Ljones, T., & Burris, R. H. (1978) *Biochem. Biophys. Res. Commun. 80*, 22.
Lockshin, A., & Burris, R. H. (1965) *Biochim. Biophys. Acta 111*, 1.
Ludden, P. W. (1977) Ph.D. Thesis, University of Wisconsin—Madison, Madison, WI.
Ludden, P. W., & Burris, R. H. (1978) *Biochem. J. 175*, 251.
Ludden, P. W., Okon, Y., & Burris, R. H. (1978) *Biochem. J. 173*, 1001.
McNary, J. E., & Burris, R. H. (1962) *J. Bacteriol. 84*, 598.
Mortenson, L. E. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 272.
Newton, W. E., Corbin, J. L., & McDonald, J. W. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st 1*, 53.
Newton, W. E., Bulen, W. A., Hadfield, K. L., Stiefel, E. I., & Watt, G. D. (1977) in *Recent Developments in Nitrogen Fixation* (Newton, W. E., Postgate, J. R., & Rodriguez-Barrueco, C., Eds.) p 119, Academic Press, London.
Okon, Y., Houchins, J. P., Albrecht, S. L., & Burris, R. H. (1977) *J. Gen. Microbiol. 98*, 87.
Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123*, 537.
Schrauzer, G. N. (1976) *Proc. Int. Symp. Nitrogen Fixation, 1st 1*, 79.
Shah, V. K., & Brill, W. J. (1973) *Biochim. Biophys. Acta 305*, 445.
Stewart, W. D. P., Fitzgerald, G. P., & Burris, R. H. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 2071.
Stiefel, E. I. (1977) *Recent Dev. Nitrogen Fixation, Proc. Int. Symp., 2nd*, 69.
Stiefel, E. I., Burgess, B. K., Wherland, S., Newton, W. E., Corbin, J. L., & Watt, G. D. (1980) in *Nitrogen Fixation* (Newton, W. E., & Orme-Johnson, W. H., Eds.) Vol. 1, p 211, University Park Press, Baltimore, MD.
Sweet, W. J., Houchins, J. P., Rosen, P. R., & Arp, D. J. (1980) *Anal. Biochem. 107*, 337.
Tiedje, J. M. (1979) *Soil Sci. Soc. Am. J. 43*, 709.
Tso, M.-Y. W., Ljones, T., & Burris, R. H. (1972) *Biochim. Biophys. Acta 267*, 600.
Turner, G. L., & Bergersen, F. J. (1969) *Biochem. J. 115*, 529.
Vandecasteele, J.-P., & Burris, R. H. (1970) *J. Bacteriol. 101*, 794.
Wherland, S., Burgess, B. K., Stiefel, E. I., & Newton, W. E. (1981) *Biochemistry 20*, 5132.

# Evidence Showing That a Proline-Specific Endopeptidase Has an Absolute Requirement for a Trans Peptide Bond Immediately Preceding the Active Bond†

Lung-Nan Lin and John F. Brandts*

ABSTRACT: The proline-specific endopeptidase (EC 3.4.21.26) from *Flavobacterium meningosepticum* is specific for the cleavage of peptide bonds on the C-terminal side of prolyl residues. Such bonds will normally exist in the all-trans configuration. However, the preceding peptide bond in the sequence (i.e., on the N-terminal side of the prolyl residue) will exist as a mixture of cis and trans forms in solution. In this study, the activity of the proline-specific endopeptidase toward the substrates *N*-Cbz-Gly-Pro-MCA (where MCA = 4-methylcoumarinyl-7-amine) and *N*-Cbz-Gly-Pro-Leu-Gly has been examined. At a high ratio of enzyme activity/substrate concentration, the hydrolysis pattern for each substrate shows two well-separated kinetic phases. It is concluded that the fast kinetic phase, whose velocity depends on enzyme concentration, results from the direct hydrolysis of the active substrate bond (i.e., either the Pro–MCA or Pro–Leu bond, respectively) in molecules where the preceding Gly–Pro bond is trans. The slow phase, whose velocity is independent of enzyme concentration, is rate-limited by the cis-to-trans isomerization of those substrate molecules which initially have the preceding Gly–Pro bond in the cis configuration. That is, substrate molecules having the cis form of the Gly–Pro bond which precedes the active bond cannot be hydrolyzed directly but must first isomerize to the trans form before cleavage can occur. The amplitude, relaxation time, and activation energy for the slow phase are consistent with this interpretation. Thus, the proline-specific endopeptidase from *Flavobacterium* has an absolute requirement for a trans peptide bond at the position immediately preceding the active bond.

Previous studies from this laboratory on the isomeric specificity of proteases have demonstrated that proline-specific aminopeptidases such as prolidase and aminopeptidase P exhibit trans specificity toward the active X–Pro bond (where X is any amino acid residue); i.e., only the trans form of the N-terminal X–Pro peptide bond can be cleaved by these two enzymes. The cis form is not a hydrolyzable substrate and must isomerize to the trans form before it can be cleaved. Taking advantage of the isomeric specificity of these enzymes, the kinetic and thermodynamic properties of isomerization for several short peptides with X-Pro at the N terminus and for polyproline have been elucidated (Lin & Brandts, 1979a,b, 1980). Recently, we showed that an endopeptidase, trypsin, also exhibits trans specificity toward a following X–Pro bond; i.e., trypsin can only cleave an active Lys–X bond in a substrate with the Lys-X-Pro sequence when the following X–Pro bond is in the trans form (Lin & Brandts, 1983a). This isomeric specificity of endopeptidases enabled us to study the properties of an X–Pro peptide bond situated inside the polypeptide chain. Trypsin, in combination with aminopeptidase P, was used to examine proline-93 isomerization in oxidized RNase[1] (Lin &

† From the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003. *Received April 18, 1983.* This work was supported by Research Grant GM-11071 from the National Institutes of Health.

0006-2960/83/0422-4480$01.50/0 © 1983 American Chemical Society

Brandts, 1983a). More significantly, the isomeric specificity of trypsin has been utilized to follow proline-93 isomerization during RNAse refolding and unfolding (Lin & Brandts, 1983b). The results obtained allowed us to clarify the role that proline-93 plays in RNAse folding (Lin & Brandts, 1983c,d).

However, not every X–Pro bond in proteins can be examined by endopeptidases such as trypsin, pepsin, and chymotrypsin since their usefulness is limited by the existence of an active bond immediately preceding the X–Pro bond of interest. In a continuing effort to develop a more general isomer-specific proteolytic (ISP) method, and also confronted with the fact that there is no endopeptidase known which will cleave a peptide bond on the N-terminal side of prolyl residues, the isomeric specificity of a proline-specific endopeptidase (EC 3.4.21.26; Yoshimoto & Tsuru, 1978; Yoshimoto et al., 1980), which will cleave a peptide bond on the carboxyl side of prolyl residues, is investigated here. Besides cleaving prolyl peptide bonds at the carboxyl side, this enzyme can also hydrolyze an Ala–Ala bond in a peptide chain, but at a much slower rate.

In the present paper, the isomeric specificity of this post-proline cleaving enzyme will be tested by using the substrates *N*-Cbz-Gly-Pro-MCA and *N*-Cbz-Gly-Pro-Leu-Gly, which will each exist as a mixture of trans and cis forms because of isomerism about the Gly–Pro bonds. If this enzyme does have trans specificity toward the preceding Gly–Pro bond, two kinetic phases will be seen during the time course of hydrolysis with high enzyme activity: a fast phase corresponding to the direct attack of enzyme on the Pro–MCA or Pro–Leu bond of substrate molecules where the preceding bond is trans and a slow phase corresponding to the cis-to-trans isomerization and subsequent hydrolysis of those molecules initially having a cis Gly–Pro bond. The relative amplitude of the two phases should reflect the cis/trans ratio of substrate at the moment hydrolysis is initiated. As will be seen, the results of this study show that this proline-specific endopeptidase is absolutely specific for the trans form of the substrates; the cis form of these two substrates cannot be hydrolyzed directly.

## Materials and Methods

Proline-specific endopeptidase (EC 3.4.21.26), isolated from cell-free extracts of *Flavobacterium meningosepticum*, was purchased from Miles Laboratories as a lyophilized powder (catalog no. 32-082-12C, lot no. R053). The enzyme was purified according to the method of Yoshimoto & Tsuru (1978) and Yoshimoto et al. (1980). The purification procedures involve ammonium sulfate fractionation, CM-cellulose and hydroxyapatite column chromatography, and gel filtration on Sephadex G-150. The fluorescent substrate, *N*-Cbz-Gly-L-Pro-MCA (MCA = 4-methylcoumarinyl-7-amine) was obtained from Vega Biochemicals. Another substrate, *N*-Cbz-Gly-L-Pro-L-Leu-Gly, is the product of Protein Research Foundation (Japan). Sodium phosphate (certified ACS) and HPLC grade acetonitrile were obtained from Fisher Scientific Co. *o*-Phthalaldehyde and mercaptoethanol were purchased from Pierce Chemical Co. Dioxane (reagent grade) and methanol (HPLC grade) are the products of MCB Inc.

*Assay for the Rate of Hydrolysis of N-Cbz-Gly-Pro-MCA Catalyzed by the Proline-Specific Endopeptidase.* The fluorescence method of Yoshimoto et al. (1979) was used to follow the rate of hydrolysis, with some modifications. A Perkin-Elmer MPF-44 fluorescence spectrophotometer with a thermostated cell holder was employed. The instrument was run in the energy mode with both slits set at 5 nm. The excitation and emission wavelengths were 370 and 440 nm, respectively. The experiments were carried out at five temperatures, from 5 to 23.2 °C by using a 1-cm quartz cell. The detailed procedures are as follows: Before the reaction was initiated, 10 μL of *N*-Cbz-Gly-Pro-MCA in dioxane ($1.5 \times 10^{-3}$ M) was mixed with 3 mL of 0.1 M sodum phosphate buffer solution (pH 7.0) in a cuvette thermostated at a certain temperature, and the solution was allowed to equilibriate for 5 min or more. At zero time, 50 μL of enzyme solution, previously incubated at the same temperature, was quickly added into the substrate solution, and the contents were mixed with a spatula for a few seconds. The increase in fluorescence intensity resulting from the release of 4-methylcoumarinyl-7-amine from *N*-Cbz-Gly-Pro-MCA was then followed until completion. The rates of hydrolysis were quantitated from the fluorescence intensity at various times compared to that at final equilibrium. The same experiments were carried out at two different enzyme concentrations.

In the present study, one unit of enzyme activity corresponds to the release of 1 μmol of 4-methylcoumarinyl-7-amine/min at 18 °C (as determined from the initial slope) from *N*-Cbz-Gly-Pro-MCA under the following conditions: 50 μL of diluted enzyme solution (in 0.1 M sodium phosphate buffer, pH 7.0, at 18 °C) was mixed with an equilibrated substrate solution (at 18 °C) containing 3 mL of 0.1 M sodium phosphate buffer (pH 7.0) and 50 μL of *N*-Cbz-Gly-Pro-MCA ($1.5 \times 10^{-3}$ M) in dioxane. The initial slope of the reaction was used to determine the rate at which 4-methylcourmarinyl-7-amine was released.

*Assay for the Rate of Hydrolysis of N-Cbz-Gly-L-Pro-L-Leu-Gly Catalyzed by the Proline-Specific Endopeptidase.* When *N*-Cbz-Gly-Pro-Leu-Gly was subjected to enzyme hydrolysis, only the Pro–Leu bond was cleaved. The resulting Leu–Gly dipeptide was derivatized with OPA reagent (*o*-phthalaldehyde and mercaptoethanol) and quantitatively determined by HPLC. The detailed experimental procedures are as follows: for initiation of the hydrolysis, 150 μL of substrate ($1.5 \times 10^{-4}$ M in 0.045 M sodium phosphate buffer, pH 7.0) incubated at 5 °C was mixed with 150 μL of enzyme solution (13 units in 0.045 M phosphate buffer, pH 7.0). At suitable time intervals (30 s and 1, 2, 3, 5, 10, and 60 min), 30 μL of reaction mixture was quickly pipetted into a centrifuge tube containing 1 mL of methanol and then mixed with a vibrator to stop enzyme activity. A control run was also carried out by separately adding 15 μL of substrate solution and 15 μL of enzyme solution into a centrifuge tube containing 1 mL of methanol. After centrifuging at 7500 rpm 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 the residues were dissolved by adding 150 μL of 0.4 M boric acid buffer, pH 9.5. The samples were then ready for HPLC analysis.

The HPLC analysis of the Leu-Gly dipeptide was performed on a Varian 5020 gradient liquid chromatography with a Fluorichrom fluorescence detector and a reverse-phase Servachrom HPLC precision column (C18, 5 μm, 4.6 mm × 25 cm, SERVA Fine Biochemicals Inc.). A 100-μL sample loop was used with a Valco injector. The gradient runs started at 9% $CH_3CN$–91% 0.0125 M sodium phosphate buffer (pH 7.2) and increased to 24% of $CH_3CN$ in 5 min and to 49% $CH_3CN$ in 25 min. The flow rate was 1 mL/min. Before a sample was injected, 50 μL of the sample solution was reacted with 100 μL of OPA reagent (Lin & brandts, 1983a) for exactly 2 min. The Leu-Gly peak was eluted at 15.5 min. The peak

[1] Abbreviations: RNase, ribonuclease; Cbz, benzyloxycarbonyl.

Table I: Relaxation Times and Amplitudes of the Fast and Slow Phases Seen in Hydrolysis of Cbz-Gly-L-Pro-MCA by Proline-Specific Endopeptidase

| temp (°C) | enzyme/substrate (units/μmol) | fast phase | | | slow phase | | |
|---|---|---|---|---|---|---|---|
| | | α (%) | τ (s) | $k$ ($s^{-1}$) | α (%) | τ (s) | $k$ ($s^{-1}$) |
| 23.2 | 8 | 76 | 11 | 0.091 | 24 | 33 | 0.030 |
| | 20 | 76.5 | 4 | 0.25 | 23.5 | 26 | 0.038 |
| 18 | 8 | 76.5 | 15 | 0.067 | 23.5 | 50 | 0.02 |
| | 20 | 76 | 6 | 0.17 | 24 | 47 | 0.021 |
| 13.2 | 8 | 77 | 19 | 0.053 | 23 | 80 | 0.0125 |
| | 20 | 77 | 8 | 0.125 | 23 | 77 | 0.013 |
| 9.5 | 8 | 77 | 23 | 0.043 | 23 | 120 | 0.0083 |
| | 20 | 77 | 10 | 0.010 | 23 | 116 | 0.0086 |
| 5 | 8 | 78 | 31 | 0.032 | 22 | 185 | 0.0054 |
| | 20 | 78.5 | 14 | 0.071 | 21.5 | 180 | 0.0056 |

height was used to quantitate the degree of hydrolysis at each time interval by using the 60-min sample as the 100% reference.

## Results

As in previous studies on the isomeric specificity of prolidase (Lin & Brandts, 1979a), aminopeptidase P (Lin & Brandts, 1979b, 1980), and trypsin (Lin & Brandts, 1983a,b), the hydrolyses of *N*-Cbz-Gly-Pro-MCA and *N*-Cbz-Gly-Pro-Leu-Gly catalyzed by the proline-specific endopeptidase were carried out at high enzyme activity to ensure that hydrolysis would be very fast with respect to the hydrolyzable form of the substrate. Figure 1a shows the degree of hydrolysis for *N*-Cbz-Gly-Pro-MCA as a function of time at 18 °C at two different ratios of enzyme activity to substrate concentration [8 (circles) and 20 units/μmol (triangles)]. In spite of the very high enzyme activity being used, two well-separated kinetic phases are seen. Figure 1a also shows that the rate of the fast phase significantly depends on enzyme activity, while the rate of the slow phase is independent of enzyme concentration. Semilog plots of substrate concentration vs. time (Figure 1b) indicate that both the fast and slow phases can be treated as first-order reactions with respect to time. From Figure 1b, the relaxation time of the slow phase was found (see Table I) to be the same at the two enzyme concentrations, 48.5 ± 1.5 s, while the relaxation times of the fast phase obtained after peeling off the slow phase were estimated to be ca. 15 s when the ratio of enzyme activity to substrate concentration is 8 units/μmol and ca. 6 s when the ratio is 20 units/μmol. The data of Figure 1b also show that the relative amplitudes of the two phases are virtually independent of enzyme concentration, i.e., 24 ± 1% for the slow phase and 76 ± 1% for the fast phase. The solid lines in Figure 1 represent the simulated kinetic curves by using the kinetic parameters obtained by the peel-off method (Table I). The excellent agreement indicates that the hydrolysis kinetics can be adequately described by two kinetic phases.

The rates of hydrolysis measured at a lower temperature of 9.5 °C are plotted in Figure 2. The kinetic patterns of hydrolysis at 9.5 °C are very similar to those at 18 °C. Two kinetic phases are also seen. For the hydrolysis experiment with higher enzyme activity (triangles), the two kinetic phases, like those at 18 °C, are well separated. The relaxation times of the fast and slow phases were found to be 10 and 116 s, respectively. The relative amplitudes are 23% for the slow phase and 77% for the fast phase. It is important to note that at the same level of enzyme activity (i.e., 20 units/μmol), the rate of hydrolysis for the fast phase decreases only 67% (6 to 10 s) from 18 to 9.5 °C, while the slow phase slows down almost 2.5 times, suggesting that the slow and fast phases have much different activation energies.

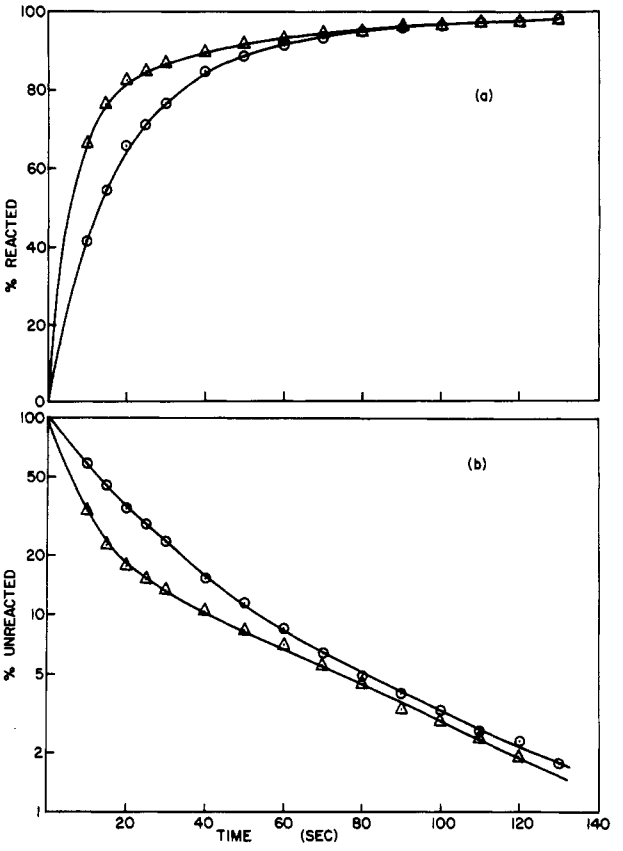


FIGURE 1: Rate of *N*-Cbz-Gly-L-Pro-MCA hydrolysis catalyzed by proline-specific endopeptidase at 18 °C: (a) the degree of hydrolysis vs. time; (b) semilog plots of substrate concentration vs. time. The ratios of enzyme activity to substrate concentration were 8 (circles) and 20 units/μmol (triangles). The solid lines represent the simulated kinetic curves for a biphasic system by using τ and α values in Table I. See text for experimental details.

Although not as well separated for the hydrolysis experiment with lower enzyme activity (circles in Figure 2), a fast and a slow phase are also evident. The amplitude and relaxation time for the slow phase obtained from Figure 2b are close to those at higher enzyme activity, as seen in Table I, while the fast phase is slowed down considerably, as expected.

The kinetic patterns of hydrolysis at other temperatures are very similar to those at 18 and 9.5 °C, and those data are listed in Table I. Arrhenius plots of $1/\tau$ values of the slow phase at two different enzyme concentrations are shown in Figure 3. An activation energy of 17 kcal/mol was found. In contrast, an activation energy of 9.4 kcal/mol was found for the fast phase in the similar plot (not shown), further suggesting that slow phase and fast phase represent different kinds of reaction.

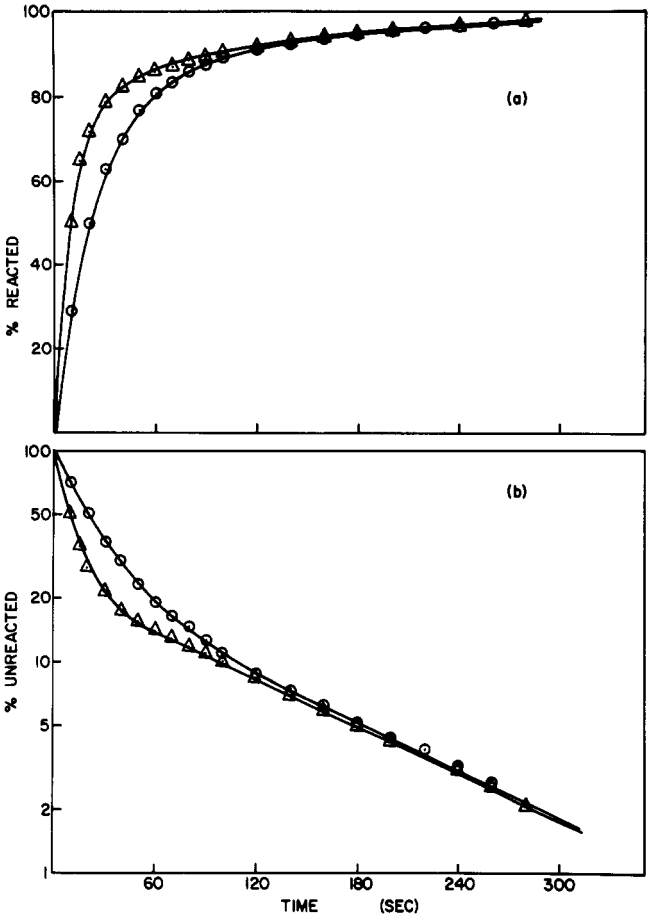


FIGURE 2: Rate of *N*-Cbz-Gly-L-Pro-MCA hydrolysis catalyzed by proline-specific endopeptidase at 9.5 °C: (a) the degree of hydrolysis vs. time; (b) semilog plots of substrate concentration vs. time. The ratios of enzyme activity to substrate concentration were 8 (circles) and 20 units/$\mu$mol (triangles). The solid lines represent the simulated kinetic curves by using $\tau$ and $\alpha$ values in Table I.

Data in Table I clearly show that the rate of the fast phase is directly proportional to enzyme activity while that of the slow phase is virtually independent of enzyme activity, suggesting that the slow phase must be rate limited by a structural change. In agreement with this, the enzyme activity has little effect on the relative amplitude of the two phases as mentioned earlier. These kinetic patterns are very similar to those reported earlier for Phe-Pro, Gly-Pro, and Gly-Pro-Ala when hydrolyzed by prolidase or aminopeptidase P (Lin & Brandts, 1979a,b) and for Gly-Gly-Lys-Phe-Pro when the active Lys–Phe bond was hydrolyzed by trypsin (Lin & Brandts, 1983a). In those cases, the isomeric specificity of proteases toward the active X–Pro bond (prolidase and aminopeptidase P) or a neighboring X–Pro bond (trypsin) was shown to be the cause of the two kinetic phases. Considering the similarity of kinetic patterns, such a conclusion can also be applied to the present results. The only active bond for the proline-specific endopeptidase in the substrate *N*-Cbz-Gly-Pro-MCA is the Pro-MCA bond, which is a monosubstituted amide bond and therefore likely to be in the all-trans form. However, from the behavior of similar model peptides, it can be surmised that the Gly–Pro bond preceding the active bond will exist as a mixture of cis and trans forms. Thus, the observation of two kinetic phases in the present study must mean that the proline-specific endopeptidase exhibits isomeric speificity toward the preceding Gly–Pro bond.

The hydrolysis of a second suubstrate, *N*-Cbz-Gly-Pro-Leu-Gly, in which a true peptide bond (i.e., Pro–Leu) is cleaved by the proline-specific endopeptidase, provides further evidence for the isomeric specificity of the enzyme toward the

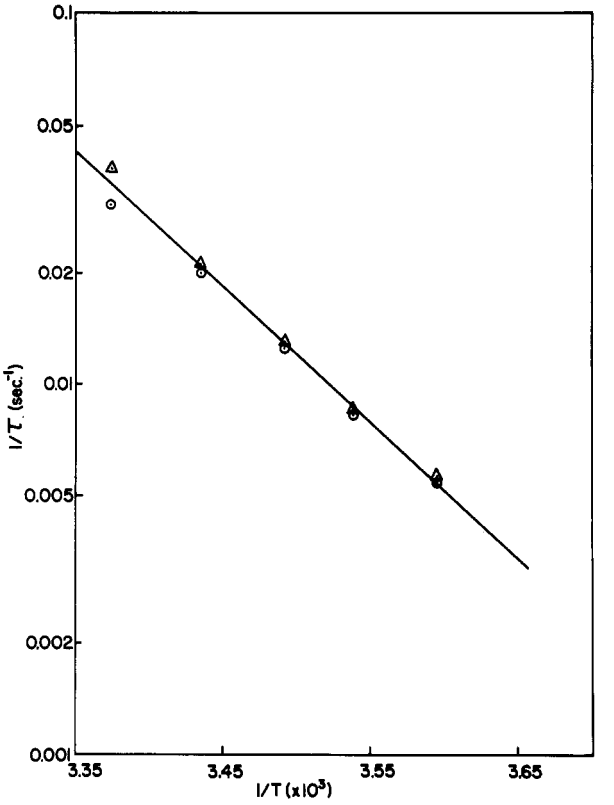


FIGURE 3: Arrhenius plot for the slow phase of hydrolysis of *N*-Cbz-Gly-Pro-MCA catalyzed by proline-specific endopeptidase. Circles and triangles represent the ratios of enzyme to substrate concentration of 8 and 20 units/$\mu$mol, respectively.

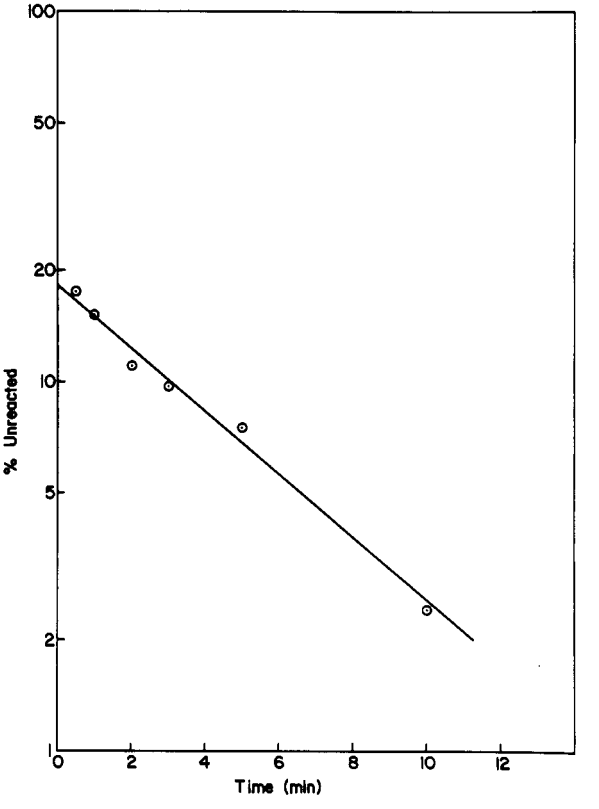


FIGURE 4: Semilog plot of the rate of hydrolysis of *N*-Cbz-Gly-L-Pro-Leu-Gly catalyzed by proline-specific endopeptidase at 5 °C. The ratio of enzyme to substrate concentration was ~580 units/$\mu$mol. See text for experimental details.

preceding X–Pro bond. The semilog plot of the kinetic data is shown in Figure 4. Two kinetic phases are clearly seen. While the rate of hydrolysis for the fast phase is too fast to be quantitatively determined, the relative amplitudes of the two phases and the relaxation time for the slow phase can be obtained from Figure 4. The relaxation time and amplitude for the slow phase were estimated to be about 5 min and 19%, respectively.

## Discussion

Studies on isomeric specificity so far suggest that protease activity is specific for trans peptide bonds. The results of the present study are consistent with such a conclusion; i.e., the proline-specific endopeptidase can only hydrolyze a Pro–Y bond (where Y is an amino acid residue) in substrates which contain the -X-Pro-Y- sequence when the *preceding* X–Pro bond is in the trans form. The cis form is not a hydrolyzable substrate and must isomerize to the trans form before it can be cleaved. This is then very analogous to the situation found to exist for trypsin, which will only hydrolyze a Lys–X bond in substrates containing a Lys-X-Pro sequence when the *following* X–Pro bond is in the trans form (Lin & Brandts, 1983a). This study further confirms our previous suggestion that the sterospecific requirement for endopeptidase activity extends beyond the active bond which is being cleaved.

This interpretation of our results (cf. Table I) suggests that in aqueous solution *N*-Cbz-Gly-Pro-MCA exists as a mixture of ca. 23% cis and 77% trans at the Gly–Pro bond in the temperature range 5–23.2 °C and that the relaxation time for cis-to-trans isomerization ranges from 30 s at 23 °C to 185 s at 5 °C. For the peptide substrate, *N*-Cbz-Gly-Pro-Leu-Gly, a mixture of ca. 19% cis and 81% trans exists in aqueous solution, and the relaxation time for cis-to-trans isomerization is 300 s at 5 °C. Because of low solubility, appropriate NMR studies cannot be carried out on these two substrates to confirm these values. However, there are NMR data in the literature regarding the cis/trans ratio and isomerization rate for peptides that are closely related to *N*-Cbz-Gly-Pro-MCA and *N*-Cbz-Gly-Pro-Leu-Gly. These data can be compared to our results determined by the ISP method. The tripeptide Gly-Pro-Ala and the tetrapeptide Gly-Gly-Pro-Ala were found to have 16–18% of the cis form at 25 °C in $D_2O$ (Grathwohl & Wüthrich, 1976). Another tripeptide, Gly-Pro-Gly, was reported to have 23% of the cis form in $D_2O$ at neutral pH at 30 °C (Fermandjian et al., 1975). Two similar tetrapeptides, Gly-Pro-Leu-Gly and Gly-Pro-Asn-Gly, were shown to have 20% of the cis form in $D_2O$ at 27 °C (Toma et al., 1980). More recently, the cis/trans ratio and rate of cis-to-trans isomerizaton of Gly-Gly-Pro-Ala were measured by Grathwohl & Wüthrich (1981). They reported that this tetrapeptide in $D_2O$ at 25 °C contains 20% of the cis form and the relaxation time of cis-to-trans isomerization at 25 °C was found to be about 33 s. Our data on the isomerism of *N*-Cbz-Gly-Pro-MCA, interpreted from hydrolysis measurements, are compatible with these NMR data, since we find 23% cis and a cis-to-trans relaxation time of ca. 30 s at 23.2 °C. The estimate of 19% cis for *N*-Cbz-Gly-Pro-Leu-Gly found by the ISP method is also in good agreement with that of Gly-Pro-Leu-Gly (20%) obtained by NMR. Furthermore, the activation energy of 17 kcal/mol found for the slow phase in the hydrolysis of *N*-Cbz-Gly-L-Pro-MCA is also in excellent agreement with values normally obtained for cis–trans isomerization of proline-containing peptides (Brandts et al., 1975; Lin & Brandts, 1979a, 1983a).

After the present study was completed, some results on the isomeric specificity of proline-specific proteases were reported by Fischer et al. (1983) by using peptidyl nitroanilides as substrates. Extensive measurements were carried out for dipeptidyl peptidase IV (EC 3.4.14), showing that this enzyme can only cleave the trans form of two dipeptide substrates, Gly-Pro-4-nitroanilide and Ala-Pro-4-nitroanilide. Another substrate, Ala-Pro-Pro-4-nitroanilide, was also studied in less detail to test the isomeric specificity of the proline-specific endopeptidase from *Flavobacterium*. The experiment was performed only at one temperature and at one enzyme concentration. The biphasic kinetics were attributed to the isomeric specificity of the enzyme, although specific conclusions about the importance of each of the two isomerizing bonds could not be made.

The substrate specificity of proline-specific endopeptidase has been extensively investigated by Yoshimoto et al. (1980). They reported that "the enzyme was inert toward denatured proteins such as egg white lysozyme, cytochrome *c* from horse muscle, and α-amylase from *Bacillus amyloliquefaciens*". However, they also showed that the proline bond near the carboxyl end in oxidized insulin B chain (30 amino acids) is readily cleaved by the proline-specific endopeptidase. This seems to indicate that this enzyme will probably cleave a proline bond of a large peptide fragment only if it is situated near the carboxyl end. A preliminary study in our lab on oxidized RNase A and RNase A has confirmed this to be the case. We have been able to investigate the isomerism of proline-114 using proline-specific endopeptidase after oxidized RNase A is subjected to a short pulse of chymotrypsin activity or after RNase A is subjected to a short pulse of pepsin and chymotrypsin activities (unpublished data). Thus, cleavage of the chain into small fragments then permits the utilization of proline-specific endopeptidase to determine the cis/trans characteristics.

In spite of the power of the ISP method, a necessary condition for the isomerism to be seen is that the cleavage of the peptide bond has to be much faster than cis-to-trans isomerization. There is at least one case where the ISP method has failed to detect the cis–trans isomerism of a proline peptide bond. It was reported by NMR that the relaxation time for the isomerization of the His–Pro bond in angiotensin II is much faster than 60 s at 5 °C (Galardy & Liakopoulou-Kyriakides, 1982). A hydrolysis study in our laboratory (unpublished results) on an angiotensin analogue ([$Sar^1$,$Ala^8$]angiotensin II), which was shown by NMR studies (Liakopoulou-Kyriakides & Galardy, 1979) to have 29% of the cis form at pH 7.5, shows only a fast cleavage phase ($\tau$ = 30 s at 5 °C) when assayed with the proline-specific endopeptidase. This confirms the results of the NMR study which showed that the rate of isomerization of the His–Pro bond in angiotensin II is very much fast than has been reported for any other proline-containing peptides in water.

In summary, this study shows that the protease activity of the proline-specific endopeptidase from *Flavobacterium* is absolutely specific for a trans peptide bond at the position immediately preceding the active bond to be cleaved. This is an important finding since it suggests that this enzyme can be utilized as the isomer-specific endopeptidase in studies designed to measure the rates of cis–trans isomerization of proline residues in proteins during unfolding and refolding reactions.

**Registry No.** *N*-Cbz-Gly-Pro-MCA, 68542-93-8; *N*-Cbz-Gly-Pro-Leu-Gly, 2646-64-2; proline-specific endopeptidase, 72162-84-6.

## References

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

Fermandjian, S., Tran-Dinh, S., Savrda, J., & Sala, E. (1975) *Biochim. Biophys. Acta 399*, 313.
Fischer, G., Heins, J., & Barth, A. (1983) *Biochim. Biophys. Acta 742*, 452.
Galardy, R. E., & Liakopoulou-Kyriakides, M. (1982) *Int. J. Pept. Protein Res. 20*, 144.
Grathwohl, C., & Wüthrich, K. (1976) *Biopolymers 15*, 2025.
Grathwohl, C., & Wüthrich, K. (1981) *Biopolymers 20*, 2623.
Liakopoulou-Kyriakides, M., & Galardy, R. E. (1979) *Biochemistry 18*, 1952.
Lin, L.-N., & Brandts, J. F. (1979a) *Biochemistry 18*, 43.
Lin, L.-N., & Brandts, J. F. (1979b) *Biochemistry 18*, 5017.
Lin, L.-N., & Brandts, J. F. (1980) *Biochemistry 19*, 3055.
Lin, L.-N., & Brandts, J. F. (1983a) *Biochemistry 22*, 553.
Lin, L.-N., & Brandts, J. F. (1983b) *Biochemistry 22*, 559.
Lin, L.-N., & Brandts, J. F. (1983c) *Biochemistry 22*, 564.
Lin, L.-N., & Brandts, J. F. (1983d) *Biochemistry 22*, 573.
Toma, F., Lam-Thanh, H., Piriou, F., Heindl, M.-C., Lintner, K., & Fermandjian, S. (1980) *Biopolymers 19*, 781.
Yoshimoto, T., & Tsuru, D. (1978) *Agric. Biol. Chem. 42*, 2417.
Yoshimoto, T., Ogita, K., Walter, R., Koida, M., & Tsuru, D. (1979) *Biochim. Biophys. Acta 569*, 184.
Yoshimoto, T., Walter, R., & Tsuru, D. (1980) *J. Biol. Chem. 255*, 4786.

# Acyl Carrier Protein from *Escherichia coli* I. Aspects of the Solution Structure As Evidenced by Proton Nuclear Overhauser Experiments at 500 MHz†

K. H. Mayo, P. M. Tyrell, and J. H. Prestegard*

ABSTRACT: The downfield aromatic (6–8 ppm) and upfield ring current shifted methyl regions (1–0 ppm) in the proton nuclear magnetic resonance spectrum of acyl carrier protein (ACP) from *Escherichia coli* have been examined at 500 MHz by using nuclear Overhauser methods. The data are analyzed in terms of the secondary structural model of Rock & Cronan (1979) [Rock, C. O., & Cronan, J. E., Jr. (1979) *J. Biol. Chem. 254*, 9778–9785], which suggests the existence of four $\alpha$-helical segments joined by three $\beta$-turns, and a short coil at the C terminus of the protein. Nuclear Overhauser effects among Tyr-71, Ile-69, Ile-72, and His-75 allow refinement of the secondary structure of the C terminus. Nuclear Overhauser effects among Tyr-71, Phe-28, and three Ile's also place stringent limitations on the folding of the four $\alpha$-helices. These data allow the proposal of a tertiary structural model for ACP.

Acyl carrier proteins (ACPs)[1] are known to play a key metabolic role in the synthesis of fatty acids in both prokaryotic and eukaryotic organisms (Thompson, 1981). Although the mode of association within the fatty acid synthetase system may vary depending on a particular organism's position on the evolutionary ladder, all known ACPs share the capacity to carry a growing fatty acid chain bound through a thio ester linkage to a 4′-phosphopantetheine prosthetic group, phosphodiester linked to a serine of ACP (Thompson, 1981). ACPs interact with at least 12 different enzymes involved in fatty acid synthesis and utilization. The diversity in this set of interactions and the suggestion that structural properties of ACPs may control relative enzymatic activities among members of this set (Schultz et al., 1969) have stimulated much interest in the structural characterization of ACP.

Among all known ACPs, ACP from *Escherichia coli* is perhaps the best characterized (Prescott & Vagelos, 1972; Vanaman et al., 1968a,b). A small protein of 8847 daltons, ACP from *E. coli* contains 77 amino acid residues, a large proportion being acidic (~29%) and a small proportion (~8%), clustered at the NH terminus, being positively charged. Although the sequence of ACP has been known for some time (Vanaman et al., 1968a,b), efforts to crystallize it have failed; therefore, no X-ray crystal structure exists. From an interpretation of optical rotary dispersion (Takagi & Tanford, 1968; Prescott et al., 1969) and circular dichroism studies (Schultz, 1975), ACP seems to possess a high $\alpha$-helix content. More recently, Rock & Cronan (1979) have applied the predictive algorithm of Chou & Fasman (1974a,b, 1978a,b) to ACP; $\alpha$-helical regions are predicted to exist between residues 3–21, 26–32, 37–53, and 58–69, with $\beta$-turns interrupting the $\alpha$-helical segments, yielding a rough, first approximation to the secondary structure.

In the present study, the objective is to test and refine the proposed secondary structure and gain some insight into the tertiary structure or folding of the ACP molecule. High-resolution $^1$H NMR (500 MHz) methods, analogous to those applied to small soluble proteins by other authors over the past few years (Gordon & Wüthrich, 1978; Poulsen et al., 1980; Wagner & Wüthrich, 1979; Dobson et al., 1980), have been employed. We present, here, results based largely on cross-relaxation rate measurements (NOEs) on reduced acyl carrier protein (ACP-SH) from *E. coli* B cells. Under suitable conditions, measured cross-relaxation rates can be interpreted in terms of interresidue (proton) distances and the general characteristics of the predictive model of Chou and Fasman

† From the Department of Chemistry, Yale University, New Haven, Connecticut 06511. *Received May 3, 1983.* This work was supported by a grant from the National Science Foundation (PCM-7821101) and benefited from NMR facilities made available through Grant CHE-7916210 from the National Science Foundation.

[1] Abbreviations: ACP, acyl carrier protein; ACP-SH, reduced acyl carrier protein; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; NOE, nuclear Overhauser effect; FID, free induction decay.

0006-2960/83/0422-4485$01.50/0 © 1983 American Chemical Society