uscript records will allow the system to store over 2 years of manuscript data before older records will have to be purged onto an inactive archival cartridge.

Indexes. The reviewer file is accessed by searching the reviewer's last name/first initial or by the reviewer number. The 100 name indexes are based upon ASCII numeric equivalent of the first two letters of the last name. The reviewer number, which is automatically assigned by the computer, is directly tied to the record accession number of the main data file. Reviewers' records may also be accessed through a set of 100 indexes based upon areas of reviewer subject expertise.

Manuscript records are accessed by a corresponding author's last name/first initial or manuscript number. The 100 manuscript number indexes are based upon the last two digits of the 9-digit manuscript number. In all, there are 400 index files requiring 474K of disk storage. The computer system cannot, nor was it intended to, replace human judgment or evaluation of manuscripts received. Nevertheless, in actual operation, the system has already proven its value by recalling reviewers for a given topic, regardless of frequency of their use, and by broadening the list of reviewers most appropriate for a given manuscript. The system also expedites the flow of manuscripts and greatly facilitates all of the operations for which it was designed. Although the system was specifically designed to be used by *Biochemistry*, it could be applicable with minor modifications to other journals.

Hans Neurath
Lorrin Garson

Role of Cis–Trans Isomerism of the Peptide Bond in Protease Specificity. Kinetic Studies on Small Proline-Containing Peptides and on Polyproline†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: Aminopeptidase P, a proline-specific exopeptidase, was isolated from *Escherichia coli*, and the cis–trans specificity of its activity was critically tested by using L-phenylalanyl-L-proline, polyproline, and glycyl-L-prolyl-L-alanine as substrates, under conditions where a high ratio of enzyme activity to substrate concentration existed. The results of the study strongly suggest that aminopeptidase P, like prolidase [Lin, L.-N., & Brandts, J. F. (1979) *Biochemistry 18*, 43], can only hydrolyze the trans form of the X–L-Pro peptide bond, while the cis form has to isomerize before it can be cleaved. This study also shows that the isomeric specificity of aminopeptidase P is valuable for studying the conformation of proline-containing peptides preequilibrated in aqueous solution as well as in the solid form. The kinetic data for polyproline hydrolysis show clearly that the cis-to-trans isomerization of polyproline I, when dissolved in water, begins step by step from the N-terminal end rather than the C-terminal end, as suggested by others from NMR data. It was also found that the rate of isomerization for glycyl-L-prolyl-L-alanine in aqueous solution is ~16 times faster than that of glycyl-L-proline, suggesting that the tripeptide could be a better model for proline isomerism in proteins. Finally, the experimental results indicate that the isomeric states of the C-terminal proline residue in L-leucyl-L-phenylalanyl-L-proline can be determined by using two aminopeptidases in tandem: one of which (leucine aminopeptidase) cleaves the Leu–Phe bond and the other of which (prolidase) cleaves the Phe–Pro bond.

A previous study from this laboratory (Lin & Brandts, 1979) on the stereospecificity of prolidase, a proline-specific dipeptidase, demonstrated that this enzyme hydrolyzes only the trans form of the X–L-Pro bond. The cis isomer is not a hydrolyzable substrate and has to isomerize before it can be cleaved. One important question raised from that study is whether other proteases will exhibit the same kind of isomeric specificity. Since the amount of cis form of the peptide bond for residues other than proline is probably less than 1% (Brandts et al., 1977; Lin & Brandts, 1978), it would be very difficult to detect stereospecificity for proteases which cleave only nonproline bonds. However, other proline-specific proteases should demonstrate such preference if the trans peptide bond is a general requirement for protease action. In order to test the generality of isomeric specificity and eventually to establish the use of proteases in conformational studies of proline-containing peptides or proteins, we have isolated aminopeptidase P (Yaron & Mlynar, 1968; Yaron & Berger, 1970) from *Escherichia coli* (strain B) for the present study.

Both prolidase and aminopeptidase P are proline-specific exopeptidases which cleave the N-terminal X–Pro bond. Whereas prolidase is a dipeptidase, which can only hydrolyze X–L-proline or X–L-hydroxyproline bonds (Davis & Smith, 1957), aminopeptidase P can cleave the bond between any N-terminal amino acid (including proline) and a subsequent L-proline residue (but not hydroxyproline) irrespective of the size of polypeptide. In this communication, the stereospecificity of aminopeptidase P will be critically tested by using L-phenylalanyl-L-proline, polyproline, and glycyl-L-prolyl-L-alanine as substrates. As in the previous paper (Lin & Brandts, 1979), this study will be carried out at a high ratio of enzyme activity to substrate concentration. The crystalline substrates as well as those preequilibrated in aqueous solution will be subjected to hydrolysis. NMR data (Grathwohl & Wüthrich, 1976) have shown that L-phenylalanyl-L-proline and glycyl-L-prolyl-L-alanine exist as a mixture of cis and trans

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isomers in water, while only one form, either cis or trans, will exist in the solid state. If aminopeptidase P activity is trans specific, two kinetic phases will therefore be seen for samples preequilibrated in water: a fast phase corresponding to the direct attack of aminopeptidase P on the trans peptide bond and a slow phase corresponding to the cis-to-trans isomerization and subsequent hydrolysis. The amplitude ratio of the two phases should be equal to the cis/trans ratio in equilibrated solution, determined from NMR studies. However, only one kinetic phase should be seen for crystalline substrates which are quickly dissolved immediately before exposure to protease action. The observed relaxation time for hydrolysis will tell whether the solid substrate is in the cis or trans form.

Polyproline also serves as an interesting substrate for the study of isomeric specificity of aminopeptidase P. The conformational isomerization of polyproline has been extensively studied (Carver & Blout, 1967; Mandelkern, 1967; Von Hippel & Schleich, 1969). One of the conclusions resulting from previous investigations is that when the *all-cis*-polyproline I is dissolved in H_2O, it will undergo cis-to-trans isomerization along the peptide chain and eventually will become the *all-trans*-polyproline II. The rate of isomerization is slow and depends on the size of polyproline. In the present study the time course of hydrolysis of polyproline catalyzed by aminopeptidase P will be investigated with the initial state of polyproline in either the all-cis or the all-trans form.

Another question raised in the previous paper (Lin & Brandts, 1979) is whether stereospecificity might be observed for a nonproline protease which cleaves a bond *adjacent* to an X–Pro bond (such as Y–X–Pro, where X and Y are amino acids other than proline). That is, does the cis–trans nature of the X–Pro bond affect the ability of a nonproline protease to cleave the Y–X bond? It is shown that for leucine aminopeptidase, the answer is in the negative.

Finally, data in this paper show that it is possible to determine the isomeric state of the C-terminal proline residue in L-leucyl-L-phenylalanyl-L-proline by using two aminopeptidases in tandem: one of which (leucine aminopeptidase) will not cleave proline and one of which (prolidase) will cleave only proline peptide bonds. Since the percent cis is significantly different for this tripeptide than for the dipeptide L-phenylalanyl-L-proline, this suggests that the isomeric state may depend on interactions occurring over rather long distances. An approach similar to this (i.e., using two proteases in tandem) could be used for longer oligopeptides and possibly for proteins, as well.

Materials and Methods

Materials. E. coli, strain B (ATCC No. 11303), was purchased from Grain Processing Corp. (Muscatine, IA) as frozen cell pastes (three-fourths log growth phase, enriched media). Prolidase (EC 3.4.3.7) (from porcine kidney, lot 57 C-0109) and leucine aminopeptidase (EC 3.4.11.1) (cytosol from porcine kidney, lot 78 C-8115) were obtained from Sigma Chemical Co. as suspensions in ammonium sulfate. The enzymes were dialyzed against a large volume of 0.16 M Tris solution (pH 8.0) immediately before use. Poly-L-proline (M_r ~6000), L-phenylalanyl-L-proline, glycyl-L-prolyl-L-alanine, and their constituent amino acids were obtained from Sigma Chemical Co. Also, *N*-Cbz-leucine, L-phenylalanine ethyl ester hydrochloride, and proline benzyl ester hydrochloride, which were used to synthesize L-leucyl-L-phenylalanyl-L-proline, were also purchased from Sigma Chemical Co. Chelex 100 (100–200 mesh, sodium form) was obtained from Bio-Rad Laboratories. Sephadex G-50 and G-200 and DEAE-Sephadex A-25 were purchased from Pharmacia. All other chemicals were reagent grade. All the solutions used for preparation of aminopeptidase P and for its assay were passed through Chelex 100 in order to remove trace amounts of heavy metals.

Preparation of Aminopeptidase P. Aminopeptidase P was purified from *E. coli* (strain B). The preparation procedures described by Yaron & Berger (1970) were employed, except DEAE-Sephadex instead of DEAE-cellulose was used for ion-exchange columns. The purified enzyme was concentrated by passing through a DEAE-Sephadex column [eluted with buffer containing 0.4 M $(NH_4)_2SO_4$, 0.1 M sodium acetate, and 0.002 M sodium citrate solution at pH 5.6] and was stored at −20 °C. The preparation showed a specific activity of 80 units/mg, which is comparable to the 116 units/mg reported by Yaron & Berger (1970) and to the 55 units/mg reported by Yaron & Mlynar (1968). The purified aminopeptidase P was examined for possible prolidase contamination by using glycyl-L-hydroxyproline as a substrate. No prolidase activity could be found.

Synthesis of L-Leucyl-L-phenylalanyl-L-proline. L-Leucyl-L-phenylalanyl-L-proline was synthesized by the following procedures. (1) *N*-Cbz-leucine was coupled in the cold with L-phenylalanine ethyl ester hydrochloride in the presence of ethyl chlorocarbonate and tri-*n*-butylamine in ethyl acetate solution (Greenstein & Winitz, 1961). (2) The *N*-Cbz-L-leucyl-L-phenylalanine ethyl ester obtained from the preceding step was then coupled with proline benzyl ester via the azide method (Greenstein & Winitz, 1961). (3) The resulting *N*-Cbz-L-leucyl-L-phenylalanyl-L-proline benzyl ester was hydrogenated in the presence of palladium black in HCl–MeOH solution to remove the protecting groups at both ends. The filtrate from the catalyst treatment was dried in vacuo and recrystallized from water–ethanol.

The overall yield of the preparation was estimated to be ~30%. The identity and purity of the prepared tripeptide were established by its proteolysis by leucine aminopeptidase and prolidase. The purity of the tripeptide was found to be more than 95%, estimated from the free proline released when the peptide was subjected to the combined activity of leucine aminopeptidase and prolidase.

Transformation of Polyproline II into Polyproline I. The polyproline (M_r ~6000) purchased from Sigma Chemical Co. was in the trans form (II) and contaminated with low molecular weight species. To make it suitable for use, we dissolved small amounts of polyproline (≃0.1 g) in 2 mL of water and the solution was applied to a Sephadex G-50 column (1.2 × 60 cm, pretreated with a 0.05 M EDTA solution and then with deionized H_2O) and eluted with deionized H_2O at a flow rate of 10 mL/h, collecting in 1-mL fractions. The absorbance was monitored at 245 nm with a Cary 14 spectrophotometer, and the fractions corresponding to the center two-thirds of the first peak were pooled and lyophilized. This fractionated polyproline was still in the trans form at this point. For conversion to the cis form, it was dissolved in 0.5 mL of formic acid and then diluted with 5 mL of propanol. The solution was allowed to stand for 48 h at room temperature. The resulting polyproline I was precipitated out by the addition of 10 mL of ether and then dried in vacuo.

Circular Dichroic Measurements of the Rate of Cis-to-Trans Isomerization of Polyproline. A Cary 60 spectropolarimeter with a 6002 CD accessory was employed to follow the rate of cis-to-trans isomerization. A known amount of the cis form of polyproline obtained from the preceding procedure was quickly dissolved in H_2O, and the sample was intermittently scanned from 280 to 200 nm by using a cell of 1-mm

path length. The ellipticity value at 215 nm was used to calculate the percentage of cis and trans forms at various times. The ellipticity value for the all-cis form was determined by extrapolating to zero time, while that for the all-trans form was obtained by scanning the sample after standing at room temperature for 24 h.

Assay for the Rate of Hydrolysis of L-*Phenylalanyl*-L-*proline and Polyproline Catalyzed by Aminopeptidase P.* The acid ninhydrin colorimetric method (Troll & Lindsley, 1955; Sarid et al., 1959) was employed to measure the rate of hydrolysis. The experimental procedure was very similar to that described earlier (Lin & Brandts, 1979). The aminopeptidase P was solubilized in veronal buffer containing DTT, manganous chloride, and sodium citrate. The preequilibrated substrate was added directly to this enzyme solution at 23 °C. In the case of L-phenylalanyl-L-proline, preequilibration was carried out either in the crystalline state or in solution as the cationic form (pH 1.9) or as the zwitterionic form (pH 5.6). For polyproline hydrolysis, either the cis form (I) was added directly to the enzyme solution or it was first converted to the trans form (II) by allowing it to incubate in water for 24 h before adding it to the enzyme solution.

Assay for the Rate of Hydrolysis of Glycyl-L-*prolyl*-L-*alanine Catalyzed by Aminopeptidase P.* Since no free proline is released when the Gly–Pro bond in the tripeptide is cleaved by aminopeptidase P, the acid ninhydrin colorimetric method could not be employed. Therefore, the amount of free glycine released was determined by converting it into formaldehyde (with ninhydrin) and then reacting with chromotropic acid to form a colored product (Alexander et al., 1945; Dehm & Nordwig, 1970). The detailed procedures are as follows. At zero time, 100 μL of 0.043 M glycyl-L-prolyl-L-alanine (pH 5.6 at 5 °C) was mixed with 1200 μL of the aminopeptidase P solution and incubated at 5 °C. At suitable time intervals, 100-μL aliquots of incubation solution were quickly pipetted into a Corex glass centrifuge tube containing 400 μL of 5% trichloroacetic acid and mixed with a vibrator. The resulting precipitate was centrifuged at 7500 rpm for 30 min. Then, 250 μL of supernatant was added to a glass-stoppered test tube containing 100 μL of 2% ninhydrin (in H_2O) and 100 μL of 20% KH_2PO_4 solution at pH 5.5. The solution was heated in a boiling water bath for 30 min. After the solution was cooled to room temperature, 2 mL of 0.5% chromotropic acid in 20 N H_2SO_4 was added and the solution was again heated in the boiling water bath for 30 min. The absorbance at 570 nm was measured on a Cary 14 spectrophotometer after the solution cooled to room temperature. The enzyme and the unhydrolyzed substrate were analyzed in an identical way as controls, and the results were used to quantitate the kinetic data. For a study of the rate of hydrolysis of solid glycyl-L-prolyl-L-alanine, 1200 μL of the above enzyme solution incubated at 5 °C was directly added to 1 mg of solid glycyl-L-prolyl-L-alanine. The assay method was the same as that for solutions of the substrate.

Assay for the Rate of Hydrolysis of L-*Leucyl*-L-*phenylalanyl*-L-*proline Catalyzed by Leucine Aminopeptidase.* When L-leucyl-L-phenylalanyl-L-proline is hydrolyzed by leucine aminopeptidase, L-leucine and L-phenylalanyl-L-proline are released. The resulting Phe–Pro bond can then be hydrolyzed by prolidase after leucine aminopeptidase is removed by precipitation. The experimental procedures are as follows. For initiation of the hydrolysis, 800 μL of 0.015 M L-leucyl-L-phenylalanyl-L-proline in H_2O was added to 800 μL of leucine aminopeptidase solution (in 0.16 M Tris and 0.02 M Mn^{2+} at pH 8.1), which had previously incubated at 23 °C (or 5.5 °C). At suitable time intervals 100-μL aliquots of incubation solution were quickly pipetted into a tube containing 1 mL of absolute alcohol and mixed with a vibrator. The resulting precipitate was centrifuged at 7500 rpm for 30 min. Then, 1.0 mL of supernatant was transferred to a test tube and all ethanol was removed in vacuo at room temperature. After this, 1.0 mL of 0.16 M Tris and 0.02 M Mn^{2+} buffer solution at pH 8.0 was added to the tube and the tube was incubated at 40 °C for 5 min. Then, 50 μL of prolidase solution (5 units in 0.16 M Tris at pH 8.0, at 40 °C) was pipetted into the above solution. After 20 min, hydrolysis was stopped by adding 2 mL of acid ninhydrin reagent. The acid ninhydrin colorimetric method was then followed (Lin & Brandts, 1979). The degree of hydrolysis of L-leucyl-L-phenylalanyl-L-proline can be calculated from the amount of free proline released.

Assay for the Rate of Hydrolysis of L-*Leucyl*-L-*phenylalanyl*-L-*proline Catalyzed by the Combined Activities of Leucine Aminopeptidase and Prolidase.* Since leucine aminopeptidase can rapidly cleave the Leu–Phe bond of L-leucyl-L-phenylalanyl-L-proline regardless of the conformation of the Phe–Pro bond (see Results), the following procedure was employed to determine the cis–trans conformation of L-leucyl-L-phenylalanyl-L-proline in solution as well as that of the resulting Phe–Pro fragment. For initiation of the hydrolysis, 800 μL of L-leucyl-L-phenylalanyl-L-proline in water was quickly pipetted into a solution containing 800 μL of leucine aminopeptidase (240 units) and 800 μL of prolidase (360 units) incubated at 23 °C (in 0.16 M Tris and 0.02 M Mn^{2+}, pH 8.1). At suitable time intervals, hydrolysis was interrupted by pipetting 150-μL aliquots into 2 mL of acid ninhydrin reagent. Then, the earlier procedures for assaying Phe–Pro were followed (Lin & Brandts, 1979). In another similar experiment, the substrate and leucine aminopeptidase were first mixed and then prolidase was added into the mixture after the hydrolysis of L-leucyl-L-phenylalanyl-L-proline by leucine aminopeptidase had proceeded for 2 or 60 min.

Results

The rates of hydrolysis of L-phenylalanyl-L-proline, polyproline, and glycyl-L-prolyl-L-alanine catalyzed by aminopeptidase P were measured under conditions where a high ratio of enzyme activity to substrate concentration existed. Figure 1a shows plots of the degree of hydrolysis for L-phenylalanyl-L-proline as a function of time for the cationic form (preequilibrated at pH 1.9), the zwitterionic form (preequilibrated at pH 5.6), and the solid form at 23 °C. Even though all hydrolyses were done with the same ratio of enzyme activity to substrate concentration (15 units/μmol) and in the same assay buffer, quite different kinetic patterns are observed. Both a fast and a slow phase were seen for the hydrolysis of the cationic and zwitterionic forms, while only a slow phase was observed when the substrate was preequilibrated as the solid. However, the relative amplitudes of the fast and slow phases for the cationic and zwitterionic forms are quite different. Semilog plots of substrate concentration vs. time (Figure 1b) show that both the fast and slow phases can be treated as first-order processes with respect to time. The relaxation times of the slow phase for all three forms of L-phenylalanyl-L-proline obtained from Figure 1b are identical within the experimental error, ~44 min, while the slow-phase amplitude was 100% for the solid form, 76% for the zwitterionic form, and 26% for the cationic form. Later in the Discussion section, it will be shown that these amplitudes correlate very nicely with the known amount of cis form which is present in each instance.

The rate of hydrolysis of both the cis (metastably present as the initial state) and trans forms of polyproline, catalyzed

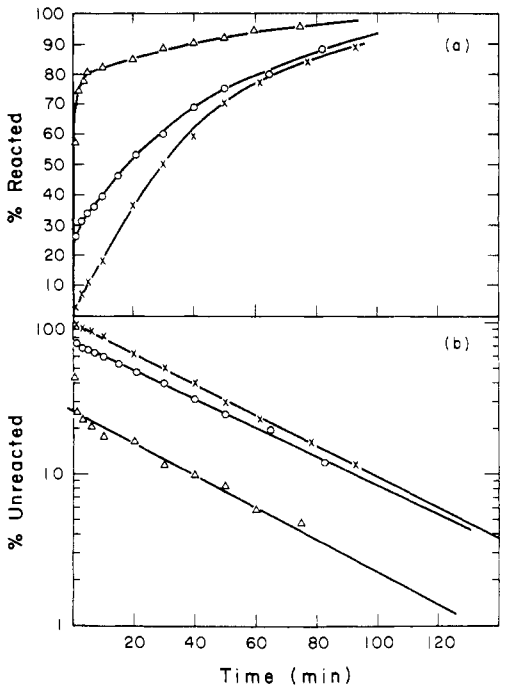

FIGURE 1: Rate of hydrolysis of L-phenylalanyl-L-proline catalyzed by aminopeptidase P at 23 °C. (a) Degree of hydrolysis vs. time; (b) semilog plot of substrate concentration vs. time. The ratio of aminopeptidase P activity to substrate concentration was 15 units/μmol. The concentration of L-phenylalanyl-L-proline was 2.1 mM in 1.23 mL of 0.05 M veronal buffer containing 2.5×10^{-4} M DTT, 8.1×10^{-3} M manganous chloride, and 3.3×10^{-2} M sodium citrate at pH 8.6. Triangles, circles, and crosses represent the cationic, zwitterionic, and solid forms of substrate, respectively, which were present when hydrolysis was initiated.

by aminopeptidase P at 23 °C, is given in Figure 2. Also shown in Figure 2 is the rate of cis-to-trans isomerization (in terms of percent trans) obtained from CD data when the all-cis form of polyproline was dissolved in H_2O. The ratio of aminopeptidase P activity to polyproline concentration was 80 units/mg for both the cis and trans forms of polyproline. Even so, a large difference in the rates of hydrolysis is evident. In less than 30 min, 80% of the proline was released from *trans*-polyproline, while it took more than 7 h to release the same amount of proline when the cis form was dissolved in water and immediately added to aminopeptidase P solution. The most interesting feature shown in Figure 2 is that the kinetic pattern of the cis-to-trans isomerization (measured from CD) is very similar to that of hydrolysis for the cis form. The data clearly indicate that the slow cleavage of polyproline preequilibrated in the cis form is rate limited by the cis-to-trans isomerization.

Figure 3a shows the rate of hydrolysis (5 °C) of glycyl-L-prolyl-L-alanine preequilibrated in H_2O at pH 5.6 (zwitterionic form) and as a solid. The ratio of enzyme activity (aminopeptidase P) to substrate concentration was 20 units/μmol for both forms of the substrate. Two kinetic phases were observed for the hydrolysis of glycyl-L-prolyl-L-alanine preequilibrated in H_2O, and only a fast kinetic phase was seen when the initial form of substrate was solid. Semilog plots of substrate concentration vs. time are given in Figure 3b. The relaxation time and amplitude of the slow phase for glycyl-L-prolyl-L-alanine preequilibrated as the zwitterionic form were estimated to be about 5 min and 17%, respectively. The relaxation time of the fast phase for both forms of the substrate is ~25 s. The above observations indicate that only the hydrolyzable form exists in the solid, while both the hydrolyzable form (83%) and the unhydrolyzable form (17%) coexist in

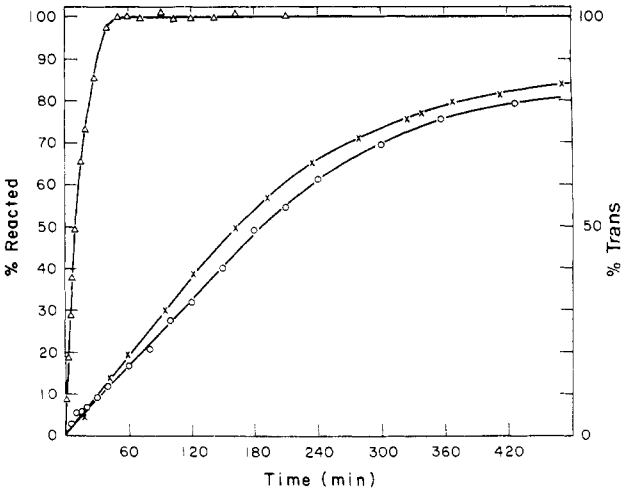

FIGURE 2: Rate of hydrolysis and cis-to-trans isomerization for polyproline at 23 °C. Triangles and circles represent the degree of hydrolysis (left ordinate) vs. time when the initial state of polyproline was the all-trans and the all-cis form, respectively. The ratio of aminopeptidase P activity to substrate concentration was 80 units/mg. The concentration of polyproline was 0.022% in 2.3 mL of 0.07 M veronal buffer containing 1.3×10^{-4} M DTT, 4.3×10^{-3} M manganous chloride, and 1.7×10^{-2} M sodium citrate at pH 8.6. Crosses represent the rate of cis-to-trans isomerization (right ordinate) of polyproline obtained from CD data when the all-cis form was dissolved in water. The concentration of polyproline was 0.013% in water.

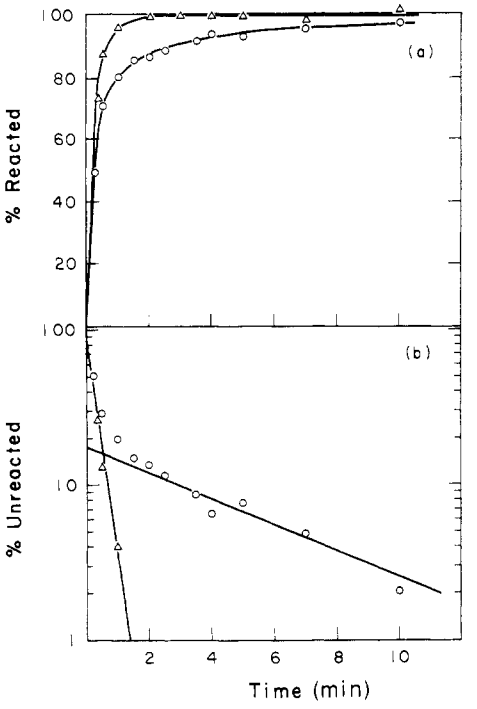

FIGURE 3: Rate of hydrolysis of glycyl-L-prolyl-L-alanine catalyzed by aminopeptidase P at 5 °C. (a) Degree of hydrolysis vs. time; (b) semilog plot of substrate concentration vs. time. The ratio of aminopeptidase P activity to substrate concentration was 20 units/μmol. The concentration of glycyl-L-prolyl-L-alanine was 2.6 mM in 1.48 mL of 0.05 M veronal buffer containing 2.0×10^{-4} M DTT, 8.4×10^{-3} manganous chloride, and 3.4×10^{-2} M sodium citrate at pH 8.6. Circles and triangles represent the zwitterionic and solid forms of substrate, respectively, which were present when hydrolysis was initiated.

water.

The rates of hydrolysis of L-leucyl-L-phenylalanyl-L-proline (as the zwitterionic form) catalyzed by leucine aminopeptidase, where only the Leu–Phe bond is cleaved, and by the combined activities of leucine aminopeptidase and prolidase, where both

the Leu–Phe and Phe–Pro bonds are cleaved, are given in parts a and b of Figure 4. As shown in Figure 4a, the Leu–Phe bond is cleaved very fast at both 23 and 5.5 °C and no slow phase is seen. Since no slow phase is seen, this shows that leucine aminopeptidase activity is not affected strongly by the cis–trans conformation of the adjacent Phe–Pro bond. Figure 4b shows the rate of free proline released from L-leucyl-L-phenylalanyl-L-proline when the combined activities of leucine aminopeptidase and prolidase are used for hydrolysis. Two well-separated kinetic phases are observed under three different experimental conditions (see Figure 4b legend for an explanation). The relaxation times for the slow phase of hydrolysis are the same (~44 min) for all three experiments. However, the amplitudes of the slow phase are identical (~51%) only under the conditions when leucine aminopeptidase and prolidase were simultaneously added into the substrate or when the prolidase was added very shortly after (i.e., 2 min) leucine aminopeptidase and substrate were mixed. The amplitude of the slow phase of hydrolysis was shifted from 51 to 76% when the addition of prolidase was delayed until 60 min after leucine aminopeptidase and L-leucyl-L-phenylalanyl-L-proline were mixed. Since prolidase cleaves only the trans form of the Phe–Pro bond (Lin & Brandts, 1979), the slow phase must correspond to the cis-to-trans isomerization of the Phe–Pro bond and subsequent hydrolysis. The shift of amplitude indicates that the cis fraction for L-leucyl-L-phenylalanyl-L-proline equilibrated in solution (51%) is different from that of the resulting dipeptide, L-phenylalanyl-L-proline (76%).

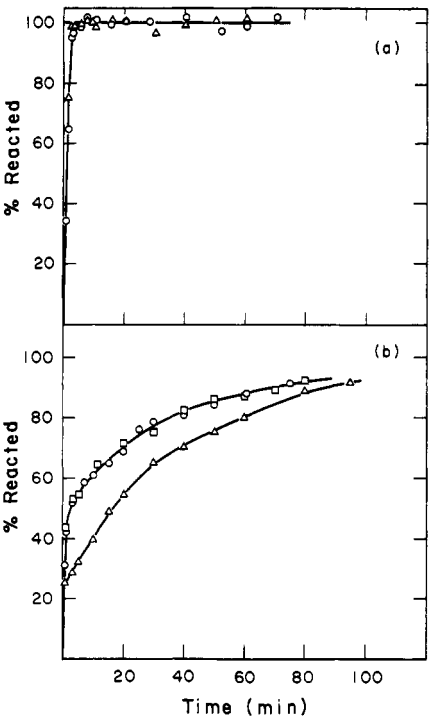

FIGURE 4: (a) Rate of hydrolysis of the Leu–Phe bond of L-leucyl-L-phenylalanyl-L-proline catalyzed by leucine aminopeptidase at 23 (circles) and 5.5 °C (triangles). The ratio of leucine aminopeptidase activity to substrate concentration was 20 units/μmol at 23 °C and 30 units/μmol at 5.5 °C. The concentration of L-leucyl-L-phenylalanyl-L-proline was 7.5 mM in 1.6 mL of 0.08 M Tris–0.01 M $MnCl_2$ solution at pH 8.0. (b) Rate of release of proline from L-leucyl-L-phenylalanyl-L-proline catalyzed by the combined activities of leucine aminopeptidase and prolidase at 23 °C. The ratio of enzyme activity to substrate concentration was 20 units/μmol for leucine aminopeptidase and 30 units/μmol for prolidase. The concentration of L-leucyl-L-phenylalanyl-L-proline was 7.5 mM in 2.4 mL of 0.11 M Tris–0.013 M $MnCl_2$ solution at pH 8.0. Circles represent the condition where leucine aminopeptidase and prolidase were simultaneously added into the substrate solution, while squares and triangles represent the conditions where prolidase was added after leucine aminopeptidase hydrolysis had proceeded for 2 and 60 min, respectively. Zero time indicates the time at which prolidase was added. See text for details.

Discussion

The kinetic patterns shown in Figure 1 for hydrolysis of the zwitterionic and cationic forms of L-phenylalanyl-L-proline catalyzed by aminopeptidase P are very similar to those seen with prolidase and reported in an earlier paper (Lin & Brandts, 1979). The relaxation times for the slow phase of hydrolysis by aminopeptidase P for the three forms of L-phenylalanyl-L-proline are virtually identical with those reported earlier for prolidase, ~44 min at 23 °C. Furthermore, the amplitudes for the slow phase of hydrolysis seen for the zwitterionic form (76%) and the cationic form (26%) are also in good agreement with those seen with prolidase, and they also correlate nicely with the known pH variation in the percent of the cis form from NMR (Grathwohl & Wüthrich, 1976). The above observations strongly suggest that aminopeptidase P, in spite of its different specificity and origin, does have the same kind of isomeric specificity as prolidase. Thus, aminopeptidase P, like prolidase, can only hydrolyze the trans form of the Phe–Pro bond. The fast phase seen for hydrolysis then corresponds to direct hydrolysis of the trans form, and the slow phase corresponds to the cis-to-trans isomerization and subsequent hydrolysis. However, when solid L-phenylalanyl-L-proline (initial state) was subjected to hydrolysis by aminopeptidase P, only a slow phase with an identical relaxation time (Figure 1) was seen, showing that L-phenylalanyl-L-proline crystallizes in the cis form.

The isomeric specificity of aminopeptidase P is also evident from the hydrolysis study of polyproline. As shown in Figure 2, aminopeptidase P can cleave polyproline much faster when it is preequilibrated in the trans form than when the initial state of polyproline is cis. Other experimental data (to be published) further show that the rate of hydrolysis of the trans form of polyproline strongly depends on aminopeptidase P activity, while higher aminopeptidase P activity has little effect on the rate of hydrolysis for the cis form, suggesting that in the latter case a process other than hydrolysis is rate limiting. The only process which logically could be the rate-limiting one is the well-documented cis-to-trans isomerization of polyproline.

Since the hydrolysis by aminopeptidase P of the cis form (initial state) of polyproline proceeds at nearly the same rate as the cis-to-trans isomerization (from CD data), we can only conclude that each trans residue which forms is immediately available for cleavage by the N-terminal exopeptidase. This shows that the formation of the trans form of the polyproline helix from the cis form must begin at the N-terminal end and progress to the C terminus. This is contrary to the mechanism suggested by Torchia & Bovey (1971) from NMR data whereby cis-to-trans conversion was proposed to begin at the C-terminal end and progress in a residue-by-residue manner to the N-terminal end. However, their mechanism is completely inconsistent with our hydrolysis data, since we would then expect to see no hydrolysis whatsoever until all of the CD change had occurred rather than seeing the parallel changes in CD and hydrolysis as were observed.

The two kinetic phases seen for hydrolysis of glycyl-L-prolyl-L-alanine, preequilibrated in neutral solution, can also be attributed to the isomeric specificity of aminopeptidase P activity. The slow phase again corresponds to the cis-to-trans isomerization and subsequent hydrolysis. A relative amplitude of 17% seen for the slow phase of hydrolysis (Figure 3b) is in good agreement with NMR data (Grathwohl & Wüthrich,

1976), showing 15% cis form. However, only a fast kinetic phase was seen for the hydrolysis of solid substrate, suggesting that this tripeptide crystallizes in the trans form. The present study also indicates that the cis-to-trans isomerization of the Gly-Pro bond in glycyl-L-prolyl-L-alanine is much faster than that for the dipeptide, glycyl-L-proline. The relaxation time of cis-to-trans isomerization for glycyl-L-prolyl-L-alanine at 5 °C is ~5 min, compared with ~80 min for glycyl-L-proline at 4.5 °C (Lin & Brandts, 1979). It seems likely that the tripeptide could be a better model for proline isomerism in proteins than the dipeptide, possibly because dipeptides in the zwitterionic state may form ringlike structures which slow down the process of isomerism (Evans & Rabenstein, 1974).

As shown in Figure 4a, leucine aminopeptidase can cleave the Leu–Phe bond fast regardless of the conformation of the adjacent Phe–Pro bond in the tripeptide, indicating that the isomeric specificity for leucine aminopeptidase does not extend to the peptide bond adjacent to the bond being cleaved. Nevertheless, it still seems possible that some endopeptidases might show some kind of isomeric specificity to a neighboring X–Pro bond.

In spite of the lack of isomeric specificity of leucine aminopeptidase toward the Phe–Pro bond in L-leucyl-L-phenylalanyl-L-proline, Figure 4b clearly demonstrates that by combining the activities of leucine aminopeptidase and prolidase the conformation of L-leucyl-L-phenylalanyl-L-proline can be determined. The relative amplitude of 51% for the slow phase of hydrolysis (Figure 4b) will be equal to the amount of the cis form of L-leucyl-L-phenylalanyl-L-proline in solution, since both the Leu–Phe bond and the trans form of the Phe–Pro bond are cleaved very fast. The relative amplitude of 76% for the slow phase of hydrolysis, obtained when prolidase was added 60 min after leucine aminopeptidase and substrate were mixed, should then correspond to the amount of cis form of the Phe–Pro dipeptide equilibrated in solution. There are no data in the literature regarding the cis/trans ratio for L-leucyl-L-phenylalanyl-L-proline in H_2O. However, the similar tripeptide L-alanyl-L-phenylalanyl-L-proline was reported to have 57% cis form in D_2O observed from NMR data (Grathwohl & Wüthrich, 1976). Also, the 76% of the cis form seen for the released Phe–Pro is in excellent agreement with that found from NMR studies on Phe–Pro in D_2O (Grathwohl & Wüthrich, 1976). This result shows that the isomeric state of prolyl residues may be strongly influenced by its location in a polypeptide chain and by the neighboring residues.

In summary, this study not only provides strong evidence to support the argument that aminopeptidase P, like prolidase, cleaves only the trans form of the X–Pro bond but also demonstrates how the isomeric specificity of the enzymes can be utilized to deduce the conformation of a proline-containing peptide in solution as well as in the solid state. The use of a nonproline protease and a proline exopeptidase in tandem can yield information on the isomeric state of nonterminal proline, thereby circumventing problems associated with the nonavailability of a high-activity proline endopeptidase. Using this technique in conjunction with unfolding and refolding experiments, it may be possible to determine the isomeric state of proline in native proteins.

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