resulting tridecapeptide to form a heptapeptide and a hexapeptide, which in turn were hydrolyzed to other products. These results, which would indicate that the collagenase acts preferentially at the amino-terminal end of the substrate, are in almost complete disagreement with the trends found here. It is possible that the neutral proteinase and aminopeptidase activities that contaminate most commercial preparations may be responsible for this observation.

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Isomer-Specific Proteolysis of Model Substrates: Influence That the Location of the Proline Residue Exerts on Cis/Trans Specificity

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ABSTRACT: In an effort to further develop the technique of isomer-specific proteolysis, a number of proline-containing substrates were subjected to hydrolysis in the presence of chymotrypsin, trypsin, or prolidase. The objective was to determine whether direct hydrolysis of the cis form of the substrate could occur and, if so, the extent to which it is slower than the hydrolysis of the equivalent trans form. It is shown that for both peptide and amide substrates, which contain proline at the P_2 position, the cis form can be hydrolyzed directly by either chymotrypsin or trypsin, in contrast to earlier suggestions in the literature. For similar amide substrates, it was found that chymotrypsin has a lower catalytic efficiency for the cis form, relative to the trans form, by a factor of 20 000 while, for trypsin and its substrate, the cis form was cleaved about 2000 times less efficiently. Results for a trypsin substrate with proline at the P_2' position, rather than the P_2 position, were quite different however, since there was no indication that the cis form could be directly cleaved even at the highest enzyme concentration. There was also no indication that prolidase could cleave the dipeptide Phe-Pro when the active bond itself is in the cis form. These collective results suggest that the ability of proteases to cleave a substrate with a cis peptide bond depends strongly on the location of the cis bond relative to the active bond that is being cleaved.

Proline isomerization now appears to play an exceedingly important role in the control of protein conformation (Brandts et al., 1975). Our understanding of the process has been severely restricted, however, due to the lack of an experimental technique that is capable of detecting isomerization when it occurs in proteins or large polypeptides. Recently, this laboratory has been involved in the development of the technique [for a review, see Brandts & Lin (1985)] called isomer-specific proteolysis (ISP), which takes advantage of the fact that many proteolytic enzymes show a strong preference for cleaving peptide substrates when the active bond and other bonds close to the active bond are in the trans configuration. The ISP method shows considerable promise for yielding useful information on proline isomerization, which should help to formulate a better understanding of its importance in protein structure.

Before the ISP method is used on proteins, however, a clear understanding of the specificity of proteases toward proline-containing substrates must be obtained from studies on model peptides. In examining the isomeric specificity of proteases, we have previously suggested that proline-specific aminopeptidases, such as prolidase and aminopeptidase P, will only cleave the trans form of an active X–Pro band (X being any amino acid residue). The cis form must first isomerize to the trans form before it can be cleaved (Lin & Brandts, 1979a,b, 1983a). Later, it was also shown that the configuration of an X–Pro bond located immediately adjacent to the active bond also plays an important role in peptide-bond hydrolysis. That is, trypsin can only readily cleave an active Lys–X bond in a substrate with the Lys-X-Pro sequence (i.e., proline at the P_2' position) when the following X–Pro bond is in the trans form (Lin & Brandts, 1983a,b), while proline-specific endopeptidase can only cleave an active Pro–X bond in a substrate with the -X-Pro-Y sequence (i.e., proline at P_1 position), when the preceding X–Pro bond is in the trans form (Lin & Brandts, 1983c). More recently, however, we found that chymotrypsin can rapidly cleave the Tyr–Val bond in the RNase sequence -Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}- and the Phe–Arg bond in the bradykinin sequence Arg_1-Pro_2-Pro_3-Gly_4-Phe_5-Ser_6-Pro_7-Phe_8-Arg_9 in spite of the existence of the cis form, which in these cases is two bonds away from the active bond (i.e., proline at the P_2 position; Lin & Brandts, 1984).

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On the other hand, Fischer et al. (1984) found two well-defined kinetic phases in the hydrolysis of several amide substrates that have proline at the P_2 position and suggested that the slow phase of hydrolysis is rate-limited by the cis to trans isomerization of the substrate and that the cis form itself is not a hydrolyzable substrate, although they cautioned that their data did not rule out a very low proteolytic sensitivity of the cis isomer. Since the discrepancy between our findings and those of Fischer et al. (1984) conceivably might be due to specificity differences between amide and peptide substrates with proline at the P_2 position, we present more detailed results in this paper for chymotrypsin hydrolysis of both types of substrates. In addition, trypsin hydrolysis of an amide substrate with proline at the P_2 position was also studied for comparison with chymotrypsin. As will be seen, these results show that, for all substrates examined, both enzymes will cleave the active bond when the isomerizing bond at the P_2 position is either cis or trans, although the cis form is cleaved much slower.

In view of this finding that protease specificity for the trans form of substrates can be a relative rather than an absolute requirement, we reinvestigated the cleavage of other substrates where the proline residue is located at different positions relative to the active bond and where it was previously suggested (Lin & Brandts, 1979a,b, 1983a) that there may be an absolute requirement for a trans bond. In both cases (i.e., prolidase cleavage of the dipeptide Phe-Pro where the active bond itself isomerizes and trypsin cleavage of Gly-Gly-Lys-Phe-Pro where proline is at the P_2' position), we find no indication that the cis form can be cleaved even at very high protease activity.

Materials and Methods

Chymotrypsin (catalog no. C4129, log 102F-8050), prolidase (catalog no. P0512, lot 39C-0480), and trypsin (porcine, catalog no. T0134, lot 120F-0459), purchased from Sigma Chemical Co., were used without further purification. Soybean trypsin inhibitor (catalog no. T9003, lot 41F-8000), lima bean trypsin inhibitor (catalog no. T9378, lot 127C-8011), N-Cbz-glycyl-L-prolyl-L-arginine-*p*-nitroanilide[1] (catalog no. 2276, log 69C-0057), *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (catalog no. S-4511, lot 94F-0602), and L-phenylalanyl-L-proline (catalog no. P6258, lot 53C-1430) were also obtained from Sigma Chemical Co. The peptide L-alanyl-L-alanyl-L-alanyl-L-prolyl-L-tyrosyl-L-alanyl-L-alanyl-L-alanine was the product of Serva Fine Biochemicals (catalog no. 51080). Glycylglycyl-L-lysyl-L-phenylalanyl-L-proline was previously synthesized in our laboratory (Lin & Brandts, 1983a). All other chemicals are reagent-grade, and their sources have been cited in previous papers (Lin & Brandts, 1979a,b, 1983a–c).

Assays for the Chymotrypsin Hydrolysis of N-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide and for the Trypsin Hydrolysis of N-Cbz-glycyl-L-prolyl-L-arginine-p-nitroanilide. These experiments were carried out at 10 °C in 0.1 M Tris and 0.01 M $CaCl_2$, pH 7.9–8.0. A Cary 14 spectrophotometer with a thermostated cell holder was employed. Hydrolysis was monitored at 410 nm. An extinction coefficient of 8480 M^{-1} cm^{-1} (at 410 nm) for *p*-nitroaniline was used to determine the substrate concentration at the end of hydrolysis (Delmar et al., 1979). The detailed procedures are as follows: A 2.5-mL sample of substrate solution (concentration ranged from 1.5×10^{-5} to 1.5×10^{-4} M, in 0.1 M Tris and 0.01 M $CaCl_2$, pH 8.0) in a 1-cm quartz cell was first equilibrated at 10 °C. At zero time, a known amount of enzyme (in 0.1 M Tris, 0.01 M $CaCl_2$, pH 8.0, at 10 °C) was quickly pipetted into the substrate solution, and the contents were mixed with a spatula for a few seconds. The increase in absorbance at 410 nm resulting from the release of free *p*-nitroaniline was followed until completion. The mixing dead time was about 10 s. The rates of hydrolysis were quantitated from the net absorbance change at various times compared to that at final equilibrium. The peel-off method was used to resolve the separate phases.

Assay for Chymotrypsin Hydrolysis of L-Alanyl-L-alanyl-L-alanyl-L-prolyl-L-tyrosyl-L-alanyl-L-alanyl-L-alanine. These experiments were also carried out at 10 °C in order to slow down the rate of isomerization. The detailed experimental procedures are as follows: For initiation of the hydrolysis, 400 μL of substrate (4×10^{-4} M, in 0.02 M sodium phosphate buffer, pH 7.8, at 10 °C) were mixed with 400 μL of chymotrypsin (concentration ranged from 4×10^{-6} to 4×10^{-4} M, in 0.02 M sodium phosphate buffer, pH 7.8, at 10 °C). At suitable time intervals, 50 μL of reaction mixture was quickly pipetted into a centrifuge tube containing 2.2 mL of methanol to stop hydrolysis. After being centrifuged at 7000*g* for 30 min, the supernatants were quantitatively transferred to test tubes and heated at 85 °C for 5 min to destroy any residual enzyme activity. The solutions were dried by nitrogen and then dissolved in 250 μL of deionized water prior to HPLC analysis. Two HPLC techniques were used to quantitate the hydrolysis data. In the first, the amount of released Ala-Ala-Ala was determined by a reverse-phase HPLC technique (C_{18}, 10 μm, 4.6 × 250 mm, Serva Fine Biochemicals) coupled with postcolumn fluorescamine derivatization (Schlabach, 1983). In the second method, free tyrosine was released from the hydrolysis product, Ala-Ala-Ala-Pro-Tyr, by long-time incubation with a low activity of proline-specific endopeptidase. The amount of tyrosine was then determined by precolumn derivatization with OPA reagent followed by reverse-phase HPLC (Lin & Brandts, 1984).

Assay for the Trypsin Hydrolysis of Glycylglycyl-L-lysyl-L-phenylalanyl-L-proline. The detailed experimental procedures have been described previously (Lin & Brandts, 1983a). Here, the hydrolysis studies were carried out under higher enzyme (up to 3×10^{-3} M) and lower substrate (1.15×10^{-3} M) concentrations than earlier studies. During acid–ninhydrin assay, 1 mL of acid–ninhydrin reagent and glacial acetic acid was added. All experiments were carried out in 0.1 M Tris buffer, pH 7.9, at 25 °C.

Assay for the Prolidase Hydrolysis of L-Phenylalanyl-L-proline. The experimental procedures have been described previously (Lin & Brandts, 1979a). Here, the hydrolyses were carried out at 25 °C in 0.1 M Tris buffer and 0.01 M $MnCl_2$, pH 8.0, with higher enzyme activity than previously used.

Results

Hydrolysis of N-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide Catalyzed by Chymotrypsin. Hydrolysis studies were performed at 10 °C with a wide range of enzyme concentrations. Figure 1 shows the semilog plot of the degree of hydrolysis as a function of time at enzyme concentrations from 8.8×10^{-4} to 8.4×10^{-7} M. The substrate concentration was kept almost constant at 1.1×10^{-4} to 1.6×10^{-4} M. Data in Figure 1 implicitly indicate two well-separated kinetic phases. In spite of 1000-fold differences in enzyme concentrations, the relative amplitudes of the two phases can be considered, within experimental errors, to be

[1] Abbreviations: Cbz, carbobenzyloxy; Tris, tris(hydroxymethyl)-aminomethane; HPLC, high-pressure liquid chromatography.

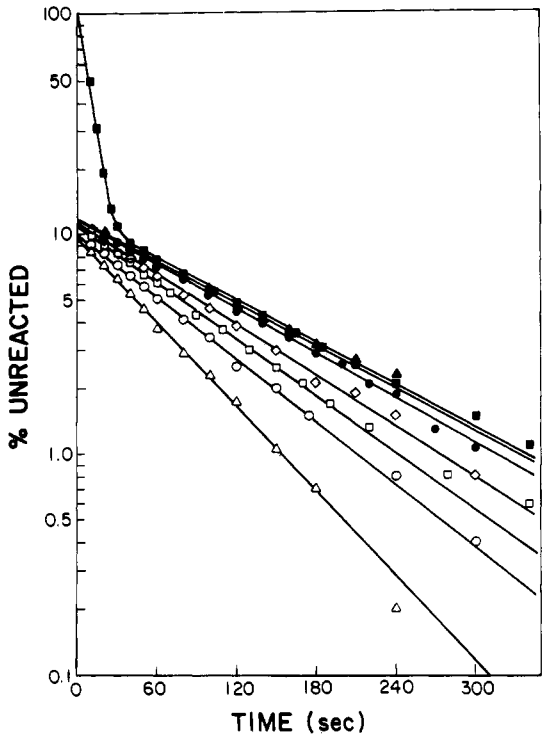

FIGURE 1: Semilog plots of the rates of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide hydrolysis catalyzed by chymotrypsin. The experiments were carried out in 0.1 M Tris buffer and 0.01 M $CaCl_2$, pH 7.9, at 10 °C. The substrate concentrations range from 1.1×10^{-4} to 1.6×10^{-4} M. The enzyme concentrations are 8.4×10^{-7} (■), 4.2×10^{-6} (▲), 1.6×10^{-5} (●), 8.8×10^{-5} (◇), 1.7×10^{-4} (□), 3.5×10^{-4} (○), and 8.8×10^{-4} M (△).

constant. The slow phase accounts for 10.5 ± 1% of the total amplitude. The relaxation time for the fast phase is about 8 s at the lowest enzyme concentration of 8.4×10^{-7} M but is too fast to measure at the higher concentrations. As seen, the slow phase is very nearly first order at all enzyme concentrations and its relaxation time does decrease significantly, from about 130 to 65 s, over the range of enzyme concentrations that could be studied. As will be shown later, the reciprocal relaxation times vary nearly linearly, rather than logarithmically, with enzyme concentration so there is no apparent change in slope of the curves in Figure 1 until the chymotrypsin concentration goes above ca. 1.6×10^{-5} M.

If chymotrypsin were completely unable to cleave the cis form of this substrate, then the relaxation time for the slow phase of hydrolysis would be totally independent of enzyme concentration and equal to the relaxation time for cis to trans isomerization. Since there is significant dependence of the slow phase on enzyme concentration, we conclude that the cis form of the substrate can in fact be cleaved directly by chymotrypsin. Further consideration will be given to this point under Discussion.

*Hydrolysis of N-Cbz-glycyl-*L*-prolyl-*L*-arginine-p-nitroanilide Catalyzed by Trypsin.* The results of these studies, shown in Figure 2, are very similar to those discussed above (cf. Figure 1) for chymotrypsin, as would be expected. Because of the greater activity of trypsin toward its substrate, a larger effect of enzyme concentration on the slow phase can be seen. The relaxation time decreases from about 90 to 10 s at trypsin concentrations from 6×10^{-7} to 8×10^{-5} M. Again, the reciprocal relaxation time varies linearly with enzyme concentration (see Discussion) so most of the slope change seen in Figure 2 occurs at trypsin concentrations above 1×10^{-5} M. At a concentration of 4×10^{-4} M (not shown), the "slow phase" is completed within the mixing dead time. The fast

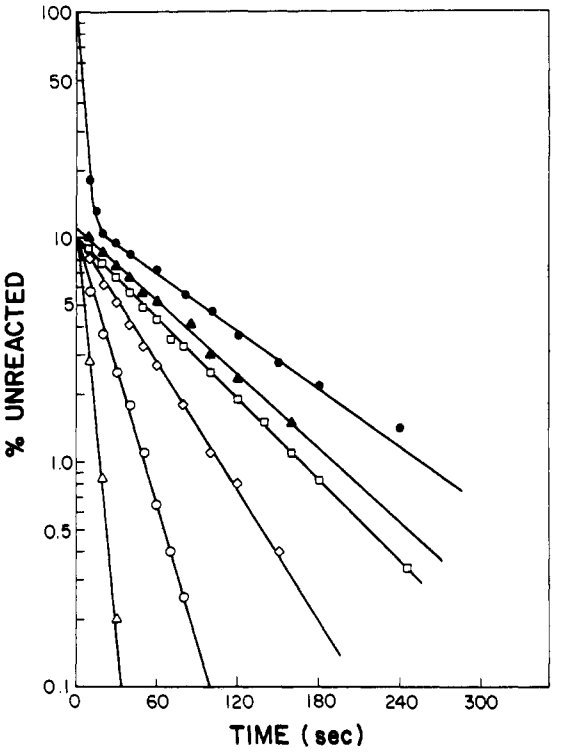

FIGURE 2: Semilog plots of the rates of *N*-Cbz-Gly-Pro-Arg-*p*-nitroanilide hydrolysis catalyzed by trypsin. The experimental conditions are the same as those in Figure 1. The substrate concentrations range from 0.7×10^{-4} to 1.2×10^{-4} M. The enzyme concentrations are 1.2×10^{-7} (●), 6.0×10^{-7} (▲), 3.0×10^{-6} (□), 1.0×10^{-5} (◇), 2.8×10^{-5} (○), and 8.0×10^{-5} M (△).

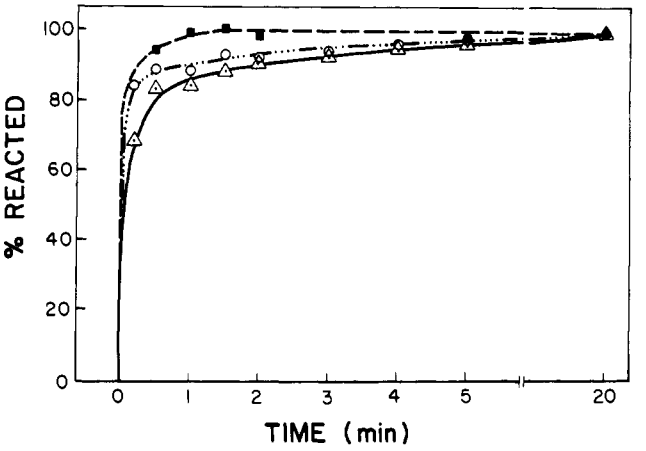

FIGURE 3: Rates of Ala-Ala-Ala-Pro-Tyr-Ala-Ala-Ala hydrolysis catalyzed by chymotrypsin. The experiments were carried out in 0.02 M sodium phosphate buffer, pH 7.9, at 10 °C. The substrate concentration is 2×10^{-4} M. The enzyme concentrations are 2×10^{-6} (▲), 2×10^{-5} (○), and 2×10^{-4} M (■).

phase can only be partially resolved at the lowest concentration of 1.2×10^{-7} M, where the relaxation time is ca. 4 s. The amplitude for the slow phase suggests a cis content of about 11% for this substrate. More importantly, the variation in relaxation time of the slow phase with enzyme concentration suggests that trypsin also is able to directly cleave the cis form of this amide substrate, as found above for chymotrypsin.

Hydrolysis of L*-Alanyl-*L*-alanyl-*L*-alanyl-*L*-prolyl-*L*-tyrosyl-*L*-alanyl-*L*-alanyl-*L*-alanine Catalyzed by Chymotrypsin.* The time dependence of hydrolysis of the Tyr–Ala bond in this peptide substrate is shown in Figure 3. Experiments were carried out at 10 °C with the same substrate concentration (2×10^{-4} M) and three chymotrypsin concentrations differing by a factor of 100. Figure 3 shows that only at the lowest

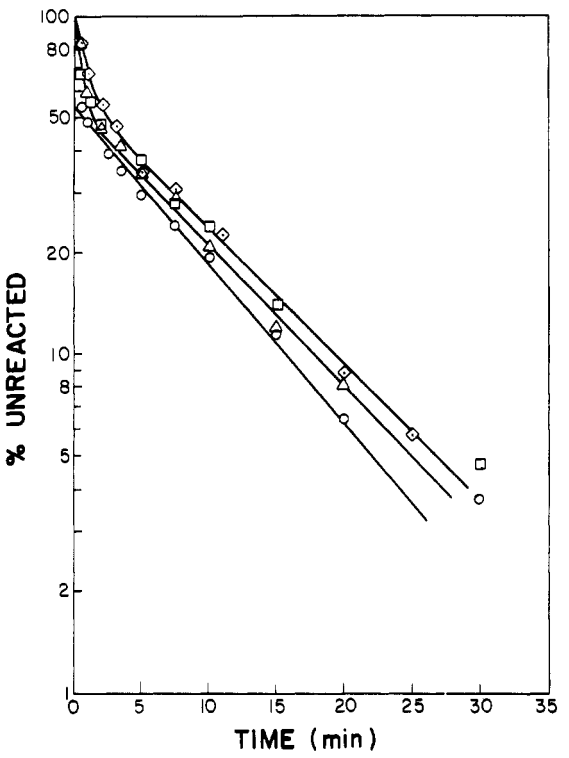

FIGURE 4: Semilog plots of the rates of Gly-Gly-Lys-Phe-Pro hydrolysis catalyzed by trypsin. The experiments were carried out in 0.1 M Tris buffer, pH 7.9, at 25 °C. The substrate concentration is 1.15×10^{-3} M. The enzyme concentrations are 3.5×10^{-4} (◆), 6.0×10^{-4} (△), 1.9×10^{-3} (□), and 3.0×10^{-3} M (○).

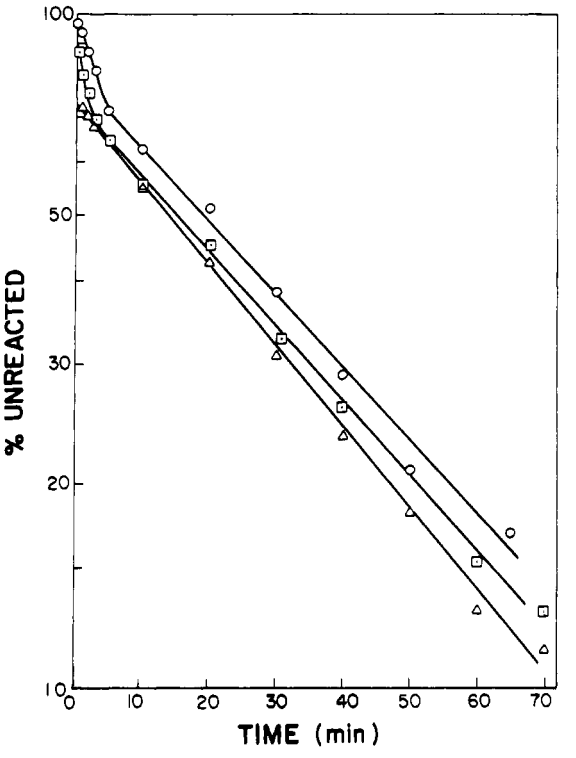

FIGURE 5: Semilog plots of the rates of Phe-Pro hydrolysis catalyzed by prolidase. The experiments were carried out in 0.1 M Tris buffer, pH 8.0, at 25 °C. The substrate concentration is 3.5×10^{-3} M. The enzyme concentrations are 9.0×10^{-7} [20 units, (○)], 4.5×10^{-6} [100 units (⊡)], and 1.8×10^{-5} M [400 units, (△)].

enzyme concentration (triangles) are both the fast and slow phases seen. The amplitude and relaxation time of the slow phase can be estimated to be about 20% and 2.8 min, respectively, while the relaxation time of the fast phase is approximately 10 s. When the enzyme concentration increases 10 times to 2×10^{-5} M (circles), the fast phase becomes faster than the mixing dead time, while the slow phase is still seen, with an amplitude of 18% and a relaxation time of 2.2 min. When the enzyme concentration increases 100 times to 2×10^{-4} M, the rate of the slow phase is significantly accelerated, since all substrate is hydrolyzed in about 1 min and τ can be estimated to be about 30 s. These kinetic data are very similar to those in Figures 1 and 2 for amide substrates and show that the cis form of this peptide substrate can also be directly hydrolyzed by chymotrypsin.

*Hydrolysis of Glycylglycyl-*L*-lysyl-*L*-phenylalanyl-*L*-proline Catalyzed by Trypsin.* The kinetics of the trypsin hydrolysis of this substrate have been investigated previously (Lin & Brandts, 1983a) and it was concluded that trypsin can only cleave the Lys–Phe bond when the following Phe–Pro bond (i.e., proline at the P_2' position) is in the cis form. In light of the above finding that chymotrypsin and trypsin are able to cleave the active bond with a cis proline at the P_2 position, we thought it necessary to reinvestigate the trypsin hydrolysis of Gly-Gly-Lys-Phe-Pro with higher enzyme concentration and lower substrate concentrations to be more certain that the cis form of Gly-Gly-Lys-Phe-Pro is not a hydrolyzable substrate.

The results are shown in the semilogarithmic plots of Figure 4. The experiments were carried out at 25 °C with the same substrate concentration (1.15×10^{-3} M), while the enzyme concentration ranged from 3×10^{-3} to 3.5×10^{-4} M. In spite of nearly a 10-fold difference in enzyme concentration, the relaxtion time of the slow phase is not significantly changed. The small decrease from 12 min of the lowest concentration to 10 min of the highest concentration can probably be attributed to the significant slow down in cleavage of the trans form at low concentrations, seen in the data for the fast phase. There is no indication in these data that the cis form can be cleaved directly by trypsin, and the slow phase seems to be rate-limited by cis to trans isomerization even at the very high concentration of 3×10^{-3} M, which is about 7% trypsin by weight.

Hydrolysis of L*-Phenylalanyl-*L*-proline Catalyzed by Prolidase.* The kinetics of prolidase hydrolysis of Phe-Pro has also been reported in a previous paper (Lin & Brandts, 1979a). It was suggested that the cis form of Phe-Pro is not a hydrolyzable substrate. In the present study at higher activity, the hydrolysis studies were extended to include three enzyme concentrations differing by a factor of 20. The results are plotted in Figure 5. They show that the relaxation times for the slow phase, ranging from 36 to 40 min, are nearly independent of enzyme concentration if we take into account the effect of slow down in the fast phase at lower enzyme concentration. These data once again show no evidence for direct cleavage of the cis form and suggest that the slow phase is rate-limited by cis to trans isomerization up to the highest enzyme concentration that was employed.

DISCUSSION

For substrates that exist in both cis and trans configurations, hydrolysis can occur in two kinetic phases. The fast phase corresponds to the direct cleavage of the trans form, and under conditions of high enzyme and low substrate concentrations used in these studies, this will be a pseudo-first-order process whose rate is closely proportional to the enzyme concentration (Lin & Brandts, 1979a). The reciprocal relaxation time will be

$$1/\tau_{fast} = C_t E_{tot} \quad (1)$$

where E_{tot} is the total concentration of enzyme and where C_t

Table I: Experimental Parameters Determined for the Chymotrypsin Hydrolysis of *N*-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide and the Trypsin Hydrolysis of *N*-Cbz-glycyl-L-prolyl-L-arginine-*p*-nitroanilide

	$1/k_{ct}$ (s)	C_t (L mol^{-1} s^{-1})	C_c (L mol^{-1} s^{-1})	C_t/C_c
chymotrypsin hydrolysis	125	0.15×10^6	8.2	18 300
trypsin hydrolysis	95	2.1×10^6	1100	1 900

is a constant that reflects the catalytic efficiency toward the trans form of the substrate.

The slow phase arises from the cleavage of the cis form, and this phase will, in the general case, be the sum of two component processes: the direct cleavage of the cis form of the substrate in a process whose rate will be proportional to E_{tot} and the indirect cleavage of the cis form after it first isomerizes to the trans form in a process whose rate will be independent of E_{tot} (i.e., assuming that the rate of isomerization is slow compared to the rate of cleavage of the trans form). The reciprocal relaxation time for the slow phase will be

$$1/\tau_{slow} = C_c E_{tot} + k_{ct} \quad (2)$$

where C_c is a constant that reflects the catalytic efficiency of the enzyme toward the cis form and where k_{ct} is the one-way, cis to trans rate constant.

Data on the slow phase for the chymotrypsin hydrolysis (from Figure 1) and the trypsin hydrolysis (from Figure 2) of amide substrates have been plotted according to eq 2 in Figure 6, and it is seen that they conform quite well to the expected linear relationship. There are small deviations in the chymotrypsin case at low enzyme concentration, but these are expected since at low concentration the cleavage of the trans form is still slow enough to retard the slow phase, as seen in Figure 1. The parameters from eq 1 and 2 have been determined from the plots in Figure 6 and are shown in Table I. It is seen that, although the cis form can be cleaved directly by either of these proteases, the catalytic efficiency is considerably less than that for the trans form. The ratio C_t/C_c is about 20 000 for chymotrypsin hydrolysis and 2000 for trypsin hydrolysis of these amide substrates. The 10-fold larger ratio for chymotrypsin could be a reflection of constraints introduced at the active site when the substrate containing the two bulky rings of phenylalanine and proline is in the cis configuration.

It appears then that the cis form of these amide substrates with proline in the P_2 position can be directly hydrolyzed by both chymotrypsin and trypsin, although with a much lower efficiency than the trans form. It also seems certain that the cis form of the peptide substrate with proline in the P_2 position, whose cleavage is shown in Figure 3, can also be directly hydrolyzed by chymotrypsin, although the data are not complete enough in this case to obtain quantitative estimates of the kinetic parameters of eq 1 and 2. One possible reason for the failure of Fischer et al. (1984) to detect the direct cleavage of the cis form for similar P_2 substrates, as mentioned earlier, is the relatively small range of chymotrypsin concentrations that they used. Using our eq 1 and 2 to analyze data in Table II of their paper and assuming a C_c/C_t ratio of 20 000, it can be shown that the highest concentration of chymotrypsin that they employed would produce less than a 5% change in the time constant for the slow phase for each of the four substrates that they examined. Such small changes would not be experimentally detectable.

In view of the 20 000-fold lower efficiency, the possibility also exists that the direct cleavage of the cis form seen in our

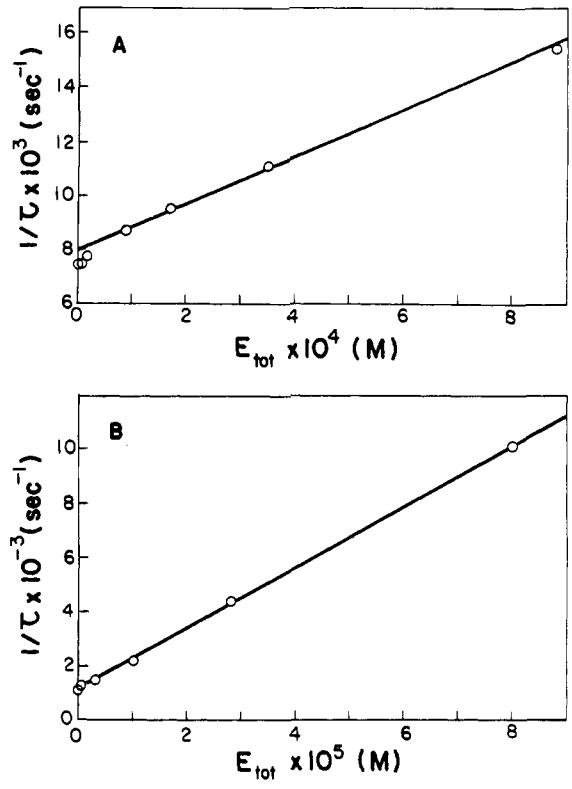

FIGURE 6: Plots of the reciprocal relaxation times ($1/\tau$) for the slow phase vs. the total enzyme concentration (E_{tot}): (A) chymotrypsin hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniline; (B) trypsin hydrolysis of *N*-Cbz-Gly-Pro-Arg-*p*-nitroanilide. The relaxation times were obtained from Figures 1 and 2, respectively. The total enzyme concentrations are listed in the legends to Figures 1 and 2.

studies is not due to chymotrypsin itself but due to a contaminating protease present in very small amounts. Although this cannot be completely ruled out, the supposed contaminant would have to have "chymotrypsin-like" specificity to cleave on the carboxy side of an aromatic residue. The same argument would also have to apply in the trypsin cleavage of its substrate, where the supposed contaminant would have to have "trypsin-like" specificity to cleave on the carboxy side of the arginine residue.

Our present findings are consistent with previous hydrolysis data on the cleavage of the Phe_8–Arg_9 bond in bradykinin and [Tyr^5]bradykinin and the cleavage of the Tyr_{115}–Val_{116} bond in RNase A, where complete hydrolysis takes place in a short time in spite of the existence of a cis proline bond at the P_2 position (Lin & Brandts, 1984).

On the other hand, the new data presented here on the trypsin-catalyzed hydrolysis of Gly-Gly-Lys-Phe-Pro, where the proline is at the P_2' position, and on the prolidase-catalyzed hydrolysis of Phe-Pro, where the active bond is the isomerizing bond, show no evidence that the cis form can be cleaved directly. This was in spite of the fact that the trypsin concentration was taken over 100 times higher than that needed to produce a large effect for the trypsin substrate with proline in the P_2 position. It should be noted that the isomerizing bond is only one peptide bond removed from the active bond at the P_2' position but is two peptide bonds removed at the P_2 position, so the proximity effect might be important. It should also be pointed out that the trans form of substrates with proline at the P_2' position are much poorer substrates for trypsin and chymotrypsin than are the trans forms of substrates with a proline at the P_2 position, and this might also involve factors that would make it very difficult to hydrolyze the cis form of Gly-Gly-Lys-Phe-Pro even at very high trypsin concentrations.

Irrespective of the factors that might produce the effect, it does appear to be very difficult or impossible to directly cleave the cis form of P_2' substrates at enzyme concentrations that are realistically attainable, in agreement with our earlier suggestions (Lin & Brandts, 1983a).

In summary, the slow phase of proteolysis for substrates with proline at the P_2' position or at the active bond will probably always be rate-limited by the isomerization step. However, for substrates with proline at the P_2 position the cis form can be hydrolyzed, but at a much slower rate than the trans form so that the isomerization step may be rate-limiting at low enzyme concentration but not at high enzyme concentration. Consequently, certain precautions must be exercised in the interpretation of ISP data on such substrates. Fischer et al. (1984) have suggested that prolines as remote from the active bond as the P_4 and P_5 positions may exert an isomer-specific effect on the rate of hydrolysis even though there is no absolute preference for the trans form. Because of this, it appears likely that cleavage of active bonds in substrates with more than one proline residue located nearby may be very complicated.

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Revised Mechanism for Inactivation of Mitochondrial Monoamine Oxidase by *N*-Cyclopropylbenzylamine†

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ABSTRACT: A mechanism previously proposed for inactivation of monoamine oxidase (MAO) by *N*-cyclopropylbenzylamine (N-CBA) [Silverman, R. B., & Hoffman, S. J. (1980) *J. Am. Chem. Soc. 102*, 884-886] is revised. Inactivation of MAO by N-[1-^{3}H]CBA results in incorporation of about 3 equiv of tritium into the enzyme and release of [^{3}H]acrolein. Treatment of inactivated enzyme with benzylamine, a reactivator for N-CBA-inactivated MAO, releases only 1 equiv of tritium as [^{3}H]acrolein concomitant with reactivation of the enzyme. Even after MAO is inactivated by N-[1-^{3}H]CBA, the reaction continues. At pH 7.2, a linear release of [^{3}H]acrolein is observed for 70 h, which produces 55 equiv of [^{3}H]acrolein while 2.3 equiv of tritium is incorporated into the enzyme. At pH 9, only 3.5 equiv of [^{3}H]acrolein is detected in solution after 96 h, but 40 equiv of tritium is incorporated into the enzyme, presumably as a result of greater ionization of protein nucleophiles at the higher pH. *N*-[1-^{3}H]Cyclopropyl-α-methylbenzylamine (N-CαMBA) produces the same adduct as N-CBA but gives only 1-1.35 equiv of tritium bound after inactivation of the enzyme. Denaturation of labeled enzyme results in reoxidation of the flavin without release of tritium, indicating attachment is not to the flavin but rather to an amino acid residue. Enzyme inactivated with N-[1-^{3}H]CαMBA is reactivated by benzylamine with the release of 1 equiv of [^{3}H]acrolein, which must have come from an adduct attached to an active site amino acid residue. About half of the tritium remains bound to the enzyme if the N-[1-^{3}H]CαMBA-inactivated enzyme is treated with a low concentration of sodium borohydride prior to benzylamine treatment. The adducts formed when N-CBA, N-CαMBA, and *N*-cyclopropyl-*N*-methylbenzylamine inactivate MAO appear to be identical. They have identical rates of reactivation by benzylamine at pH 7.2 and 9. These results suggest that N-CBA is oxidized by one electron to the amine radical cation followed by homolytic cyclopropyl ring cleavage and attachment to an active site radical, producing a 3-(amino acid residue)propanal adduct.

The compound *N*-cyclopropylbenzylamine (N-CBA)[1] was shown to be a mechanism-based (enzyme-activated) inactivator of mitochondrial monoamine oxidase (MAO, EC 1.4.3.4) (Silverman & Hoffman, 1980). A mechanism-based inactivator is an unreactive compound that is structurally related to the substrate or product of an enzyme and is converted by the enzyme to a species that inactivates the enzyme without prior release from the active site (Silverman & Hoffman, 1984; Walsh, 1984; Abeles & Maycock, 1976; Rando, 1974). The

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[1] Abbreviations: N-CBA, *N*-cyclopropylbenzylamine; MAO, monoamine oxidase; N-CαMBA, *N*-cyclopropyl-α-methylbenzylamine; N-CMBA, *N*-cyclopropyl-*N*-methylbenzylamine; 2,4-DNPH, 2,4-dinitrophenylhydrazine; Me_2SO, dimethyl sulfoxide; Tris-HCl, tris(hydroxymethyl)aminomethane.

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