Biochem. J. (1990) 271, 559–562 (Printed in Great Britain)

Slow tight-binding inhibition of prolyl endopeptidase by benzyloxycarbonyl-prolyl-prolinal

Alice V. BAKKER, Stanley JUNG, Robin W. SPENCER, Fredric J. VINICK and W. Stephen FARACI*
Department of Medicinal Chemistry, Central Research Division, Pfizer Inc., Groton, CT 06340, U.S.A.

Prolyl endopeptidase is a serine proteinase that specifically cleaves peptides on the carboxy side of proline residues. Wilk & Orlowski [(1983) J. Neurochem. **41**, 69–75] have shown that benzyloxycarbonyl-prolyl-prolinal (Z-prolyl-prolinal) is a potent inhibitor of prolyl endopeptidase. We show that Z-prolyl-prolinal is a slow-binding inhibitor of mouse brain prolyl endopeptidase with K_i 0.35±0.05 nM. Kinetic analysis indicates that the mechanism is a simple, but slow, reversible equilibrium between free and bound enzyme ($E+I \rightleftharpoons EI$) with rate constants for association (k_{on}) and dissociation (k_{off}) of 1.6×10^5 $M^{-1}\cdot s^{-1}$ and approx. 4×10^{-5} s^{-1} respectively. Slow-binding inhibition is dependent on the presence of the aldehyde group since the alcohol (Z-prolyl-prolinol) is a rapid and 50000-fold poorer inhibitor (K_i 19 μM). Prolyl endopeptidase from human brain is also inhibited by Z-prolyl-prolinal with kinetics similar to those of the mouse brain enzyme.

INTRODUCTION

Prolyl endopeptidase is a serine proteinase that specifically cleaves peptidyl proline bonds (Wilk, 1983). The enzyme is known to hydrolyse many biologically active peptides, including substance P, neurotensin, angiotensin II, oxytocin and bradykinin (Orlowski *et al.*, 1979). Although a serine proteinase, the enzyme is quite sensitive to thiol-blocking reagents (e.g. *p*-mercuribenzoate), which suggests that a cysteine residue is essential for activity. The enzyme is ubiquitous in mammalian tissue, the highest concentrations being found in the liver, testes, skeletal muscle and brain (Yoshimoto *et al.*, 1979). Prolyl endopeptidase has been purified from rabbit brain (Orlowski *et al.*, 1979) and bovine brain (Yoshimoto *et al.*, 1983), and inhibition by Z-prolyl-prolinal (Fig. 1) (Wilk & Orlowski, 1983; Friedman *et al.*, 1984) and other prolinal derivatives (Tsuru *et al.*, 1988) has been investigated. Wilk & Orlowski (1983) suggested that Z-prolyl-prolinal is a non-competitive transition-state inhibitor with K_i 14 nM.

We now show here that Z-prolyl-prolinal is, in fact, a competitive slow-binding inhibitor of mouse brain and human brain prolyl endopeptidases with K_i values of 0.35 nM and 0.5 nM respectively.

EXPERIMENTAL

Materials

The following materials were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.): Trizma base, dithiothreitol, EDTA and $(NH_4)_2SO_4$. Sephadex G-100, Sephadex G-75 and DE-52 DEAE-cellulose were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Z-glycyl-prolyl-NH-Mec was obtained from Bachem Bioscience Inc. (Philadelphia, PA, U.S.A.). CD-1 male mice were obtained from Charles River Laboratories (Boston, MA, U.S.A.). Human brain samples were obtained from the National Disease Research Interchange (Philadelphia, PA, U.S.A.). Prolyl endopeptidase was purified from mouse brain by following the procedure of Orlowski *et al.* (1979).

Synthesis of Z-prolyl-prolinol

Z-prolyl-prolinol was prepared by using a modification of the procedure of Wilk & Orlowski (1983). 1-Hydroxybenzotriazole hydrate (4.05 g, 30 mmol) was added as a neat solid to benzyloxycarbonyl-proline (7.47 g, 30 mmol) dissolved in 100 ml of dichloromethane. L-Prolinol (3.0 ml, 30 mmol) and dicyclohexylcarbodi-imide (6.2 g, 30 mmol) were then added and the resulting solution was stirred for 48 h at 25 °C. Precipitated dicyclohexylurea was removed by filtration, and the filtrate was washed successively with 1 M-NaOH, 1 M-HCl and saturated NaCl, and then dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the product was crystallized from diethyl ether/pentane to yield 7.2 g (72%) of a white solid. ^{1}H n.m.r. (250 MHz) δ (p.p.m.) ([^{2}H]chloroform) 1.70–2.30 (8H, m), 3.26–3.80 (6H, m), 4.22–4.73 (2H, m), 4.78–5.20 (3H, m), 7.05–7.48 (5H, m); i.r. (KBr) 3500, 1705 and 1640 cm^{-1}; m.s. *m/z* 332 (M^+); m.p. 113–115 °C.

Synthesis of Z-prolyl-prolinal

The aldehyde was prepared by oxidation of Z-prolyl-prolinol (7.2 g) according to the method of Mancuso *et al.* (1978) and purified by flash chromatography on silica (ethyl acetate eluent).

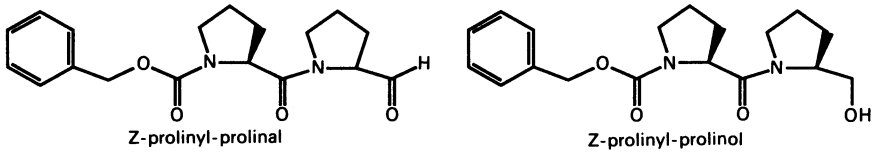

Fig. 1. Structures of Z-prolyl-prolinal and Z-prolyl-prolinol

Abbreviations used: Z-, benzyloxycarbonyl-; -NH-Mec, 7-(4-methyl)coumarinylamide.
*To whom correspondence should be addressed.

This material was crystallized from diethyl ether with an isolated yield of 3.1 g (43%). ^{1}H n.m.r. (250 Mz) δ (p.p.m.) ([^{2}H]-chloroform) 1.50–2.26 (8H, m), 3.22–3.72 (4H, m), 4.12–4.63 (2H, m), 4.85–5.22 (2H, m), 7.10–7.50 (5H, m), 9.31–9.53, (1H, m); i.r. (KBr) 1723, 1700 and 1639 cm^{-1}; m.s. m/z 330 (M^+).

Analytical methods

Prolyl endopeptidase activity was measured in 2.0 ml of 0.1 M-Tris/acetate buffer, pH 7.3, at 25 °C containing 1 mM-dithiothreitol and 1.25–2.5 μM-Z-glycyl-prolyl-NH-Mec as substrate. The reaction was initiated by adding sufficient enzyme to liberate approx. 5 nmol of 7-amino-4-methylcoumarin/min. Activity was measured by monitoring the resulting increase in fluorescence (380 nm excitation, 460 nm emission). Slow-binding inhibition (Morrison & Walsh, 1988) was apparent in the progress curves of inhibition by 5–50 nM-Z-prolyl-prolinal under these assay conditions. Each progress curve was fitted to the equation:

$$y = A \cdot e^{-kt} + B + C \cdot t$$

by non-linear least-squares regression (Bevington, 1969; Copp *et al.*, 1987). The steady-state (final) rate equals C, the initial rate is $-A \cdot k + C$ and the observed rate constant is k. To determine the type of inhibition, substrate (ranging in concentration from 0.1 to 100 μM) was incubated in buffer with Z-prolyl-prolinal (ranging in concentration from 0 to 100 μM) to which enzyme was added, and fluorescence was measured as a function of time. Collective constants ($V_{max.}$, K_m and K_i) were determined with the programs HYPER and COMP (Cleland, 1979).

Return of activity

To determine if the inhibition of prolyl endopeptidase by Z-prolyl-prolinal is reversible, activity was assayed upon dilution of the enzyme–inhibitor complex to well below the K_i. Prolyl endopeptidase solution was inhibited with 70 nM-Z-prolyl-prolinal, a 2 μl portion was diluted into 2 ml of assay buffer containing substrate, and activity was measured as a function of time. A control rate was also measured in which enzyme was incubated in the absence of inhibitor and a portion was diluted into an assay cuvette.

A return-of-activity experiment was also performed with Z-prolyl-prolinol. Enzyme was inhibited by 500 μM-Z-prolyl-prolinol and a 2 μl portion was diluted into 2 ml of assay buffer containing substrate.

RESULTS AND DISCUSSION

Prolyl endopeptidase was purified to approx. 75% homogeneity (determined by PAGE) by following the method of Orlowski *et al.* (1979). Although the source of the enzyme in this study was mouse brain (as opposed to rabbit or cow brain), the M_r value was found to be similar (67700 for the mouse brain enzyme as compared with 66000 for that from rabbit brain). Prolyl endopeptidase activity from human brain was measured in a crude homogenate.

Inhibition of prolyl endopeptidase by Z-prolyl-prolinal

Z-prolyl-prolinal meets the criteria for slow-binding inhibition proposed by Morrison & Walsh (1988), in that addition of inhibitor to an enzyme/substrate assay mixture leads to a first-order decrease in the rate of substrate hydrolysis and a final non-zero rate (Fig. 2).

There are three mechanisms that are proposed to describe slow-binding inhibition (Morrison, 1982; Erion & Walsh, 1987). The first is slow initial binding between enzyme and inhibitor to form an enzyme–inhibitor complex (mechanism A in Scheme 1).

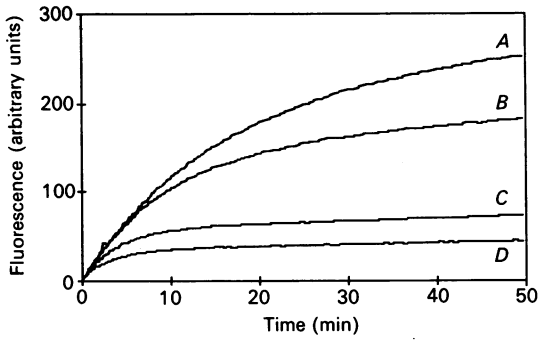

Fig. 2. Progress curves of prolyl endopeptidase inhibition by (curve *A*) 5 nM-, (curve *B*) 10 nM-, (curve *C*) 20 nM- and (curve *D*) 30 nM-Z-prolyl-prolinal in assays containing 1.25 μM substrate

Mechanism A: $E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI$

Mechanism B: $E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI^*$

Mechanism C: $E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E^* \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI^*$

Scheme 1. Three mechanisms that can account for slow-binding inhibition

The second is fast initial binding to form an intermediate EI, which slowly isomerizes to give EI* (mechanism B in Scheme 1). A third possible mechanism is slow isomerization of one free enzyme form to another, followed by rapid binding of the inhibitor (mechanism C in Scheme 1). These three mechanisms of inhibition can be distinguished by the kinetic experiments discussed below.

Inhibition of enzyme was performed at different Z-prolyl-prolinal concentrations. The rates of approach to the new steady state ($k_{obs.}$) predicted by mechanisms A, B and C in Scheme 1 are given by eqns. (1), (2) and (3) respectively (Erion & Walsh, 1987), in the limit $[S] \ll K_m$ under which these experiments were performed ($[S] = 1.25$ μM, $K_m = 16 \pm 1$ μM):

$$k_{obs.} = k_{-1} + k_{+1}[I] \quad (1)$$

$$k_{obs.} = k_{-2} + k_{+2}[I]/([I] + K_i) \quad (2)$$

$$k_{obs.} = k_{+1} + k_{-1}K_i/([I] + K_i) \quad (3)$$

The behaviour of $k_{obs.}$ as a function of [I] will be different in each case. Mechanisms A and B predict that increasing [I] will lead to an increase in $k_{obs.}$, whereas mechanism C predicts that decreasing [I] leads to this effect. Fig. 3 shows a linear increase in $k_{obs.}$ with increasing inhibitor concentration, consistent with mechanisms A and B, but not mechanism C, ruling out slow enzyme isomerization as a possible inhibition mechanism.

Mechanisms A and B can be distinguished by the effect that [I] has on the initial slope (v_0) and $k_{obs.}$ of a progress-curve assay. If mechanism A is correct, v_0 will be the same regardless of [I], and a plot of $k_{obs.}$ versus [I] will be linear. For mechanism B, v_0 will decrease with increasing [I], and a plot of $k_{obs.}$ will saturate in [I]. Our data show that the initial rate remains constant (Fig. 2), and a plot of $k_{obs.}$ versus [I] is linear to $[I] \approx 100 \times K_i$ (Fig. 3). Therefore

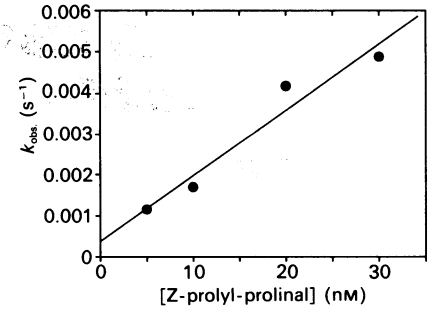

Fig. 3. Rate of approach to the steady state of prolyl endopeptidase inhibition ($k_{obs.}$) as a function of Z-prolyl-prolinal concentration

we conclude that mechanism A is the simplest scheme consistent with the kinetics of prolyl endopeptidase inhibition.

The slope in Fig. 3 is the second-order rate constant for inhibition (k_{+1}), $(1.6 \pm 0.3) \times 10^5$ $M^{-1} \cdot s^{-1}$. Analysis of v_f/v_0 (final rate/initial rate, determined by exponential + linear fits to progress curves) plotted versus [I] gives a K_i value of 0.35 ± 0.05 nM.

Wilk & Orlowski (1983) state that Z-prolyl-prolinal inhibition is non-competitive, which suggests the existence of a ternary enzyme–substrate–inhibitor complex. Since the structural similarity of Z-prolyl-prolinal to typical substrates is significant, and since the pre-steady-state analysis is consistent with a simple but slow equilibrium (see above), we suggest instead that Z-prolyl-prolinal is a competitive inhibitor, but with such slow kinetics and potent inhibition that either (1) traditional assay methods are inadequate because true steady states are not achieved and/or (2) the Michaelis–Menten assumption (that [E] $\ll$ [S], [I]) is easily violated with sub-nanomolar inhibitors.

One can also address the issue of competitive versus non-competitive or uncompetitive inhibition by analysis of the effect of substrate concentration on the observed rates of Z-prolyl-prolinal inhibition ($k_{obs.}$). When [S] approaches K_m, eqn. (1) expands to eqn. (4), eqn. (5) or eqn. (6), depending on whether inhibition is competitive, uncompetitive or non-competitive respectively (these equations are derived with the assumption that $E + S \rightleftharpoons ES$ equilibration is rapid relative to other rates):

$$k_{obs.} = k_{-1} + k_{+1}[I]/(1 + [S]/K_m) \quad (4)$$

$$k_{obs.} = k_{-1} + k_{+1}[I]/(1 + K_m/[S]) \quad (5)$$

$$k_{obs.} = k_{-1} + k_{+1}[I] \quad (6)$$

At substrate concentrations increasing from 1.6 μM to 80 μM ($0.1 \times K_m$ to $5 \times K_m$), the measured $k_{obs.}$ decreased, consistent only with eqn. (4) (Fig. 4). Thus Z-prolyl-prolinal is a competitive slow-binding inhibitor of prolyl endopeptidase.

Mechanism A in Scheme 1 implies that the value for k_{-1} (k_{off}) is simply calculated as $K_i \cdot k_{on}$. From the above data, $k_{off} = (6 \pm 2) \times 10^{-5}$ s^{-1}. This calculated value can be compared with the directly measured value obtained by dilution of the enzyme–inhibitor complex into an assay mixture. Such an experiment is shown in Fig. 5; from these data $k_{off} = (3 \pm 1) \times 10^{-5}$ s^{-1}. This agreement (within error) of calculated and experimental values of k_{off} is further support for mechanism A in Scheme 1. Inhibition of prolyl endopeptidase from human brain by Z-prolyl-prolinal followed similar kinetics; K_i was calculated to be 0.5 nM with k_{on} and k_{off} values of 0.7×10^5 $M^{-1} \cdot s^{-1}$ and 3×10^{-5} s^{-1} respectively.

Inhibition of prolyl endopeptidase by the alcohol, Z-prolyl-prolinol, is quite different from that by the aldehyde in that no slow-binding inhibition is apparent. Steady-state inhibition is achieved within cuvette mixing time, with $K_i = 19 \pm 1$ μM, i.e. less potent that the aldehyde by 50000-fold. The mechanism of

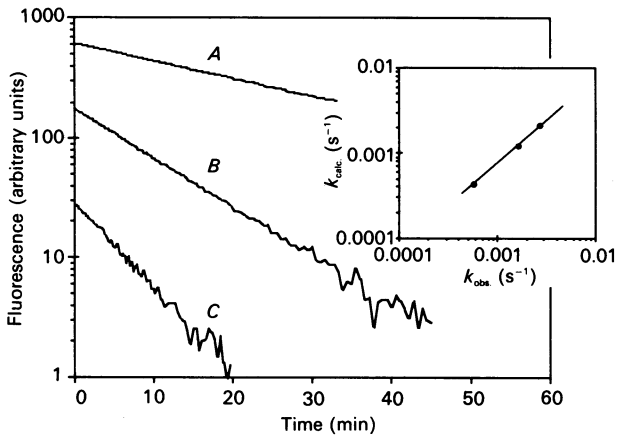

Fig. 4. Rate of approach to the steady state of prolyl endopeptidase inhibition at substrate concentrations of (curve *A*) 80 μM ($5 \times K_m$), (curve *B*) 16 μM (K_m) and (curve *C*) 1.6 μM ($0.1 \times K_m$) at a fixed (15 nM) Z-prolyl-prolinal concentration

Progress curves were fitted to

$$y = A \cdot e^{-kt} + B + C \cdot t$$

and for this Figure $\log(y - B - C \cdot t)$ is plotted versus t to show the variation of $k_{obs.}$ (the slopes of the semi-logarithmic data). Inset: $k_{calc.}$ from eqn. (4) is plotted versus $k_{obs.}$

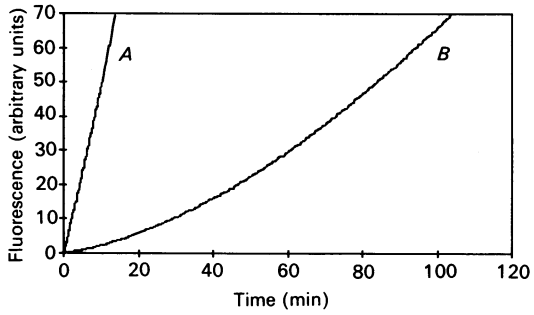

Fig. 5. Return of enzyme activity on dilution of an enzyme/inhibitor mixture

Curve *A*, control (no inhibitor); curve *B*, [Z-prolyl-prolinal] = 70 nM ($200 \times K_i$) before dilution, 0.07 nM ($0.2 \times K_i$) after dilution into the assay.

inhibition is competitive, as determined by analysis of the plots of initial rate versus [S] at various values of [I] by the program COMP (Cleland, 1979). The inability of Z-prolyl-prolinol to form a long-lived enzyme complex with prolyl endopeptidase demonstrates the importance of the aldehyde group in slow-binding inhibition.

The question that remains is the structure of the inhibited enzyme complex. Stein *et al.* (1987) have shown that inhibition of human leucocyte elastase by trifluoromethyl ketones occurs by nucleophilic attack of serine-195 on the trifluoromethyl ketone group to give a hemiketal. Formation of a hemiacetal by serine addition to aldehyde inhibitors has also been shown with α-chymotrypsin and *N*-benzoyl-L-phenylalaninal (Kennedy & Schultz, 1979). Prolyl endopeptidase, a serine proteinase, should also be capable of nucleophilic attack on the aldehyde carbonyl group to give a hemiacetal. We suggest that slow-binding inhibition by Z-prolyl-prolinal is due to such hemiacetal formation, and Z-prolyl-prolinol is a much more rapid and weaker inhibitor because it cannot form such an adduct. However, kinetic analyses of plots of the rate constants k_{on} and k_{off} versus

pH are not completely supportive of his hypothesis. For both elastase inhibition (Stein *et al.*, 1987) and chymotrypsin inhibition (Kennedy & Schultz, 1979), an increase in k_{on} and a decrease in k_{off} are observed with increasing pH. For inhibition of prolyl endopeptidase by Z-prolyl-prolinal, neither k_{on} nor k_{off} changes significantly from pH 6 to pH 9, though the enzyme alone displays a typical serine proteinase titration of $V_{max.}/K_m$ with $pK_a = 6.5$ (Fersht, 1977). Thus, although hemiacetal formation may account for the slow-binding inhibition by Z-prolyl-prolinal, prolyl endopeptidase remains distinct from other serine proteinases in its cytosolic location, sensitivity to thiol-blocking reagents and pH-dependence of inhibition kinetics.

We are grateful to Dr. Michael Snider and the National Disease Research Interchange (N.D.R.I.) for assistance in obtaining human brain samples.

REFERENCES

Bevington, P. R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York
Cleland, W. W. (1979) Methods Enzymol. **63**, 103–138
Copp, L. J., Krantz, A. & Spencer, R. W. (1987) Biochemistry **26**, 169–178
Erion, M. D. & Walsh, C. T. (1987) Biochemistry **26**, 3417–3425
Fersht, A. (1977) Enzyme Structure and Mechanism, pp. 145–155, W. H. Freeman, New York
Friedman, T. C., Orlowski, M. & Wilk, S. (1984) J. Neurochem. **42**, 237–241
Kennedy, W. P. & Schultz, R. M. (1979) Biochemistry **18**, 349–356
Mancuso, A. J., Jiamg, S. L. & Swern, D. (1978) J. Org. Chem. **43**, 2480–2482
Morrison, J. F. (1982) Trends Biochem. Sci. **7**, 102
Morrison, J. F. & Walsh, C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. **60**, 201–301
Orlowski, M., Wilk, E., Pearce, S. & Wilk, S. (1979) J. Neurochem. **33**, 461–469
Stein, R. L., Strimpler, A. M., Edwards, P. D., Lewis, J. J., Mauger, R. C., Schwartz, J. A., Stein, M. M., Trainor, D. A., Wildonger, R. A. & Zottola, M. A. (1987) Biochemistry **26**, 2682–2689
Tsuru, D., Yoshimoto, T., Koriyama, N. & Furukawa, S. (1988) J. Biochem. (Tokyo) **104**, 580–586
Wilk, S. (1983) Life Sci. **33**, 2149–2157
Wilk, S. & Orlowski, M. (1983) J. Neurochem. **41**, 69–75
Yoshimoto, T., Ogita, K., Walter, R., Koida, M. & Tsuru, D. (1979) Biochim. Biophys. Acta **569**, 184–192
Yoshimoto, T., Nishimura, T., Kita, T. & Tsuru, D. (1983) J. Biochem. (Tokyo) **18**, 1206–1212

Received 18 April 1990; accepted 16 August 1990