjected to long-time incubation with aminopeptidase P.

The kinetic patterns of hydrolysis at other temperatures are very similar to those shown in Figures 1 and 2. The relaxation times, reciprocal relaxation times, and amplitudes of the slow phase at various temperatures are summarized in Table I. These data indicate that the amplitudes of hydrolysis are slightly dependent on temperature as well as on the state of protonation. The relaxation times depend strongly on temperature, and an activation energy of 18 kcal/mol was obtained for the slow phase of hydrolysis, from an Arrhenius plot of reciprocal relaxation time (Figure 3).

Oxidized RNase A. We are interested here in examining the state of isomerization of a specific Tyr–Pro peptide bond in RNase, which occurs in the sequence -Lys_{91}-Tyr_{92}-Pro_{93}-. When oxidized RNase is subjected to trypsin activity, a total of 13 peptide fragments are ultimately released (Hirs et al., 1960). However, only one of these degradation products, fragment 92–98, has a prolyl residue located immediately adjacent to an amino-terminal residue. Therefore, fragment 92–98 is the only molecule in the entire trypsin hydrolysate which should be a substrate for APP, since it cleaves only amino terminal X–Pro bonds. The hydrolysis of fragment 92–98 by APP releases free tyrosine which can be readily detected by HPLC. This is seen in Figure 4, where the elution pattern on a C-18 column is shown for oxidized RNase treated first with trypsin (followed by STI addition to inactivate the trypsin) and then with APP. The control was treated identically except that no APP was added. The resolution of the column is excellent under the conditions used, since a commercial preparation containing 16 different amino acids leads to a chromatogram having 15 completely separated peaks. The profiles in Figure 4 show that the major difference between the sample and control, in the region where free amino acids migrate, is the large peak occurring at the position expected for free tyrosine, shown by the arrows. The small peak in the control, which occurs close to the tyrosine position, is due to a contaminant in the trypsin. Its concentration varies considerably for different lots of trypsin, and it is in all likelihood an autolysis product. If oxidized RNase is subjected to long-term trypsin treatment followed by long-term APP treatment, then this results in the release of 1.0 ± 0.05 mol

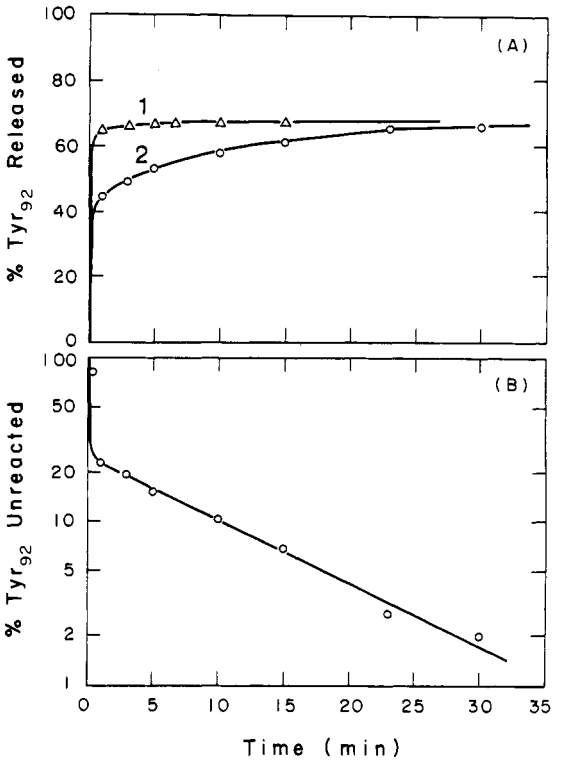
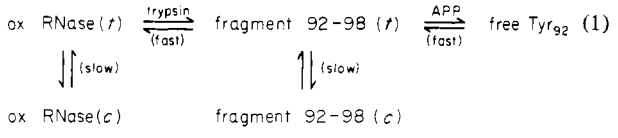


FIGURE 5: Rate of hydrolysis of fragment 92–98 by aminopeptidase P at 10 °C. Oxidized RNase was first subjected to a trypsin pulse for 30 s and then the trypsin activity was inactivated by STI. Triangles represent the hydrolysis data when APP was added immediately after stopping trypsin activity. Circles represent the hydrolysis data when APP was added after waiting for 60 min after stopping trypsin activity. The ratio of trypsin activity to oxidized RNase concentration is 5×10^5 BAEE units/μmol. The ratio of APP activity to oxidized RNase is 6×10^2 units/μmol. The amount of tyrosine released from a sample incubated with trypsin for 60 min was used for the 100% reference value in order to quantitate the hydrolysis data.

of tyrosine/mol of RNase. There seems little doubt then that, in spite of the enormous complexity of this hydrolysate which contains degraded RNase, trypsin, STI, and APP, it is still possible to quantitatively follow the specific release of Tyr_{92} which results from the cleavage of two peptide bonds.

By examining the time dependence of the release of tyrosine-92, it is possible to confirm the involvement of cis–trans isomerism in the cleavage process. Assume for the moment that trypsin will only cleave the Lys_{91}–Tyr_{92} peptide bond in RNase if the Tyr_{92}–Pro_{93} peptide bond is trans, in agreement with our results on the model pentapeptide. Since all hydrolyses are carried out at very high activities of trypsin and APP, the pertinent kinetic scheme will be



where the *t* and *c* designations refer to the trans or cis nature of the peptide bond between Tyr_{92} and Pro_{93}. The peptide isomerizations, occurring vertically, are then considered to be slow while the hydrolyses, occurring horizontally, by both trypsin and APP are assumed to be fast and completely specific to the trans form of the prolyl peptide bond. All of our results on oxidized RNase can be understood in terms of this scheme.

Curve 1 in Figure 5A shows results obtained from the following two-step treatment of oxidized RNase: first, a 30-s pulse of trypsin activity (i.e., add trypsin, wait 30 s, add STI), followed *immediately* by a pulse of APP activity whose duration is indicated on the abscissa. Under these conditions, there is scarcely any slow phase seen in the release of Tyr_{92} by APP. The total amount of tyrosine that is released only accounts for 67% of the total amount of Tyr_{92}. No more tyrosine is released if the trypsin concentration during the 30-s pulse is increased substantially (results not shown). This shows that the 33% of Tyr_{92} which is not released must be in a structural form different from the 67% which is released. Referring to eq 1, we then conclude that oxidized RNase exists 67% in the trans form and 33% in the cis form at equilibrium. The 30-s pulse of trypsin cleaves only the trans form, since the cis–trans equilibrium is too slow to readjust appreciably during the short trypsin pulse. It is understandable then that subsequent immediate treatment with APP would show no slow phase in the release of Tyr_{92} since all of the newly formed fragment 92–98 would be in the correct (i.e., trans) isomeric state for rapid APP hydrolysis because of the trypsin specificity.

The same experiment described for curve 1 of Figure 5A has been repeated to obtain curve 2, with the single exception that a long-time incubation (60 min) was allowed between the end of the trypsin pulse and the start of the APP pulse. In this case, there was still only 67% release of Tyr_{92} but, as seen in Figure 5A, there is both a fast phase and a slow phase for APP hydrolysis. These account for 63% and 37% of the total amplitude, respectively. Thus, it appears that the 60-min wait between pulses allows fragment 92–98 to change from its nearly 100% trans state immediately after trypsin cleavage to its equilibrium mixture of 63% trans and 37% cis which then causes the two-phase characteristics seen in the APP hydrolysis. The semilogarithmic plot of these data in Figure 5B leads to a relaxation time of 11.0 min, which then corresponds, according to the scheme of eq 1, to the reciprocal of the rate constant for the one-way cis-to-trans isomerization of proline-93 in fragment 92–98.

Since the above kinetic results reflect only properties of the second APP hydrolysis, they will give information only on the isomerization in fragment 92–98 and tell us nothing about relaxation times for isomerization of proline-93 in oxidized RNase itself. This information can be obtained from a different experiment shown in Figure 6, which treats the trypsin pulse time as the variable and the APP pulse time as the constant. This consists of the following two steps: first, a trypsin pulse of variable duration as indicated on the abscissa of Figure 6 and, second, a very long (60-min) pulse of APP activity that is sufficient to hydrolyze all of the fragment 92–98 that is released by trypsin cleavage. As expected in terms of the schemes of eq 1, both a fast and slow phase are seen in the trypsin hydrolysis kinetics in Figure 6A. A fast phase of 65% amplitude is seen, confirming our previous interpretation from the independent data of Figure 5, as is a slow phase of 35% amplitude. The combined release from both phases corresponds to 1.0 mol of Tyr_{92}/mol of oxidized RNase. The semilogarithmic plot of these data in Figure 6B leads to a relaxation time of 4.8 min for the slow phase. According to eq 1, this corresponds to the reciprocal rate constant for the one-way, cis-to-trans conversion of proline-93 in oxidized RNase. Thus, the isomerization of proline-93 occurs more than twice as fast in oxidized RNase as in fragment 92–98, even though they contain nearly equivalent cis content at equilibrium.

Discussion

The results of this study show conclusively that trypsin can only hydrolyze a Lys–X bond in substrates which contain a -Lys-X-Pro sequence when the following X–Pro bond is in the

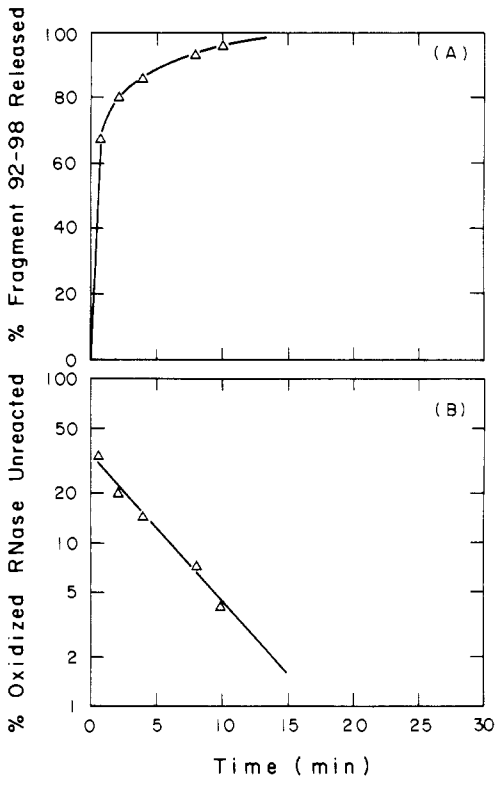


FIGURE 6: Rate of hydrolysis of oxidized RNase catalyzed by trypsin activity at 10 °C. Oxidized RNase was subject to trypsin activity for the times indicated. The released fragment 92–98 was assayed by APP. The ratio of trypsin activity to oxidized RNase concentration is the same as that of Figure 5. The ratio of APP activity to oxidized RNase is 30 units/μmol. The amount of tyrosine released from a sample incubated with trypsin for 60 min was used for the 100% reference value in order to quantitate the hydrolysis data.

trans form. The cis form is not a substrate and must isomerize to the trans form before being cleaved. This is in contrast to the situation for at least one other protease (Lin & Brandts, 1979b), since leucine aminopeptidase has been shown to cleave substrates rapidly no matter whether the following peptide bond is cis or trans. Although the evidence is not extensive enough to be certain, it is possible that this may reflect a basic difference between the active sites of exopeptidases and endopeptidases. It is known that aminopeptidases generally will cleave dipeptides just as rapidly as they will cleave corresponding substrates with longer chains, showing that the activity is unaffected by the presence or absence of a peptide bond following the active bond. This is not normally the case for endopeptidases. For example, trypsin can cleave Gly-Gly-Lys-Gly-Gly about 100 times faster than Gly-Gly-Lys-Gly (Yamamoto & Izumiya, 1967), indicating that the bond following the active bond may be very important in the enzyme–substrate complex. Certainly, the results we have obtained on the model pentapeptide and on oxidized RNase show definitely that the peptide bond following the active bond plays a very critical role in the mechanism of action of trypsin.

The kinetic and thermodynamic characteristics of the cis–trans isomerization of the model pentapeptide, determined here by the ISP method, are quite similar to those of other related peptides. The zwitterionic form of the pentapeptide has a somewhat lower cis content (58% at 32 °C) than does the corresponding dipeptide Phe-Pro (75%), but comparable to that of Leu-Phe-Pro (51%) (Lin & Brandts, 1979b). Both the pentapeptide and Phe-Pro show a reduction in cis content in going from the zwitterionic to the anionic form, although the change is less pronounced for the pentapeptide. Temperature was found to have a significant effect on the equilibrium for the pentapeptide, since the cis content increases from 49% at 17 °C to 67% at 40 °C, leading to a calculated enthalpy change of 2.0 kcal/mol. This is almost identical with the value of 1.8 kcal/mol reported for the dipeptide Pro-Hyp from NMR studies (Roques et al., 1977) but in contrast to results on Phe-Pro, where no temperature dependence was detected, and on Gly-Pro, where a value of −2.0 kcal/mol was found (Cheng & Bovey, 1977).

The activation energy of 18 kcal/mol found for the slow phase in the hydrolysis of the pentapeptide is very close to that expected if the hydrolysis is rate limited by proline isomerization (Brandts et al., 1975). The one-way, cis-to-trans relaxation time (16 min at 32 °C) is somewhat larger than for the dipeptide Phe-Pro (7.2 min), but the calculated two-way relaxation times for the two are identical (4.1 min) within errors. These relaxation times are much slower than for isomerization of the similar bond in oxidized RNase and fragment 92–98, as is normally found for peptides in which the prolyl residue is in the C-terminal position (Evans & Rabenstein, 1974; Lin & Brandts, 1979b).

While the cis contents for the Tyr_{92}–Pro_{93} bond in oxidized RNase and fragment 92–98 are comparable (33% vs. 35%), the isomerization occurs twice as fast in the unsevered chain (relaxation time of 5 min at 10 °C vs. 11 min in the fragment), suggesting that long-range chain dynamics could be important. This viewpoint will be reinforced by results presented in a later paper, where it will be shown that the thermodynamics and kinetics for isomerization of Pro_{93} in RNase with disulfide bonds intact are considerably different than for oxidized RNase (Lin & Brandts, 1983).

These studies show that a non-proline-specific endopeptidase, such as trypsin, can be used in tandem with a proline-specific aminopeptidase, such as APP, to determine both the thermodynamic and kinetic aspects of isomerization of prolyl residues located internally in long randomly coiled polypeptide chains such as oxidized RNase. It will be shown in a later paper (Lin & Brandts, 1983) that this methodology can also be applied successfully to native RNase if one important modification is made. This involves treatment of the native protein with pepsin as the first step in the protease sequence followed in succession by trypsin treatment and APP treatment. The pepsin treatment is necessary since native RNase, in contrast to oxidized RNase, is not an effective substrate for trypsin and APP.

Registry No. Proline, 147-85-3; trypsin, 9002-07-7; RNase A, 9001-99-4; glycylglycyl-L-lysyl-L-phenylalanyl-L-proline, 83998-90-7.

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Isomerization of Proline-93 during the Unfolding and Refolding of Ribonuclease A†

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ABSTRACT: Using the method of isomer-specific proteolysis, the isomerization of proline-93 has been monitored directly during the time course of the unfolding and refolding reactions of RNase A. It has been found that proline-93 is 100% cis in the native protein and 70% cis in the reversibly unfolded protein. During the unfolding reaction, the change from 100% to 70% cis occurs as a first-order process with a relaxation time of 140 s in 8.5 M urea, 10 °C. For refolding, the change from 70% to 100% cis also occurs as a first-order process, with a relaxation time (10 °C) of 90 s in 0.3 M urea, 130 s in 1.0 M urea, and 310 s in 2.0 M urea. Parallel experiments which measured the recovery of enzyme activity during refolding were also conducted. These show that 30% of the activity recovers in a slow phase with a first-order relaxation time (10 °C) of 100 s in 0.3 M urea. Because of the excellent agreement of both the amplitude and relaxation time for trans-to-cis isomerization and for activity recovery, it is concluded that the slowest phase in the recovery of enzyme activity is rate limited by the isomerization of proline-93. These results demonstrate that proline-93 must be cis before refolding to the active form can take place, in contrast to previous suggestions, and argue against the existence of a nativelike intermediate form on the refolding pathway which contains proline-93 in the incorrect trans configuration.

It was first proposed in 1975 (Brandts et al., 1975) that the unfolding and refolding of globular proteins are rate limited by cis–trans isomerization of those peptide bonds that contain the imide nitrogen of prolyl residues. According to this suggestion in its simplest form, the slow phase in folding reactions arises because a sizable fraction of denatured molecules have one or more proline residues in the incorrect configuration. The actual conformational changes (i.e., changes in ψ and ϕ angles) were assumed to occur rapidly once all of the prolines are in the correct native configuration. Although a simple process in itself, the possible occurrence of isomerization can prove to be an enormous experimental complication when trying to elucidate the "pathway" of folding for proteins containing proline. Being both very slow and highly visible in terms of phase amplitudes, proline isomerization in the unfolded chain can either obscure or be mistaken for other kinetic phases which might be attributable to true structural intermediates along the folding pathway.

Although considerable indirect evidence now suggests that proline isomerization acts to slow down protein folding and unfolding (Brandts et al., 1977; Lin & Brandts, 1978; Schmid & Baldwin, 1978; Cook et al., 1979), there is still some question as to the precise way in which this might occur for particular proteins. Most of the critical data has been obtained on RNase A. Nall et al. (1978) and Cook et al. (1979) concluded that, in contrast to unfolding, the slow phase in refolding does not possess the appropriate characteristics for a reaction which is rate limited by proline isomerization. In particular, they suggest that the relaxation time is 40-fold faster than expected, the activation energy in low Gdn·HCl is much too small, and the strong dependence of relaxation time on Gdn·HCl is inconsistent with data on the isomerization of small model compounds. Their data were interpreted in terms of a model whereby unfolded RNase refolded quickly to a nativelike intermediate I_N which contains one or more prolines in an incorrect nonnative configuration. This proposed intermediate is able to bind 2′-CMP with high affinity, suggesting that the active site is intact. Furthermore, it was assumed that the pathway through the nativelike intermediate was responsible for a large acceleration in the rate of isomerization during refolding, as well as for the small activation energy for the slow phase. Later, it was suggested that proline-93 is in the incorrect trans configuration in I_N (Schmid, 1981; Schmid & Blaschek, 1981; Rehage & Schmid, 1982).

Results from the preceding paper (Lin & Brandts, 1983a) show that, by using trypsin and aminopeptidase P (APP) in tandem, it is possible to quantitatively measure the cis/trans ratio for proline-93 in oxidized RNase because of the stereospecificity of trypsin proteolysis. This technique does not work directly for native RNase or for reversibly unfolded RNase, since they are not good substrates for trypsin in the native-state buffers which must be used to maintain high trypsin activity. However, the technique will work if the native or reversibly unfolded RNase is treated first with pepsin in a buffer (4.5 M urea, pH 2) which is a native-state buffer for pepsin but a denaturing buffer for RNase. Thus, the technique which is employed here will consist of treatment of RNase with a short pepsin pulse, followed by a trypsin pulse of variable duration followed by a long APP pulse. The pepsin pulse causes irreversible unfolding and limited degradation, thereby facilitating the cleavage of RNase by trypsin in a native-state buffer. Trypsin will then cleave the Lys_{91}-Tyr_{92} bond only for those RNase molecules having the Tyr_{92}–Pro_{93} bond in the

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