Evidence Suggesting That Some Proteolytic Enzymes May Cleave Only the Trans Form of the Peptide Bond†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: The rates of hydrolysis of glycyl-L-proline and L-phenylalanyl-L-proline, catalyzed by prolidase, have been measured at several temperatures under conditions where a high ratio of prolidase activity to substrate concentration existed. Two well-separated kinetic phases, which can be adequately treated as two first-order reactions, were observed for the hydrolysis. The relative amplitudes of the two phases are nearly independent of temperature, but strongly dependent on the initial state of protonation of the dipeptides. It was found that the amplitude of the slow phase is strictly proportional to the known amount of cis isomer, while the amplitude of the fast phase correlates with the amount of the trans isomer. Furthermore, the relaxation time and activation energy of the slow phase of hydrolysis are in good agreement with the same parameters determined for cis–trans isomerization of the dipeptides, as measured by a pH-jump method for samples not being hydrolyzed. These results lead us to the conclusion that the slow phase seen for hydrolysis is rate limited by cis–trans isomerization of the X–Pro peptide bond. Thus, this proline-specific protease appears to have an absolute requirement for the trans form of the peptide bond and appears not to cleave the cis form or to cleave it extremely slowly.

Because of its unique cyclic structure, proline occupies an exceptional place among the naturally occurring amino acids. Much interest has been focused on the effect of proline residues upon the structure and conformation of peptides and proteins in solution. For example, it is generally believed that proline has a rather unique function in helix termination and in the formation of β bends, so that this residue likely assumes a disproportionate role in dictating features of globular protein structure, relative to its rather low abundance. NMR studies (Thomas & Williams, 1972; Evans & Rabenstein, 1974; Wüthrich & Grathwohl, 1974; Grathwohl & Wüthrich, 1976a,b; Cheng & Bovey, 1977) have shown that proline-containing peptides exist as a mixture of two isomers in solution and the proportion of cis and trans isomers depends not only on the state of protonation of peptides but also on the neighboring amino acid residues. Recently, the rates of cis–trans isomerization for several dipeptides were measured using the pH-jump method and rates were found to be strongly dependent on steric factors associated with neighboring groups (Brandts et al., 1975). A critical role for proline residues in the kinetics of protein folding and unfolding was suggested. Strong evidence (Brandts et al., 1977; Lin & Brandts, 1978) has now been obtained, showing rather clearly that proline isomerism is the rate-limiting step seen in the folding of proline-containing proteins.

Although these unique attributes of proline are sufficient to indicate its importance, it seems likely that there are still other as-yet-undocumented cases where proline will be found to confer kinetic regulation on conformational processes. For example, it has already been suggested (Brown et al., 1977) that concanavalin A has two similar "native states" having quite different saccharide-binding activity and that the slow equilibrium between the two involves proline isomerism. Also, the X-ray structure of immunoglobulin light chains shows that this protein can exist in a linear form or in a form having a sharp bend at proline-212 (Firca et al., 1978). Thus, cis–trans isomerism may be a frequently used means of kinetically regulating the structure of *well-folded* proteins, quite possibly being used to control the intensity of various physiological activities in the process.

The difficulty in determining those cases where proline could be critically involved is that the isomerism process is very hard to "see". The only direct solution method for distinguishing the cis and trans isomers of prolyl residues is NMR, and this has not yet proved to be effective for large proteins where overlapping resonances are a serious problem. In an effort to develop an indirect method for distinguishing cis prolyl residues from trans residues, we have undertaken an investigation of the proteolysis of X–Pro bonds. So far, two exopeptidases have been reported to be able to specifically catalyze the cleavage of the imide bond. One is prolidase (EC 3.4.3.7; Davis & Smith, 1957; Sjöström et al., 1973), a dipeptidase which can hydrolyze the X–L-proline or X–L-hydroxyproline bond (where X is an amino acid other than proline). The other is aminopeptidase P (Yaron & Mlynar, 1968; Yaron & Berger, 1970), which cleaves the bond between any N-terminal amino acid residue (including proline) and a subsequent L-proline residue irrespective of the size of the substrate. However, one important question regarding the specificity of these two enzymes has never been asked before: Are these enzymes specific for one of the two isomer forms of the X–L-Pro peptide bond? In this communication the cis–trans specificity of prolidase activity will be critically tested using glycyl-L-proline and L-phenylalanyl-L-proline as substrates. The study will be carried out using very high enzyme concentration. If prolidase activity is specific, then only one form (cis or trans) of the dipeptide will be rapidly hydrolyzed. The other form of the dipeptide will slowly isomerize to the form which prolidase can attack. Thus, when the whole time course of hydrolysis is studied, two kinetic phases will be seen: a fast phase corresponding to the direct attack of prolidase on the hydrolyzable form and a slow phase corresponding to the cis–trans isomerization and subsequent hydrolysis. The amplitude ratio of the two phases should be nearly independent of temperature and equal to the cis/trans ratio in equilibrated solution, observed from NMR studies. Furthermore, the rate constant of the slow phase for hydrolysis and its activation energy should be identical with those for cis–trans isomerization reported from pH-jump studies. As will be seen, the results of this study strongly suggest that prolidase activity is absolutely specific for the trans form of the X–L-Pro bond.

Materials and Methods

Materials. Prolidase (from porcine kidney) was purchased from Sigma Chemical Co. (Lot 7C-0109) as a suspension in 2.7 M $(NH_4)_2SO_4$. The specific activity of the enzyme was about 187 units/mg (one unit will hydrolyze 1.0 μmol of

† From the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003. *Received June 28, 1978.* This work was supported by a grant (GM-11071) from the National Institutes of Health.

0006-2960/79/0418-0043$01.00/0

glycyl-L-proline/min at pH 8.0 at 37 °C). The enzyme was dialyzed against a large volume of 0.16 M Tris solution (pH 7.9) immediately before assaying. Glycyl-L-proline, L-phenylalanyl-L-proline, their constituent amino acids, tris(hydroxymethyl)aminomethane, and ninhydrin (crystalline) were also purchased from Sigma Chemical Co. Phosphoric acid, glacial acetic acid, and manganous sulfate, all analytical grades, were obtained from Fisher Scientific Co. Absolute alcohol (low UV absorption) is the product of U.S. Industrial Chemicals Co. All reagents were directly used without further purification.

Two methods have been employed to follow the rate of dipeptide hydrolysis catalyzed by prolidase. A spectrophotometric method similar to that of Josefsson & Lindberg (1965) was used for glycyl-L-proline. This method is based upon the decrease of absorption at low wavelength as peptide bonds are hydrolyzed, while the interference of enzyme and buffer is removed by precipitating them out by the addition of ethanol. Because of the interference of phenylalanine absorption, the spectrophotometric method cannot be applied for L-phenylalanyl-L-proline. Thus, an acid ninhydrin colorimetric method described by Troll & Lindsley (1955) and Sarid et al. (1959) was employed for the study of L-phenylalanyl-L-proline hydrolysis. The method is based on the measurement of the characteristic red color (515 nm) produced when free proline released from dipeptide interacts with ninhydrin in acetic acid–phosphoric acid solution. The details for these two methods are described below.

UV Spectrophotometric Assay. In this method, 250 μL of glycyl-L-proline solution (pH 5.6, about 1.0×10^{-2} M) was delivered with a pipet into a 10-mL test tube thermostated by circulating water from a water bath. Just before the assay started, prolidase with known activity was incubated for 10 min in a 0.16 M Tris/0.02 M $MnSO_4$ solution at pH 7.9 at the same temperature. At zero time, 250 μL of prolidase solution was quickly pipetted into the substrate solution and mixed thoroughly with a stirring bar for 5 s. At suitable time intervals, 50-μL aliquots of incubation solution were quickly pipetted into a glass centrifuge tube containing 1.0 mL of absolute ethanol and mixed with a vibrator. The resulting precipitate was centrifuged at 7500 rpm for 30 min. The absorption measurements of the supernatant were carried out in a semimicrocuvette with 1.0 cm path length on a Cary 14 recording spectrophotometer at both 220 and 300 nm (for base line determination). The absorbances of various solutions containing glycyl-L-proline, glycine, and proline of known concentrations were also determined in the identical way and were used for quantitating the kinetic results. The degree of hydrolysis at each time interval could then be calculated.

For the hydrolysis studies of substrate which was preequilibrated at pH 1.9, a slightly modified procedure was used. After a long equilibration at pH 1.9, a small amount of concentrated Tris buffer was added to the substrate to bring its pH to 7.9. The hydrolysis was immediately initiated by adding the prolidase solution, and the kinetics were monitored in the same way as for the substrate which was preequilibrated at pH 5.6.

Acid Ninhydrin Assay. This procedure is very similar to that described above except that (1) 500 μL of 0.008 M L-phenylalanyl-L-proline was mixed with 500 μL of prolidase solution and (2) at suitable time intervals hydrolysis was interrupted by pipetting 50-μL aliquots into 2 mL of ninhydrin reagent [3 g of ninhydrin dissolved in a mixture of glacial acetic acid (60 mL) and 6 M phosphoric acid (40 mL)]. For determination of proline released during the hydrolysis, 3 mL of glacial acetic acid was added and the solution was heated in a boiling-water bath for 30 min. After cooling to room temperature, the absorbance was measured on a Cary 14 at 515 nm. A series of similar measurements for solutions with known amounts of proline, phenylalanine, and L-phenylalanyl-L-proline were also carried out. Plots of corrected absorbance vs. proline concentration were found to be linear within 1%, so proline concentrations in the unknown could be accurately determined.

pH-Jump Method for Studying the Kinetics of Isomerism of L-Phenylalanyl-L-proline. The details of this method have been described in a previous paper from this laboratory (Brandts et al., 1975). An aqueous L-phenylalanyl-L-proline solution (about 0.25%) was first titrated to pH 1.8 with 6 M HCl and allowed to equilibrate for at least 60 min. A 3-mL aliquot of this solution was placed in a small test tube. At zero time, a predetermined amount of KOH solution (1 M) was quickly added to L-phenylalanyl-L-proline solution with a pipet and mixed thoroughly. The slow pH transient was then followed as a function of time with a digital Radiometer pH meter (type PHM 64), from which pH values were read to 0.001 unit. The relaxation time for cis–trans isomerization was determined by the semilog plotting of ΔpH vs. time. The ΔpH amplitude was of the order of 0.15 to 0.2 pH unit for the slow equilibration.

Results

The rates of hydrolysis were measured over the temperature range from 4.5 to 32 °C for glycyl-L-proline and from 14.8 to 40.3 °C for L-phenylalanyl-L-proline. Since it is known that the ratio of the cis to trans form varies markedly with the titration of the terminal carboxyl group of proline-containing dipeptides (Evans & Rabenstein, 1974), both the zwitterionic form (preequilibrated at pH 5.6) and the cationic form (preequilibrated at pH 1.9) of each dipeptide were hydrolyzed by prolidase using the same final incubation buffer (0.08 M Tris, pH 7.9, 0.01 M $MnSO_4$). A high ratio of enzyme activity to substrate concentration (88 units/μmol of L-phenylalanyl-L-proline and 57 units/μmol of glycyl-L-proline) assured that hydrolysis would be first order with respect to the concentration of the hydrolyzable form(s) of substrate. Figures 1a and 2a show plots of the degree of hydrolysis for L-phenylalanyl-L-proline and glycyl-L-proline as a function of time for the cationic forms and the zwitterionic forms at 23.5 °C. In spite of the high prolidase activity, two well-separated kinetic phases were seen for both substrates. It is evident from these data that the rate of the slow phase for L-phenylalanyl-L-proline is much slower (ca. eight times slower) than for glycyl-L-proline at the same temperature, even though higher prolidase activity was used for the hydrolysis of L-phenylalanyl-L-proline. It is also seen that the relative amplitudes of the fast and slow phases are strongly dependent on the initial state of protonation of the dipeptides. In both cases, the zwitterionic form gives a larger amplitude for the slow phase. However, the relative amplitude of the slow phase for L-phenylalanyl-L-proline is much different from that for glycyl-L-proline for both cationic and zwitteronic forms. Treatment of the data in Figures 1a and 2a shows that both the fast and slow phases can be treated as first-order reactions with respect to time. Semilog plots of substrate concentrations vs. time are given in Figures 1b and 2b for L-phenylalanyl-L-proline and glycyl-L-proline, respectively. From such plots both the amplitude and relaxation time of the two phases were determined. For L-phenylalanyl-L-proline, the slow-phase relaxation time was 43 min for the zwitterionic form and 45 min for the cationic form, which are identical within the

Table I: Relaxation Time and Amplitude of Slow Phase Seen in Hydrolysis of Glycylproline and Phenylalanylproline by Prolidase[a]

	glycylproline							phenylalanylproline					
	initial pH at 1.90			initial pH at 5.6				initial pH at 1.90			initial pH at 5.6		
temp, °C	α, %	τ, min	k, min^{-1}	α, %	τ, min	k, min^{-1}	temp, °C	α, %	τ, min	k, min^{-1}	α, %	τ, min	k, min^{-1}
4.5	17.5	88.2	0.011	40.0	75.4	0.013	14.8	27.0	136.0	0.0073	74.0	137.0	0.0073
13.5	18.0	24.0	0.042	40.0	21.8	0.046	23.5	30.0	45.0	0.022	73.0	43.0	0.023
23.5	18.0	5.4	0.185	39.0	5.6	0.179	32.0	31.0	17.5	0.057	75.0	16.1	0.062
32.0	19.0	2.4	0.417	38.5	2.3	0.435	40.3	31.5	5.7	0.175	75.0	5.9	0.169

[a] See text for detailed experimental procedures and explanations.

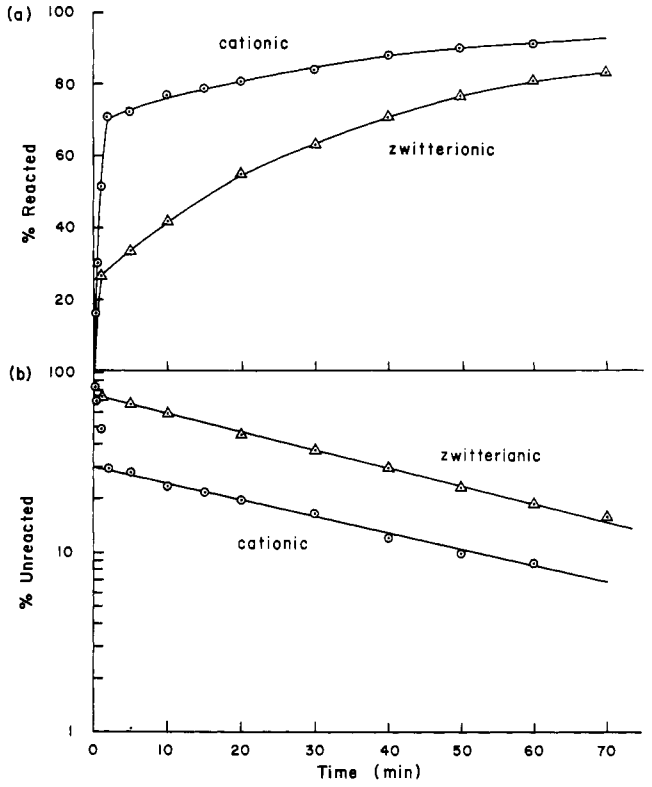

FIGURE 1: The rate of L-phenylalanyl-L-proline hydrolysis catalyzed by prolidase at 23.5 °C: (a) the degree of hydrolysis vs. time, (b) semilog plot of substrate concentration vs. time. The ratio of prolidase activity to substrate concentration was 88 units/μmol. The concentration of L-phenylalanyl-L-proline was 4 mM in 1 mL of 0.08 M Tris–0.01 M $MnSO_4$ solution at pH 7.9. Triangles and circles represent the zwitterionic and cationic forms of substrate, respectively, which were presented when hydrolysis was initiated. See text for details.

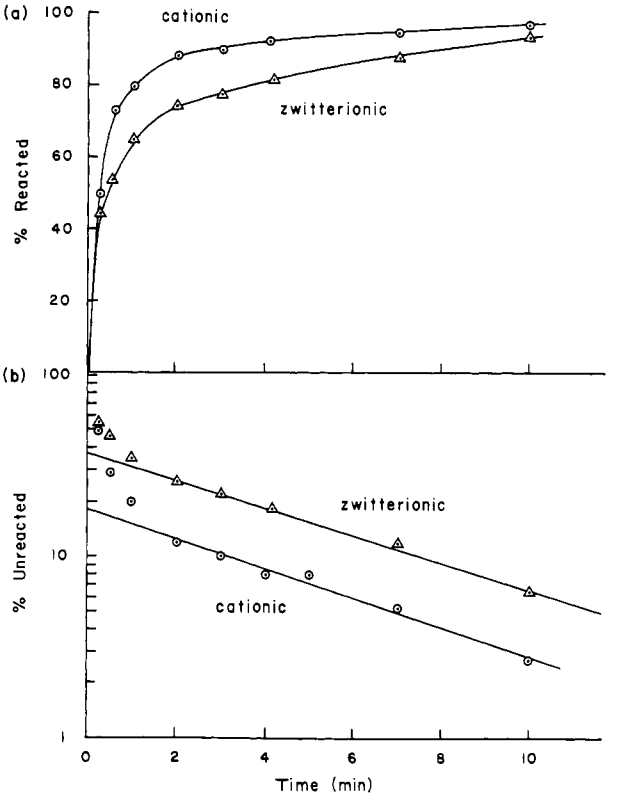

FIGURE 2: The rate of glycyl-L-proline hydrolysis catalyzed by prolidase at 23.5 °C: (a) the degree of hydrolysis vs. time, (b) semilog plot of substrate concentration vs. time. The ratio of prolidase activity to substrate concentration was 57 units/μmol. The concentration of glycyl-L-proline was 5.3 mM in 0.5 mL of 0.08 M Tris–0.01 M $MnSO_4$ solution at pH 7.9. For other details, see the legend for Figure 1.

experimental errors. The slow-phase amplitude was 73% for the zwitterionic form and 30% for the cationic form. For glycyl-L-proline, the relaxation times for the slow phase were 5.4 min for the zwitterionic form and 5.6 min for the cationic form, while the amplitudes were 39% for the zwitterionic form and 18% for the cationic form. The kinetic pattern of hydrolysis at other temperatures was very similar to that shown in Figures 1 and 2. The relaxation times, reciprocal relaxation times (k), and amplitudes of the slow phase for both substrates at various temperatures are summarized in Table I. These data show that the amplitudes are essentially independent of the temperature but show a large dependence on the state of protonation. Arrhenius plots of the k values for both substrates are shown in Figure 3. The same activation energy (21 500 cal/mol) was obtained for the slow phase of hydrolysis for both glycyl-L-proline and L-phenylalanyl-L-proline, despite the large difference in their relaxation time. Thus, it indicates that a common mechanism might be used to explain the slow phase for both substrates.

It has been demonstrated (Sjöström, 1974) that the activity of prolidase, which was isolated from pig intestine (Sjöström et al., 1973), can be competitively inhibited by proline. The values of the Michaelis–Menten constant and inhibitor constant for the glycyl-L-proline/proline system were reported to be 0.13 and 0.21 mM. An important question posed by our earlier results concerns the slow phase and whether it reflects some intrinsic property of the enzyme or whether it can be assigned to isomerism of the substrates. If the former is the case, then the relaxation time of the slow phase should be increased by the presence of inhibitor, whereas it should not be if the latter explanation is correct. Consequently, we carried out a hydrolysis study of glycyl-L-proline in the presence of large amounts of proline at an initial pH of 5.6 and 13.5 °C. The concentrations of glycyl-L-proline and proline were both 0.005 34 M. The ratio of prolidase activity to the concentration of glycyl-L-proline was 110 units/μmol. The results are shown in Figure 4, and they lead to a fairly definite conclusion. In the presence of a large amount of proline the fast phase of hydrolysis for glycyl-L-proline was slowed down to about one-half of that with no added proline. However, proline

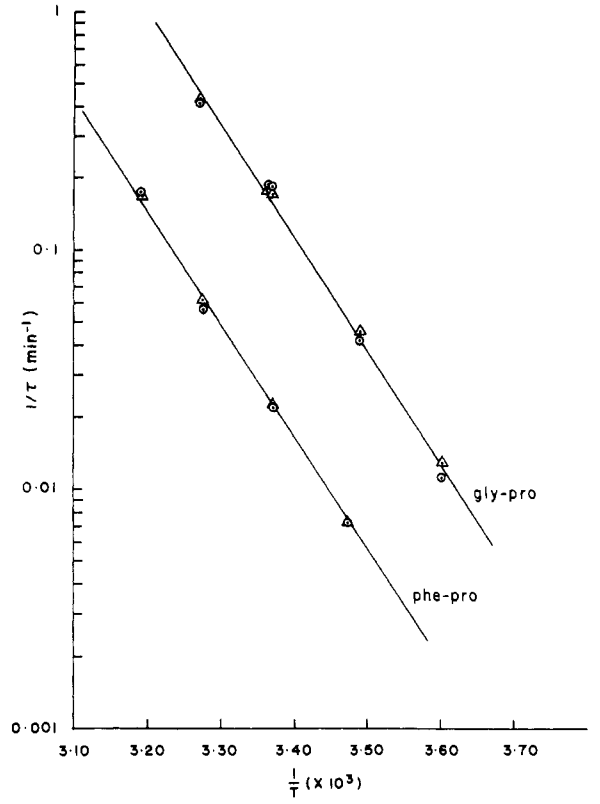

FIGURE 3: Arrhenius plots for the slow phase of hydrolysis. Triangles and circles represent the zwitterionic and cationic forms of substrates, respectively, which were present when hydrolysis was initiated. The experimental conditions were the same as those of Figures 1 and 2.

Table II: Results of pH-Jump Studies on Phenylalanylproline and Glycylproline Isomerism at 22.5 °C

phenylalanylproline[a]			glycylproline[a]		
final pH	τ, min	k_1, min^{-1}	final pH	τ,[b] min	k_1, min^{-1}
8.4	10.6	0.023	4,5	4.0	0.162
7.8	10.4	0.023	4.0	3.7	0.176
4.2	8.7	0.028	3.8	3.8	0.171
3.2	7.4	0.032	3.4	3.0	0.216

[a] Initial pH value for both substrates was 1.8. [b] Data taken from Brandts et al. (1975).

concentration has no measurable effect on the relaxation time of the slow phase, which is 23 ± 1 min in the presence and absence of proline. Futhermore, the relative amplitude of the two phases also is unaffected by the inhibitor. These results strongly suggest that the slow phase is rate limited by a structural change occurring in the substrate and not by the actual process of bond cleavage.

The results of a pH-jump study on L-phenylalanyl-L-proline isomerization at 22.5 °C are reported in Table II. Also listed in Table II are the relaxation times for glycyl-L-proline isomerization previously reported from this laboratory (Brandts et al., 1975). It can be seen that the rate of isomerization for glycyl-L-proline is much faster than that of L-phenylalanyl-L-proline, consistent with the previous conclusion that the rate of isomerization becomes slower as the bulkiness of the side chain of the N-terminal residue increases. The single rate constant for isomerization in the cis to trans direction (i.e., k_1) was calculated from the relaxation times and the cis/trans ratio determined from NMR (Grathwohl & Wüthrich, 1976a) and both are included in Table II.

Discussion

Because high enzyme activity was employed in the hydrolysis studies for both dipeptides, only one fast kinetic phase should be seen if prolidase can cleave both cis and trans forms of substrates directly with approximately the same speed. However, our data (Figures 1 and 2) show that two well-separated kinetic phases are observed for the hydrolysis of both L-phenylalanyl-L-proline and glycyl-L-proline. The relaxation times of the two phases are separated by a factor of about 20 for glycyl-L-proline and by a factor of 50 for L-phenylalanyl-L-proline at room temperature. It might be argued that this non-first-order behavior could be due to the results of product inhibition. This seems very unlikely under our experimental conditions. As shown in Figure 4, in the presence of a large amount of proline, only the fast phase was slowed down and that only by a factor of 2. The rate of the slow phase and relative amplitude of the two phases were virtually unchanged. This evidence not only can be used to rule out the possibility that product inhibition causes the complications, but also can be used to argue that the rate-limiting step in the slow phase has nothing to do with hydrolysis and that it is most probably caused by a structural change occurring in the substrate. Isomerization of the X–Pro peptide bond seems to be the only reasonable possibility.

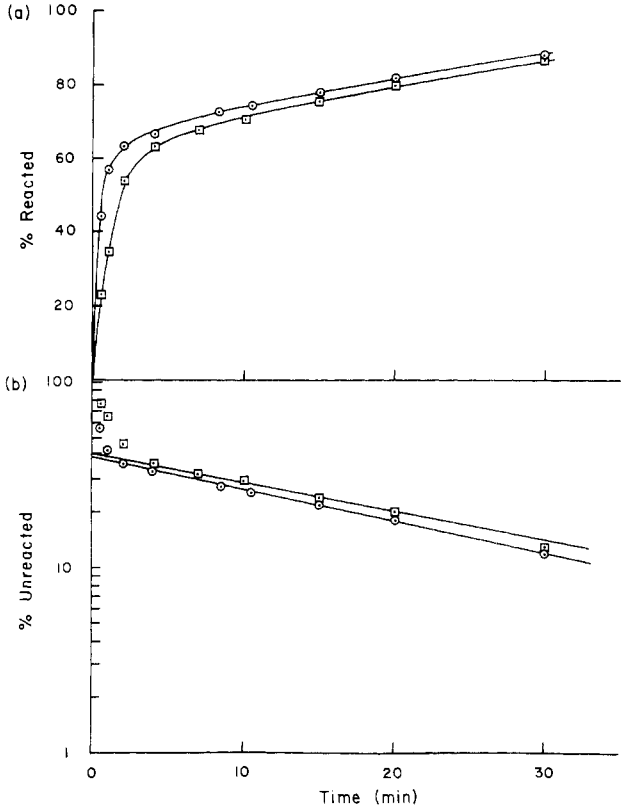

FIGURE 4: The rates of hydrolysis for glycyl-L-proline (zwitterionic form) in the presence and absence of proline at 13.5 °C: (a) the degree of hydrolysis vs. time, (b) semilog plot of substrate concentration vs. time. The ratio of prolidase activity to substrate concentration was 110 units/μmol of glycyl-L-proline. The concentrations of both proline and glycyl-L-proline were 5.3 mM in 0.5 mL of 0.08 M Tris–0.01 M $MnSO_4$ solution at pH 7.9. Triangles represent the data with no added proline. Rectangles represent the data with added proline (5.3 mM).

The simplest mechanism which is consistent with all of our data is the following:

$$\begin{array}{l} \qquad\qquad S_{cis} \\ \text{(slow) } k_{-1} \downarrow\uparrow k_1 \\ S_{trans} + E \underset{\text{(very fast)}}{\rightleftharpoons} ES_{trans} \xrightarrow[\text{(fast)}]{} E + P \end{array}$$

where S_{cis} and S_{trans} represent the two forms of the dipeptides,

Table III: Comparisons of the Experimental Amplitude and Relaxation Time for Hydrolysis with the Calculated Values

		α_{slow}, %		τ_{slow},[b] min	
		exptl	calcd[a]	exptl	calcd
glycyl-L-proline	zwitterionic	39	37	5.6	6.2
	cationic	18	15	5.4	6.2
L-phenylalanyl-L-proline	zwitterionic	73	76	43.0	44.0
	cationic	30	29	45.0	44.0

[a] ^{13}C NMR data of Grathwohl & Wüthrich (1976a). [b] Experimental data were obtained at 23.5 °C, while calculated data were derived from pH-jump data at 22.5 °C.

only one of which (S_{trans}) is a true substrate. The fast phase seen then corresponds to the direct splitting of the peptide bond of S_{trans} by prolidase, while the slow phase is rate limited by the $S_{cis} \rightarrow S_{trans}$ conversion. It should be noted that the relaxation time of the slow phase will be $1/k_1$ since the backward reaction (k_{-1}) will not occur in the presence of high prolidase activity. The relative amplitudes of the slow and fast phase of hydrolysis will be simply

$$\alpha_{slow} = \% \text{ cis}$$

$$\alpha_{fast} = \% \text{ trans}$$

which can be calculated easily from existing NMR data (Grathwohl & Wüthrich, 1976a; Evans & Rabenstein, 1974). The relaxation time for the slow phase (i.e., $1/k_1$) can also be calculated independently from the equilibrium relaxation time (i.e., $1/(k_1 + k_{-1})$) obtained from pH-jump data (Table II) coupled with the S_{cis}/S_{trans} ratio from NMR.

The data in Table III show the comparison of the experimental amplitudes and relaxation times measured for the hydrolysis of the two substrates, with the calculated values of the same parameter assuming the correctness of the above mechanism based on proline isomerism. The agreement is remarkably good and leaves little doubt that the mechanism is substantially correct. In particular, it is impressive that the large difference in relative amplitude for the cationic and zwitterionic forms of the two substrates is accounted for nicely from the known pH variation in the percent cis form from NMR. Also, the fact that the relaxation time for L-phenylalanyl-L-proline is about eightfold faster than for glycyl-L-proline is correctly predicted by the isomerism model.

The activation energy for the slow phase of hydrolysis was found to be 21 500 cal/mol for both dipeptides. This is also in excellent agreement with values normally obtained for cis–trans isomerism of proline-containing solutes [e.g., 19 800 cal/mol for L-alanyl-L-proline obtained by Brandts et al. (1975)]. Also, the nearly temperature-independent values obtained for the amplitude of the slow phase (Table I) are exactly as expected for proline isomerism.

In summary, this study provides very strong evidence to support the contention that only the trans form of dipeptides can be cleaved by prolidase. If the cis form is cleaved at all, the rate must be considerably slower. This being the case, it seems at least possible that some or all other proteases will also exhibit isomeric specificity. This steric effect will be most demonstrable for enzymes specific for X–Pro or X–Hpr bonds because of the large cis fraction under normal conditions. However, it may well occur for bonds involving residues other than proline where the amount of cis form is probably less than 1% (Brandts et al., 1977; Lin & Brandts, 1978). In addition, one must wonder if isomer specificity might also extend sometimes to the peptide bond adjacent to the bond being cleaved. Thus sequences like Pro–X∼Y or X∼Y–Pro may exhibit a slow phase in hydrolysis involving enzymes which will cleave the X–Y bond but not the imide peptide bond.

An important facet of this stereospecificity for trans peptide bonds is that it may provide a practical means of distinguishing cis prolyl residues from trans prolyl residues in a peptide chain simply on the basis of their susceptibility to protease action. As indicated in the introduction, some new method of determining the isomeric state of prolyl residues is clearly needed before we will be able to understand the real importance of cis–trans isomerism in the kinetic regulation of protein structure.

Acknowledgments

One of us (J.F.B.) wishes to acknowledge a very useful discussion with Charles Cantor, which was important to the development of this work.

References

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

Brandts, J. F., Brennan, M., & Lin, L.-N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4178.

Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883.

Cheng, H. N., & Bovey, F. A. (1977) *Biopolymers 16*, 1465.

Davis, N. C., & Smith, E. L. (1957) *J. Biol. Chem. 244*, 261.

Evans, C. A., & Rabenstein, D. L. (1974) *J. Am. Chem. Soc. 96*, 7312.

Firca, J. R., Ely, K. R., Kremer, P., Westholm, F. A., Dorrington, K. J., & Edmunson, A. B. (1978) *Biochemistry 17*, 148.

Grathwohl, C., & Wüthrich, K. (1976a) *Biopolymers 15*, 2025.

Grathwohl, C., & Wüthrich, K. (1976b) *Biopolymers 15*, 2043.

Josefsson, L., & Lindberg, T. (1965) *Biochim. Biophys. Acta 105*, 149.

Lin, L.-N., & Brandts, J. F. (1978) *Biochemistry 17*, 4102.

Sarid, S., Berger, A., & Katchalski, E. (1959) *J. Biol. Chem. 234*, 1740.

Sjöström, H. (1974) *Acta Chem. Scand., Ser. B 28*, 802.

Sjöström, H., Noren, O., & Josefsson, L. (1973) *Biochim. Biophys. Acta 327*, 457.

Thomas, W. A., & Williams, M. K. (1972) *J. Chem. Soc., Chem. Commun.*, 994.

Troll, W., & Lindsley, T. (1955) *J. Biol. Chem. 215*, 655.

Wüthrich, K., & Grathwohl, C. (1974) *FEBS Lett. 43*, 337.

Yaron, A., & Mlynar, D. (1968) *Biochem. Biophys. Res. Commun. 32*, 658.

Yaron, A., & Berger, A. (1970) *Methods Enzymol. 19*, 521.