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Inhibition of Rabbit Brain Prolyl Endopeptidase by *N*-Benzyloxycarbonyl-Prolyl-Prolinal, a Transition State Aldehyde Inhibitor

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Abstract: Prolyl endopeptidase cleaves peptide bonds on the carboxyl side of proline residues within a peptide chain. The enzyme readily degrades a number of neuropeptides including substance P, neurotensin, thyrotropin-releasing hormone, and luteinizing hormone-releasing hormone. The finding that the enzyme is inhibited by benzyloxycarbonyl-prolyl-proline, with a K_i of 50 μM, prompted the synthesis of benzyloxycarbonyl-prolyl-prolinal as a potential transition state analog inhibitor. Rabbit brain prolyl endopeptidase was purified to homogeneity for these studies. The aldehyde was found to be a remarkably potent inhibitor of prolyl endopeptidase with a K_i of 14 nM. This K_i is more than 3000 times lower than that of the corresponding acid or alcohol. By analogy with other transition state inhibitors, it can be assumed that binding of the prolinal residue to the S_1 subsite and the formation of a hemiacetal with the active serine of the enzyme greatly contribute to the potency of inhibition. The specificity of the inhibitor is indicated by the finding that a variety of proteases were not affected at concentrations 150 times greater than the K_i for prolyl endopeptidase. The data indicate that benzyloxycarbonyl-prolyl-prolinal is a specific and potent inhibitor of prolyl endopeptidase and that consequently it should be of value in *in vivo* studies on the physiological role of the enzyme. **Key Words:** Prolyl endopeptidase—*N*-benzyloxycarbonyl-prolyl-prolinal—Rabbit brain—Inhibition—Neuropeptides. **Wilk S. and Orlowski M.** Inhibition of rabbit brain prolyl endopeptidase by *N*-benzyloxycarbonyl-prolyl-prolinal, a transition state aldehyde inhibitor. *J. Neurochem.* **41**, 69–75 (1983).

Prolyl endopeptidase, an enzyme capable of degrading many neuropeptides, cleaves peptidylprolyl peptide and peptidylprolyl amino acid bonds (Orlowski et al., 1979). Immunological and biochemical studies (Hersh, 1981; Yoshimoto et al., 1981; Andrews et al., 1982) have clearly shown that prolyl endopeptidase from brain is identical to the "post-proline cleaving enzyme" first described by Walter et al. in 1971, and also to enzymatic activities designated as thyrotropin-releasing hormone (TRH) deamidase (Rupnow et al., 1979) and kininase B (Oliveira et al., 1976). The enzyme is rather uniformly distributed in brain (Orlowski et al., 1979; Andrews et al., 1980) and is found largely in the cytosolic fraction (Rupnow et al., 1979; Kreider et al., 1981; Dresdner et al., 1982). It is a serine protease (Yoshimoto et al., 1977) with a marked sensitivity to sulfhydryl reagents (Orlowski et al., 1979; Andrews et al., 1980).

Although prolyl endopeptidase may play a prominent role in neuropeptide degradation, little is known about its function *in vivo*. The availability of a selective and potent inhibitor would be a definite asset in studies investigating the physiological role of this enzyme. Serine and sulfhydryl proteases are inhibited by peptide aldehyde analogs of substrates by the formation of transition-state-like intermediates (Westerik and Wolfenden, 1972; Thompson, 1973). We report here the synthesis and properties of *N*-benzyloxycarbonyl-prolyl-prolinal

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Abbreviations used: Bz, α-*N*-Benzoyl; Z, *N*-Benzyloxycarbonyl; Hip, Hippuryl; LHRH, Luteinizing hormone-releasing hormone; 2NA, 2-Naphthylamide; *p*-NA, *p*-Nitroanilide; SM, Sulfamethoxazole; TMS, Trimethylsilane; TRH, Thyrotropin-releasing hormone.

The nomenclature of Schechter and Berger (1967) is used to describe the interaction of substrate with enzyme. Amino acid residues are designated P_1, P_2, P_3 . . . in the N-terminal direction and P_1', P_2', P_3' in the C-terminal direction of the bond undergoing cleavage. The corresponding subsites of the enzyme are designated S_1, S_2, S_3, etc.

(Z-Pro-Prolinal), a potent and specific peptide aldehyde inhibitor of prolyl endopeptidase.

MATERIALS AND METHODS

The following substances were obtained from Sigma Chemical, St. Louis, MO: Z-Pro, α-*N*-benzoyl-arginine-2-naphthylamide (Bz-Arg-2NA), α-*N*-benzoyl-arginine-*p*-nitroanilide (Bz-Arg-*p*-NA), papain type II, α-chymotrypsin type IV, and *N*-hydroxysuccinimide. Dicyclohexylcarbodiimide, 1-(3-dimethylamino propyl)-3-ethyl carbodiimide·HCl and L-prolinol were obtained from Aldrich Chemical, Milwaukee, WI. Z-Pro-Pro, Z-Ala-Pro, Pro-2NA, and Gly-Pro-2NA were products of Bachem, Torrance, CA. Trypsin was purchased from Worthington Biochemical, Freehold, NJ. Z-Gly-Gly-Leu-*p*NA, Z-D-Ala-Leu-Arg-2NA, Z-Leu-Leu-Glu-2NA, and hippuryl-Arg-Arg-Leu-2NA (Hip-Arg-Arg-Leu-2NA) were synthesized in this laboratory as previously described (Wilk and Orlowski, 1980; Orlowski and Wilk, 1981*a*). Eastman Chromogram Silica Gel Sheets were purchased from Eastman Chemical, Rochester, NY. The HPLC column used was an IBM 5 μm C18 (250 mm × 4.5 mm), IBM Instruments, Danbury, CT. Aminopeptidase M was obtained from Boehringer-Mannheim, Indianapolis, IN. Cation-sensitive neutral endopeptidase was purified from bovine pituitaries as described previously (Wilk and Orlowski, 1980). Membrane-bound neutral metalloendopeptidase was purified from rabbit kidney cortex using the procedure described by Orlowski and Wilk (1981*a*) for purification of the enzyme from pituitary. Diaminopeptidase IV was purified to apparent homogeneity from rabbit kidney cortex essentially as described by Yoshimoto and Walter (1977). Phenyl Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Piscataway, NJ.

Synthesis of Z-Pro-Prolinal

N-hydroxysuccinimide ester of Z-Pro. The *N*-hydroxysuccinimide ester of Z-Pro was prepared according to the method of Anderson et al. (1964). Into a 50-ml Erlenmeyer flask was added 10 mmol Z-L-Pro (2.49 g) dissolved in 20 ml tetrahydrofuran. The solution was cooled in an ice-salt bath and 10 mmol *N*-hydroxysuccinimide (1.15 g) was added. Next 10 mmol dicyclohexylcarbodiimide (2.06 g) was added and the solution was stirred over a magnetic stirrer in the cold for 21 h. The solution was then filtered and the precipitate was washed with tetrahydrofuran. The solvent was evaporated in a flash evaporator to yield a semisolid. Addition of 2-propanol induced crystallization of the *N*-hydroxysuccinimide active ester of Z-Pro. Yield was 2.68 g, 81%.

Z-Pro-Prolinol. Into a 50-ml Erlenmeyer flask was placed 2 mmol Z-Pro-*N*-hydroxysuccinimide ester (0.69 g) and 11 ml tetrahydrofuran. Next 2 mmol L-prolinol was added and the mixture was stirred at room temperature for 72 h. The tetrahydrofuran was removed by flash evaporation and the residue dissolved in 20 ml ethyl acetate. The ethyl acetate was washed with 10 ml 1 *M* Na_2CO_3, and then dried over anhydrous Na_2SO_4. The solvent was evaporated to yield 0.53 g (1.6 mmol) of a white solid. MP = 94–95°C. HPLC analysis: single peak Rt = 17.5 min.

Z-Pro-Prolinal. The oxidation of Z-Pro-Prolinol to Z-Pro-Prolinal was carried out essentially as described by Thompson (1977). A mixture of 0.8 mmol Z-Pro-Prolinol, 500 mg 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide·HCl, and 2 ml redistilled dimethylsulfoxide was stirred at room temperature for 10 min. Next 100 μl 2 *M* anhydrous H_3PO_4 in dimethylsulfoxide was added, and the reaction allowed to proceed for 2 h. The reaction was terminated by the addition of 5 ml of 1 *M* potassium phosphate buffer (pH 7.5) and the product extracted into 5 ml ethyl acetate. The combined ethyl acetate fractions were dried over anhydrous sodium sulfate. The ethyl acetate was evaporated to dryness, and the residue dissolved in 5 ml ethanol and added to a solution of 4 g sodium bisulfite in 7 ml water with vigorous stirring. After 10 min, the ethanol was evaporated to about 7 ml of solution. The aqueous residue was extracted twice with 5 ml ether to remove unreacted Z-Pro-Prolinol. The bisulfite adduct of Z-Pro-Prolinal was decomposed by the addition of solid Na_2CO_3 to a final pH of 9. After 5 min, the free aldehyde was extracted into two portions, 5 ml each, of ether. Evaporation of the ether gave 43 mg of an oil (0.13 mmol Z-Pro-Prolinol). Thin-layer chromatography on Eastman Chromogram Silica Gel sheets in a chloroform: acetone system (70:30) gave a single spot when viewed under an ultraviolet lamp. Spraying with 0.5% 2,4-dinitrophenylhydrazine in 2 *M* HCl gave a yellow spot coincident with the spot observed under ultraviolet light (R_f = 0.8). HPLC analysis: single peak Rt = 19.0 min. Aldehyde determination: theoretical, 0.50 μmol/ml; found, 0.55 μmol/ml.

^{1}H-NMR ($CDCl_3$, in ppm downfield from trimethylsilane (TMS)): δ9.48 (broad, J = 5 Hz, CHO), δ7.33 (S, aromatic H), δ5.10 (s, Ar–C$\underline{H}_2$–0), δ4.55 (m, N–C$\underline{H}$–CO), δ3.58 (two superimposed t, J = 5.3 Hz, N–C$\underline{H}$–C$\underline{H}_2$), δ1.99 (m, N–CH_2–C$\underline{H}_2$–C$\underline{H}_2$–).

^{13}C-NMR ($CDCl_3$, in ppm downfield from TMS): δ198 (CHO), 168 (CH–$\underline{C}$O–N), 146 (O–$\underline{C}$O–N), 128.6–127.9 (ArC), 67.1 (Ar–C$\underline{H}_2$O), 64.5 and 58.13 (NH–$\underline{C}$H–$\underline{C}$O), 46.8 (N–$\underline{C}H_2CH_2$), 29.2 (CH_2–$\underline{C}H_2$–CO–H), 25.5–24.5 (N–CH_2–$\underline{C}H_2$).

Synthesis of Z-Pro-Pro-2NA

This compound was prepared by reacting L-Pro-2NA with *N*-hydroxysuccinimide ester of Z-Pro. The active ester (1.19 mmol, 460 mg) was dissolved in 20 ml tetrahydrofuran. L-Pro-2NA·HCl (1.19 mmol, 330 mg) was added and the mixture stirred at room temperature. To this suspension was added 1.19 mmol of triethylamine and stirring was continued for 15 h. The mixture was then filtered, the precipitate washed with tetrahydrofuran, and the filtrate combined with the washings. After removal of the solvent by flash evaporation, the solid product was dissolved in ethyl acetate and transferred to a separatory funnel. The ethyl acetate solution was washed four times with 30 ml 1 *M* HCl, two times with 30 ml 0.5 *M* Na_2CO_3, and finally once with 30 ml water. The ethyl acetate solution was dried over anhydrous $MgSO_4$, filtered, and evaporated to yield the product. MP = 87°C; yield = 48%; HPLC analysis: single peak Rt = 28.1 min.

Determination of enzyme activities

The activities of all enzymes were measured by determining the release of aromatic amines from the appropri-

ate chromogenic substrates, using the diazotization procedure of Bratton and Marshall (1939) as modified by Goldbarg and Rutenberg (1958).

Prolyl endopeptidase. Activity was determined using Z-Gly-Pro-sulfamethoxazole (Z-Gly-Pro-SM) as substrate as described by Orlowski et al. (1979). The incubation mixture (final volume 0.25 ml) contained substrate (1.0 m*M*), dithiothreitol (0.4 m*M*), enzyme (0.1 unit), and Tris-HCl buffer (0.1 *M;* pH 8.3). Samples were incubated for 15 min at 37°C and the reaction was stopped by addition of 0.25 ml 10% trichloroacetic acid.

The enzymes listed below were measured at a final substrate concentration of 0.25 m*M*. Incubations were carried out in a 0.05 *M*, pH 7.5 Tris-HCl buffer at 37°C in a final volume of 0.25 ml.

Cation-sensitive neutral endopeptidase. The three activities associated with this enzyme complex were measured as described (Wilk and Orlowski, 1980). The chymotrypsinlike activity was measured with Z-Gly-Gly-Leu-*p*NA, the trypsinlike activity with Z-D-Ala-Leu-Arg-2NA, and the peptidylglutamylpeptide bond hydrolyzing activity with Z-Leu-Leu-Glu-2NA.

Membrane-bound metalloendopeptidase. This enzyme was measured in a coupled assay using Hip-Arg-Arg-Leu-2NA and aminopeptidase M as described (Orlowski and Wilk, 1981*a*).

Trypsin. Activity was determined with the substrate Bz-Arg-*p*NA.

Chymotrypsin. Activity was determined with the substrate Z-Gly-Gly-Leu-*p*NA.

Papain. Activity was determined with the substrate Bz-Arg-2NA in the presence of dithiothreitol (0.4 m*M*).

Diaminopeptidase IV. Activity was determined with the substrate Gly-Pro-2NA.

Purification of prolyl endopeptidase

Prolyl endopeptidase was purified to apparent homogeneity by addition of a phenyl Sepharose chromatographic step to the procedure described by Orlowski et al. (1979). The enzyme after step 7 of the purification procedure was dialyzed against a Tris-EDTA buffer (10 m*M;* pH 7.3) containing 10 m*M* 2-mercaptoethanol and 25% ammonium sulfate by saturation. A 0.7 cm × 17 cm column of phenyl Sepharose was equilibrated with the dialyzing buffer. The dialyzed enzyme was applied to the column and eluted with a linear gradient of 100 ml of dialyzing buffer against 100 ml of the buffer containing no ammonium sulfate. The enzyme eluted as a sharp peak at the end of the gradient.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in a Tris-EDTA buffer (0.1 *M;* pH 8.3) containing 10 m*M* 2-mercaptoethanol in 7% gels as described by Weber and Osborn (1969). A 10-μg sample of protein was layered on the surface of each gel and a current of 5 mA per tube was applied for a period of about 2 h. Gels were stained for protein with Coomassie brilliant blue.

HPLC analysis

HPLC was performed on a Perkin-Elmer Series 2 liquid chromatograph equipped with an LC-55 variable wavelength spectrophotometric detector. A 4.5 mm × 250 mm IBM 5-μm C18 reverse-phase column was equilibrated with a mixture of acetonitrile (20%) in 0.05 *M* potassium phosphate buffer, pH 2.0. Peptides were eluted with a linear acetonitrile gradient and the effluent was monitored at 210 nm. The flow rate was 1 ml/min and the concentration of acetonitrile was increased at a rate of 1%/min. Generally 10 μl of a 10 m*M* solution in methanol of the compound to be analyzed was injected onto the column, and peptides were monitored at a sensitivity of 0.5 absorbance units full scale.

Determination of kinetic constants of substrates and inhibitors

Solutions of naphthylamide substrates were prepared in dimethylsulfoxide. Dilutions were made in dimethylsulfoxide. Incubation mixtures contained 10 μl of substrate in a final volume of 250 μl (dimethylsulfoxide concentration, 4%). Prolyl endopeptidase activity is not affected by 4% dimethylsulfoxide. A stock solution of Z-Pro-Prolinal was prepared in methanol. Dilutions were made with water. All other compounds were dissolved in Tris-HCl (0.1 *M;* pH 8.3). Inhibitors were preincubated for 15 min at 37°C with the enzyme. The reaction was initiated by addition of substrate. K_m values were obtained by a computerized least-squares analysis of the Lineweaver-Burk plot. Values of k_{cat} were computed assuming a molecular weight of 66,000 for the enzyme (Orlowski et al., 1979). Inhibition constants were obtained graphically by the method of Dixon (1953). In the case of the tight binding inhibitor Z-Pro-Prolinal, inhibition constants were obtained by the method of Henderson (1972). In this method the inhibitor concentration divided by the degree of inhibition is plotted on the ordinate and the velocity without inhibitor divided by the velocity with inhibitor is plotted on the abscissa. A linear plot is generated and the mechanism of inhibition obtained from the variation of slope with substrate concentration.

Determination of aldehyde concentration

Aldehyde concentration was determined spectrophotometrically as described by Reingold and Orlowski (1979). Quantitation was based on formation of the 2,4-dinitrophenylhydrazone using butyraldehyde as standard.

NMR spectra

NMR spectra were recorded on a Varian FT 80A instrument and values are reported downfield from TMS = 0 in parts per million δ.

RESULTS

Addition of a phenyl Sepharose chromatography step to the purification procedure of prolyl endopeptidase described by Orlowski et al. (1979) was sufficient to yield an apparently homogeneous preparation of the enzyme. This is indicated by the finding of a single protein band on polyacrylamide gel electrophoresis. The recovery of activity in this hydrophobic chromatography step was about 50%. The isolated enzyme has a specific activity of 2200 units/mg protein, one unit being defined as the amount of enzyme required to release 1 μmol of SM per hour from the chromogenic substrate Z-Gly-Pro-SM.

The kinetic constants of the enzyme for the hy-

TABLE 1. *Kinetic constants for the hydrolysis of several substrates by prolyl endopeptidase*

Substrate $P_3-P_2-P_1-P_1'-$	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Z-Gly-Pro-SM	0.096	44.4	463
Z-Gly-Pro-2NA	0.13	21.4	165
Z-Phe-Pro-2NA	0.097	2.2	22.7
Z-Pro-Pro-2NA	0.10[a]	0.14	1.4

[a] Derived from the K_i value (Fig. 1) assuming that for a competitive inhibitor, $K_m = K_i$.

drolysis of several chromogenic substrates are given in Table 1. It can be seen that the K_m values for all four substrates are quite similar, but that they differ markedly with respect to their catalytic rate constants. The SM substrate is more efficiently hydrolyzed than the corresponding naphthylamide, suggesting that SM is a better leaving group than 2-naphthylamine. This justifies the use of Z-Gly-Pro-SM as substrate for the routine determination of prolyl endopeptidase activity. Replacement of the glycine residue in Z-Gly-Pro-2NA by phenylalanine markedly decreases the catalytic rate constant and a still more pronounced decrease in k_{cat} is seen when position P_2 is occupied by a proline residue, as in Z-Pro-Pro-2NA. In fact Z-Pro-Pro-2NA is so slowly hydrolyzed that this compound can serve as a competitive inhibitor. Inhibition of the hydrolysis of Z-Gly-Pro-SM by Z-Pro-Pro-2NA is shown in Fig. 1. The K_i of Z-Pro-Pro-2NA is 0.10 mM. The K_m of this compound was obtained by assuming that for a competitive inhibitor $K_m = K_i$. It is likely that the more hydrophobic phenylalanyl and prolyl groups contribute to this decrease in reaction rate. Nonproductive binding of the P_2 prolyl residue to the S_1 subsite of the enzyme is another factor to be considered as contributing to the low k_{cat} observed with Z-Pro-Pro-2NA.

The inhibitory constants of several proline-containing derivatives are given in Table 2. Previous studies on the interaction between lamb kidney prolyl endopeptidase and a series of proline-containing peptides had shown that compounds of the general structure Z-Pro-X, where X = amino acid residue, act as competitive inhibitors of the enzyme (Koida and Walter, 1976). We have found that the rabbit brain enzyme is also inhibited by such peptides. Thus, for example, Z-Pro-Ala inhibits the enzyme with a K_i in the millimolar range (Table 2). It is, however, of interest that Z-Ala-Pro is a more potent competitive inhibitor than Z-Pro-Ala. Since compounds of the general structure Z-Ala-Pro-X are substrates for the enzyme, Z-Ala-Pro can be regarded as a potential product of a prolyl endopeptidase catalyzed reaction, and accordingly the inhibition by this peptide may be a consequence of its binding to the S_1 and S_2 subsites of the enzyme. By contrast, Z-Pro-Ala is not a substrate and therefore its inhibitory action may be the result of a weak binding of the Ala residue to the S_1 subsite of the enzyme (peptides containing three to five Ala residues are weakly hydrolyzed by lamb kidney prolyl endopeptidase; Walter and Yoshimoto, 1978), or to the binding of the Pro residue to the S_2 subsite. That this last mode of binding contributes to the inhibitory potency is indicated by the finding that replacement of the Ala residue in Z-Ala-Pro by a Pro residue decreased the K_i from 160 μM to 50 μM.

The finding that Z-Pro-Pro and the corresponding alcohol Z-Pro-Prolinol are good competitive inhibitors of the enzyme induced us to synthesize Z-Pro-Prolinal as a potential transition state analog inhibitor of the enzyme. The aldehyde was efficiently synthesized from Z-Pro-Prolinol using the carbodiimide-dimethylsulfoxide reagent of Pfitzner and Moffatt (1965). The oily product obtained was pure as judged by TLC and HPLC. When a standard solution of Z-Pro-Prolinal was prepared and the aldehyde concentration of this solution was determined after conversion to the 2,4-dinitrophenylhydrazone (Reingold and Orlowski, 1979), the obtained results were in excellent agreement with the theoretical concentration. ^1H and ^{13}C NMR analysis confirmed the structure. Stock solutions are routinely stored at −20°C, and are stable under these conditions.

Z-Pro-Prolinal was found to be a potent inhibitor

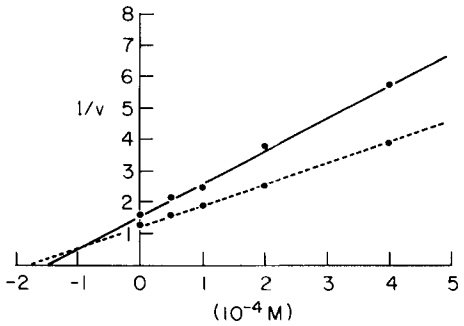

FIG. 1. A Dixon plot of the inhibition of prolyl endopeptidase by Z-Pro-Pro-2NA. The inhibitor was preincubated for 15 min at 37°C with the enzyme and the reaction was initiated by addition of Z-Gly-Pro-SM as described in Materials and Methods. Inhibitor concentration on abscissa. (– – –), concentration of substrate is 0.4 mM; (———) concentration of substrate is 0.2 mM.

TABLE 2. *Inhibitory constants of several proline-containing derivatives*

Derivative	K_i (μM)	Mode of inhibition
Z-Pro-Ala	2200	Competitive
Z-Ala-Pro	160	Competitive
Z-Pro-Pro	50	Competitive
Z-Pro-Prolinol	50	Competitive
Z-Pro-Prolinal	0.014	Noncompetitive

Kinetic constants were obtained as described in Materials and Methods.

of the enzyme. Initial analysis by the method of Dixon (1953) revealed noncompetitive inhibition with a K_i of 9 nM (Fig. 2). However, as discussed by Goldstein (1944) the boundary for zone A behavior in which the Michaelis-Menten equation can be validly applied is $E/K_i = 0.1$, where E = total concentration of enzyme. Since it can be calculated that in these studies this ratio approximates 0.2, the kinetic analysis of this tight binding inhibitor was also carried out by the method of Henderson (1972). The slopes of the Henderson plot at three substrate concentrations were essentially invariant, again indicating noncompetitive inhibition. The K_i values obtained from the slopes of these plots ranged from 12 to 16 nM. The aldehyde derivative is therefore three orders of magnitude more potent than the corresponding acid and alcohol as a prolyl endopeptidase inhibitor. Inhibition was not readily reversible. Prolonged dialysis of the aldehyde-treated enzyme led to restoration of only 20% of the original activity.

To evaluate the specificity of Z-Pro-Prolinal as an inhibitor of prolyl endopeptidase, this compound

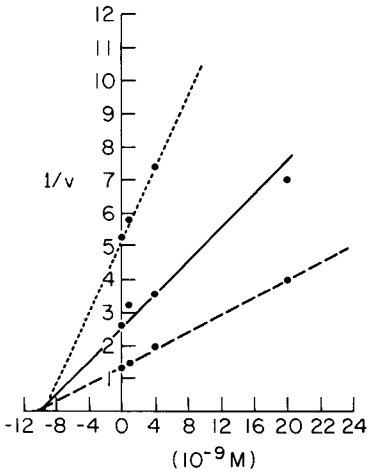

FIG. 2. A Dixon plot of the inhibition of prolyl endopeptidase by Z-Pro-Prolinal. The inhibitor was preincubated for 15 min at 37°C with the enzyme and the reaction was initiated by addition of Z-Gly-Pro-SM as described in Materials and Methods. Inhibitor concentration on abscissa. (·····) concentration of substrate is 0.1 mM; (———) concentration is 0.2 mM; (– – –) concentration of substrate is 0.4 mM.

was tested on a number of endopeptidases and on diaminopeptidase IV, also known as "postproline diaminopeptidase" (Yoshimoto and Walter, 1977). The concentration of Z-Pro-Prolinal used was 2 μM or approximately 150 times greater than its K_i for prolyl endopeptidase. At this inhibitor concentration trypsin, chymotrypsin, papain, membrane-bound metalloendopeptidase (Orlowski and Wilk, 1981*a*), diaminopeptidase IV, and the trypsinlike, chymotrypsinlike, and peptidylglutamyl-peptide hydrolyzing activities of the multicatalytic protease complex (Orlowski and Wilk, 1981*b*) were not inhibited.

DISCUSSION

Peptide aldehyde analogs of the acyl portion of protease substrates are potent competitive inhibitors of serine and sulfhydryl proteases (Westerik and Wolfenden, 1972; Thompson, 1973). Interaction of the aldehyde with the active site residue results in the formation of an analog of the tetrahedral transition state intermediate of the enzyme-substrate complex. It is notable that several protease inhibitors isolated from microbial cultures such as leupeptin, antipain, chymostatin, and elastatinal are peptide aldehydes (Umezawa and Aoyagi, 1977). The structure of the inhibitor described here is based on the specificity of prolyl endopeptidase for peptide bonds at the carboxyl end of prolyl residues, and the finding that Z-Pro-Pro and Z-Pro-Prolinol are good competitive inhibitors of the enzyme.

The observation that the K_i of Z-Pro-Prolinal is more than three orders of magnitude lower than that of the corresponding alcohol or acid is consistent with its designation as a transition state inhibitor of the enzyme. Transition state peptide aldehydes commonly inhibit serine and sulfhydryl proteases in a competitive manner, apparently by forming hemiacetals or thiohemiacetals with the "active" serine or cysteine of the enzyme. It is therefore of interest that the inhibition of prolyl endopeptidase by Z-Pro-Prolinal is noncompetitive in nature and not readily reversible by dialysis. Although it has been reported that leupeptin inhibits trypsin activity toward the amide substrate benzoyl-Arg-*p*-nitroanilide noncompetitively (Aoyagi and Umezawa, 1975), the reason for this anomaly is not known. Prolyl endopeptidase, while being a serine protease (Yoshimoto et al., 1977), contains a thiol group near the active site whose integrity is necessary for expression of enzymatic activity (Orlowski et al., 1979; Andrews et al., 1980; Yoshimoto et al., 1981). One can speculate that the prolinal residue of the inhibitor, while predominantly interacting with the "active serine" residue of the enzyme, can also interact with the sulfhydryl group near the active site to form a thiohemiacetal, and that at this mode of binding the inhibitor is not readily displaced by

the substrate. Further detailed studies are needed to elucidate the nature of the interaction of Z-Pro-Prolinal with prolyl endopeptidase.

Z-Pro-Prolinal was tested for its capacity to inhibit other proteolytic enzymes at a concentration more than 150-fold greater than its K_i for prolyl endopeptidase. At this concentration it failed to inhibit the classic serine endopeptidases trypsin and chymotrypsin, and the classic sulfhydryl protease papain. Diaminopeptidase IV, a serine protease, removes dipeptide moieties from oligopeptide substrates in which proline is present in the penultimate position (McDonald et al., 1971). This enzyme, which may be regarded as a postproline diaminopeptidase (Yoshimoto et al., 1978), is not inhibited by Z-Pro-Prolinal, presumably because this compound lacks a free α-amino group. We also tested the inhibitor on two neutral endopeptidases isolated in our laboratory from brain and pituitary. Cation-sensitive neutral endopeptidase exhibits three distinct catalytic activities and apparently represents a multicatalytic protease complex (Orlowski and Wilk, 1981*b*). None of the three activities is inhibited by Z-Pro-Prolinal. Membrane-bound metalloendopeptidase, an enzyme apparently identical to an activity described as "enkephalinase" (Almenoff et al., 1981), also is not inhibited. Z-Pro-Prolinal therefore seems to be a selective inhibitor of prolyl endopeptidase.

Assuming that prolyl endopeptidase is analogous to other serine proteases and contains a histidine group at the active site, Yoshimoto et al. (1977) prepared a series of chloromethyl ketones as alkylating agents. They found compounds of the type Z-Gly-Pro-CH_2Cl to inhibit the enzyme irreversibly. These inhibitors also appeared to be specific, as they failed to inhibit trypsin, α-chymotrypsin, elastase, and papain. It is unclear, however, whether their specificity for prolyl endopeptidase is as great as Z-Pro-Prolinal, since the concentrations of chloromethyl ketones tested were the same for the other proteolytic enzymes as for prolyl endopeptidase. Z-Pro-Prolinal did not inhibit other proteolytic enzymes at concentrations more than 150-fold greater than its K_i for prolyl endopeptidase. The chloromethyl ketones are also not as potent as Z-Pro-Prolinal. The lamb brain enzyme was reported to be 87% inhibited by Z-Gly-Pro-CH_2Cl at a concentration of $1 \times 10^{-5} M$ (Yoshimoto et al., 1981).

Prolyl endopeptidase may play a prominent role in the regulation of the biological activities of various neuropeptides. A number of peptides such as substance P, neurotensin, luteinizing hormone-releasing hormone (LHRH), TRH, bradykinin, and angiotensin II contain proline residues and are cleaved by prolyl endopeptidase (Wilk et al., 1979; Orlowski et al., 1979). Although the physical and catalytic properties of this enzyme have been the subject of intensive investigation, almost nothing is known about its physiological role. The availability of a potent and specific inhibitor of prolyl endopeptidase should facilitate studies on the physiological role of this enzyme *in vivo*. To this end we have recently demonstrated that Z-Pro-Prolinal inhibits prolyl endopeptidase *in vivo* (Friedman et al., 1983).

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