Biochem. J. (1992) 283, 871–876 (Printed in Great Britain)



# Peptidyldiazomethanes

## A novel mechanism of interaction with prolyl endopeptidase

Stuart R. STONE,* Denise RENNEX,† Peter WIKSTROM,§ Elliott SHAW and Jan HOFSTEENGE
Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

Peptidyldiazomethanes with proline in the $P_1$ position were found to be competitive slow-binding inhibitors of prolyl endopeptidase. Progress-curve experiments monitoring the increase in the degree of inhibition with time indicated that the kinetic mechanism involved an initial complex that isomerized to form a tighter complex. Reversibility of the inhibited complex was demonstrated by monitoring the regain of enzyme activity after removal of free inhibitor and dilution into an assay containing competing substrate. The kinetics of the reversal of inhibition indicated a more complicated inhibitory mechanism involving more than one pathway for reversal of the tight complex. A slow-binding mechanism of inhibition has not been previously observed with peptidyldiazomethanes. Incorporation of [$^3$H]Ac-Ala-Ala-Pro-diazomethane into prolyl endopeptidase was observed after denaturation of the inhibited complex. The peptide labelled with [$^3$H]Ac-Ala-Ala-Pro-diazomethane was isolated and found to contain the active-site serine residue.

## INTRODUCTION

Until recently, it was thought that peptidyldiazomethanes were reagents that specifically inactivated cysteine proteinases by alkylation of the active-site cysteine residue (Shaw, 1990). Serine proteinases of the chymotrypsin family, such as chymotrypsin, trypsin and elastase, are not inhibited by peptidyldiazomethanes (Green & Shaw, 1981). In fact, chymotrypsin-like serine proteinases may in some cases degrade peptidyldiazomethanes without being inhibited (Watanabe *et al.*, 1979; Zumbrunn *et al.*, 1988). A possible exception to the general resistance of chymotrypsin-like serine proteinases to diazomethanes is plasma kallikrein, which is inactivated slowly by benzyloxycarbonyl-Phe-Arg-diazomethane (Z-Phe-Arg-$CHN_2$) (Zumbrunn *et al.*, 1988). A recent report, however, indicates that serine proteinases of the subtilisin family are inactivated by diazomethanes through the formation of a covalent bond with the active-site histidine residue (Ermer *et al.*, 1990). Thus peptidyldiazomethanes do not exhibit such a clear specificity for cysteinyl proteinases as was originally throught (Green & Shaw, 1981).

Prolyl endopeptidase (PE) is a cytoplasmic serine proteinase that cleaves bonds on the *C*-terminal side of proline residues (Dresdner *et al.*, 1982; Yoshimoto *et al.*, 1980). Recently, the sequence of porcine PE has been determined by cDNA cloning and the identity of the active-site serine residue established (Rennex *et al.*, 1991). The active-site histidine residue has also been tentatively identified (Stone *et al.*, 1991). The primary structure of PE shows no sequence similarity with other serine proteinases and the sequences around the active-site serine and histidine residues are not closely related to those of other known serine proteinases.

PE has previously been shown to be inhibited by peptidyldiazomethanes (Green & Shaw, 1983), but the mechanism of inhibition was not investigated. In the present study we have examined the kinetic mechanism of inhibition of PE by peptidyldiazomethanes and have found these compounds to be reversible slow-binding inhibitors of the enzyme. Incorporation of [$^3$H]Ac-Ala-Ala-Pro$CHN_2$ into PE could be demonstrated after denaturation of the inhibited complex. A labelled peptide was isolated and found to contain the active-site serine residue of the enzyme. These results indicate that peptidyldiazomethanes exhibit different modes of interactions with different classes of proteinases.

## MATERIALS AND METHODS

### Materials

Z-Gly-Pro-pNA (where -pNA is *p*-nitroanilide) and Ala-Ala-Pro-OH were purchased from Bachem (Bubendorf, Switzerland) and tritiated acetic acid anhydride was from Amersham (Bucks., U.K.). PE was purified from either porcine kidney or skeletal muscle by using the method previously described (Rennex *et al.*, 1991). Peptidyldiazomethanes were synthesized as described previously (Green & Shaw, 1981; Stone *et al.*, 1991). All other chemicals were of the highest purity available commercially.

### Amidolytic assay of PE

Kinetic studies on the mechanism of inhibition of PE by peptidyldiazomethanes were carried out at 37 °C in 50 mM-Hepes buffer, pH 7.4, containing 0.1 M-NaCl, 1.0 mM-EDTA, 5.0 mM-dithiothreitol, 0.02% (v/v) Tween 20 detergent and 2% (v/v) methanol and the substrate Z-Gly-Pro-pNA as previously described (Stone *et al.*, 1991). Under the conditions of the assay, PE was stable at low concentrations (< 20 pM) for several hours.

### Reversal of inhibition of PE by Ac-Ala-Ala-Pro$CHN_2$

A 100 $\mu$l aliquot of PE (200 nM) was incubated at 37 °C with 5 $\mu$M-Ac-Ala-Ala-Pro$CHN_2$ for 1 h in the same buffer as used for assays, except that no methanol was added. At the end of this time, the sample was spun through a 1.0 ml column of Sephadex G-10 as described by Salvesen & Nagase (1989) in order to remove any unbound inhibitor. The results from a control experiment using [$^3$H]Ac-Ala-Ala-Pro$CHN_2$ in buffer indicated that a carry-over of about 3% of the unbound inhibitor could be

Abbreviations used: PE, prolyl endopeptidase; Ac, acetyl; pNA, *p*-nitroanilide; Z, benzyloxycarbonyl; -$CHN_2$, diazomethane.

* Present address and address for correspondence: Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH.
† Present address: Division of Hematology–Oncology, New England Medical Center, Boston, MA 02111, U.S.A.
§ Present address: Pentapharm AG, Aesch, Switzerland.

expected. The enzyme sample was then diluted $10^4$-fold into an assay mixture containing 109 $\mu$M-Z-Gly-Pro-pNA, and the recovery of the enzyme activity was monitored for 6–8 h at 37 °C as described above. In order to minimize evaporation from the cuvettes, the sample was covered with mineral oil. Control samples were treated exactly as described above, except that the initial incubation mixture did not contain Ac-Ala-Ala-Pro-$CHN_2$ and the assay was commenced 4 h after dilution into the assay buffer by the addition of substrate.

### Effect of PE on the structural integrity of Ac-Ala-Ala-Pro-$CHN_2$

In order to test whether Ac-Ala-Ala-Pro-$CHN_2$ was broken down by PE, [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ (62 $\mu$M) was incubated with PE (1.25 $\mu$M) in the assay buffer for 4 h at 37 °C. At the completion of the incubation, a sample of the mixture was chromatographed using reverse-phase h.p.l.c. on a $C_{18}$ column (Vydac, Hesperia, CA, U.S.A.). After loading the sample on to the column, which had been equilibrated with 3.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, the column was washed with the same solvent for 5 min at a flow rate of 1.0 ml/min and developed with a linear gradient of 3.5–10.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over a period of 25 min with the same flow rate. Fractions (1.0 ml each) were collected, and the radioactivity of each fraction was determined. Under these conditions a standard of Ac-Ala-Ala-Pro-$CHN_2$ was found to be eluted at 18.5 min. In view of the observation that, in some cases, serine proteinases are capable of cleaving peptidyldiazomethanes to yield the free peptide and diazomethane (Zumbrunn *et al.*, 1988), Ac-Ala-Ala-Pro-OH was synthesized as an additional standard by acetylating Ala-Ala-Pro-OH with acetic anhydride. This compound was eluted at 9.5 min under the above chromatographic conditions.

### Labelling of PE with [$^3$H-]Ac-Ala-Ala-Pro-$CHN_2$ and isolation of the labelled peptide

PE (6 $\mu$M) was incubated with a 10-fold molar excess of [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ (62 $\mu$M) in 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl, 1 mM-EDTA and 2% (v/v) methanol for 2 h at 37 °C. Incorporation of the inhibitor into the protein was analysed by SDS/PAGE (Laemmli, 1970). Samples were removed during the time course of inhibition and added to loading buffer that had been heated to 95 °C. These samples were then electrophoresed on a 10%-polyacrylamide gel and revealed by fluorography using 1 M-sodium salicylate (Chamberlain, 1979).

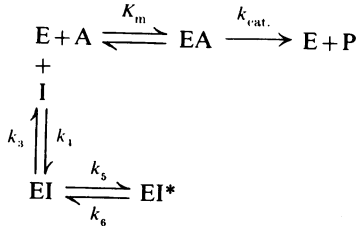
The labelled protein was dialysed against 50 mM-$NH_4HCO_3$ at 4 °C. The protein concentration was measured by amino acid analysis in order to determine the stoichiometry of incorporation of the inhibitor into the enzyme. The labelled protein was denatured by heating (10 min at 80 °C) and digested with 5% (w/w) chymotrypsin for 2 h at 37 °C. The digest was acidified with trifluoroacetic acid and chromatographed on a reverse-phase $C_{18}$ h.p.l.c. column (Vydac) using a linear gradient of 0–49% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 70 min at a flow rate of 1 ml/min. Fractions were collected and the radioactivity of each fraction was measured. The fractions comprising the major radioactive peak were re-chromatographed using a gradient of 14–28% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 100 min at a flow rate of 1 ml/min. The single radioactive peak, which contained more than one peptide, was concentrated by freeze-drying and the pH was adjusted to about 8 with dilute aq. $NH_3$. $NH_4HCO_3$ and $CaCl_2$ were added to give final concentrations of 100 mM and 5 mM respectively. The peptide was digested with 2% (w/w) thermolysin for 5 h at 37 °C. The digest was acidified by the addition of trifluoroacetic acid and chromatographed as described above, using a gradient of 0–49% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 140 min at a flow rate of 1 ml/min.

### Protein analysis

Amino acid sequence determination was performed using an Applied Biosystems gas-phase sequencer Model 470A as described previously (Rennex *et al.*, 1991). Amino acid analysis was performed by the method of Knecht & Chang (1987).

## THEORY AND DATA ANALYSIS

Peptidyldiazomethanes were found to be slow-binding inhibitors of PE. Slow-binding inhibition of enzymes can usually be explained by one of two mechanisms, termed A and B (Morrison, 1982; Morrison & Stone, 1985; Morrison & Walsh, 1988). The data for the inhibition of PE conformed to mechanism B in which an initial complex between the enzyme and inhibitor isomerizes to form a tighter complex. This mechanism is presented in the following scheme, in which relationships for the inhibition constant ($K_i$) for the first complex and the overall inhibition constant ($K_i^*$) are also given:




$$K_i = \frac{k_4}{k_3}$$

$$K_i^* = \frac{K_i k_6}{k_5 + k_6}$$

**Scheme 1**

The kinetic parameters for the slow-binding inhibition of PE by peptidyldiazomethanes were determined by monitoring the progress curve of enzyme-catalysed *p*-nitroaniline formation in the presence of various concentrations of inhibitor. Such progress curves can be descrbed by eqn. (1) (Morrison, 1982):

$$P = v_s t + \frac{(v_0 - v_s)}{k'}[1 - \exp(-k' t)] \quad (1)$$

where $P$ is the concentration of *p*-nitroaniline at time $t$, $k'$ is the apparent first-order rate constant for the transition from the initial ($v_0$) to the steady-state ($v_s$) velocity. For the above mechanism, relationships describing the dependence of $v_0$, $v_s$ and $k'$ on the concentration of inhibitor have been given (Morrison & Stone, 1985; Morrison & Walsh, 1988). These relationships were substituted into eqn. (1), and the progress-curve data were fitted to the resulting equation by non-linear regression to obtain estimates for $K_i$, $K_i^*$, $k_5$ and $k_6$.

## RESULTS

### Inhibition of PE by peptidyldiazomethanes

Ac-Ala-Ala-Pro-$CHN_2$ caused slow-binding inhibition of PE, as shown in Fig. 1; there was a time-dependent increase in the amount of inhibition caused by this compound. Cha (1976) proposed several kinetic mechanisms to account for slow-binding

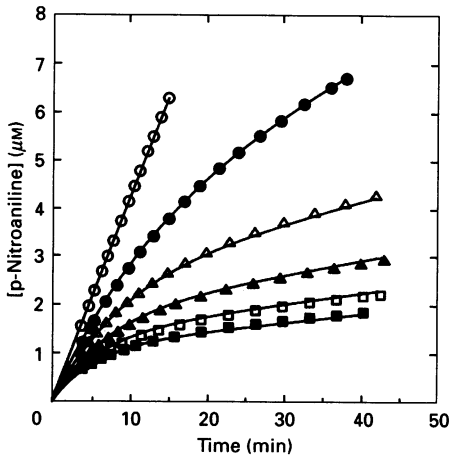



**Fig. 1. Inhibition of PE by Ac-Ala-Ala-Pro-$CHN_2$**

Assays were performed as described in the Materials and methods section, with 0.25 nM-PE and 65 μM-Z-Gly-Pro-pNA. The assays contained 0 (○), 0.2 (●), 0.4 (△), 0.6 (▲), 0.8 (□) and 1.0 (■) μM-Ac-Ala-Ala-Pro-$CHN_2$. The curves drawn in the Figure represent the fit of the data to the equation describing slow-binding inhibition mechanism B.

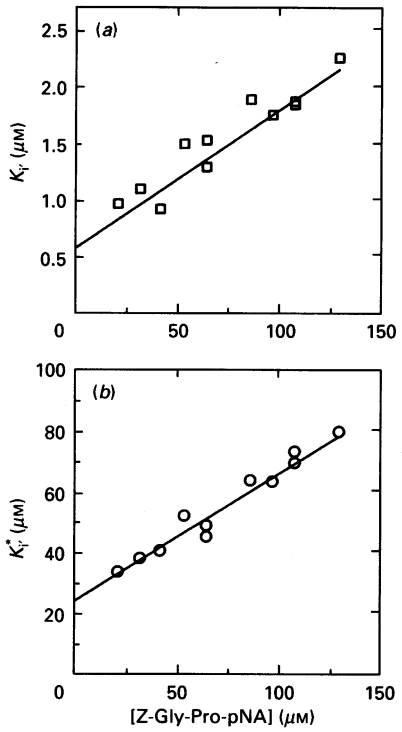



**Fig. 2. Dependence of the values of apparent inhibition constants $K_{i'}$ and $K_i^*$ on the concentration of the substrate Z-Gly-Pro-pNA**

Data similar to those shown in Fig. 1 were obtained at the indicated concentrations of Z-Gly-Pro-pNA and analysed to yield estimates for $K_{i'}$ and $K_i^*$, which are plotted in (*a*) and (*b*) respectively. The data were fitted to eqn. (2) by weighted linear regression, and the lines drawn show the results of this analysis.

inhibition, and these mechanisms have been reviewed by Morrison and his colleagues (Morrison, 1982; Morrison & Stone, 1985; Morrison & Walsh, 1988). The slow-binding inhibition caused by Ac-Ala-Ala-Pro-$CHN_2$ conformed to mechanism B. This mechanism involves the formation of an initial complex that slowly isomerizes to form a tighter complex, as outlined in Scheme 1. The data of Fig. 1 were fitted to the equation that describes this model. The lines drawn in the Figure represent the fit of the data to this equation and illustrate that the inhibition can be described well by this model. Analysis of the data of Fig. 1 and other similar data sets yielded estimates for the apparent inhibition constants $K_i$ and $K_i^*$ (defined in Scheme 1). In addition, estimates for the two rate constants, $k_5$ and $k_6$, were obtained. The values of $K_{i'}$ and $K_{i'}^*$ were found to depend on the concentration of substrate, as shown in Fig. 2. The linear dependence of these values on the substrate concentration indicates that Ac-Ala-Ala-Pro-$CHN_2$ acted as a competitive inhibitor of PE, and eqn. (2) describes this dependence:

$$K_{i'} = K_i(1 + S/K_m) \quad (2)$$

where $K_i$ and $K_{i'}$ are respectively the true and apparent values of the inhibition constant, $S$ is the concentration of the substrate and $K_m$ is its Michaelis constant. The data of Fig. 2 were fitted to eqn. (2) and estimates of $48 \pm 15$ and $59 \pm 11$ μM for $K_m$ were obtained from the data of Fig. 2(*a*) ($K_{i'}$) and 2(*b*) ($K_{i'}^*$) respectively. The value of $K_m$ determined independently from initial-velocity studies was $48 \pm 2$ μM (Rennex *et al.*, 1991). The agreement between the estimates of $K_m$ determined from the substrate dependence of $K_{i'}$ and $K_{i'}^*$ and that determined from initial-velocity studies confirms the competitive mechanism. Analysis of the data of Fig. 2 also gave estimates for the true values of $K_i$ and $K_i^*$, and these values are given in Table 1. The value for $K_i$ is 24-fold higher than that for $K_i^*$, indicating a significant increase in affinity for Ac-Ala-Ala-Pro-$CHN_2$ as a result of the isomerization step. The values of $k_5$ and $k_6$ did not vary with the concentration of substrate, as is expected for slow-binding mechanism B (Morrison & Walsh, 1988), and the values given in Table 1 represent the weighted means of 11 determinations at different substrate concentrations.

In order to test whether the slow-binding inhibition observed with Ac-Ala-Ala-Pro-$CHN_2$ was unique to this compound, the effect of other peptidyldiazomethanes with proline in the $P_1$

**Table 1. Kinetic parameters for the inhibition of PE by peptidyldiazomethanes**

Assays were performed and data were analysed as described in the Materials and methods section. The estimates of $K_i$ and $K_i^*$ for Ac-Ala-Ala-Pro-$CHN_2$ were derived from the data of Fig. 2, whereas those for $k_5$ and $k_6$ are the weighted averages of 11 determinations. The estimates of the kinetic parameters for Z-Leu-Leu-Pro-$CHN_2$ were derived from the data of Fig. 3, whereas analysis of a similar set of data yielded those for Z-Ala-Ala-Pro-$CHN_2$.

| Inhibitor | $K_i$ (μM) | $K_i^*$ (nM) | $k_5$ ($min^{-1}$) | $10^2 \times k_6$ ($min^{-1}$) |
|---|---|---|---|---|
| Ac-Ala-Ala-Pro-$CHN_2$ | $0.578 \pm 0.115$ | $24.4 \pm 2.5$ | $0.397 \pm 0.010$ | $1.54 \pm 0.03$ |
| Z-Ala-Ala-Pro-$CHN_2$ | $1.69 \pm 0.08$ | $16.3 \pm 1.4$ | $0.506 \pm 0.070$ | $0.488 \pm 0.047$ |
| Z-Leu-Leu-Pro-$CHN_2$ | $0.073 \pm 0.003$ | $24.3 \pm 0.4$ | $0.126 \pm 0.011$ | $6.23 \pm 0.46$ |

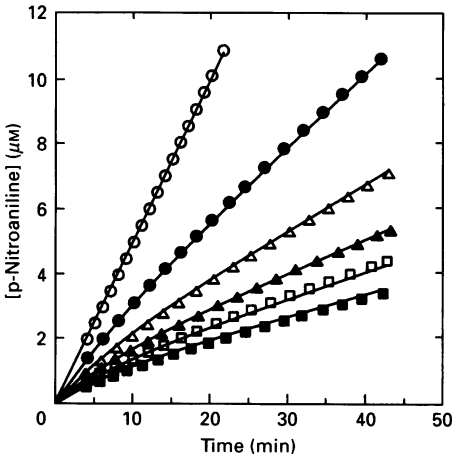



**Fig. 3. Inhibition of PE by Z-Leu-Leu-Pro-$CHN_2$**

Assays were performed as described in the Materials and methods with 0.25 nM-PE and 109 μM-Z-Gly-Pro-pNA. The assays contained 0 (○), 0.1 (●), 0.2 (△), 0.3 (▲), 0.4 (□) and 0.5 (■) μM-Z-Leu-Leu-Pro-$CHN_2$. The data were fitted to the equation describing slow-binding inhibition mechanism B, and the lines drawn represent the fit of the data to this equation.

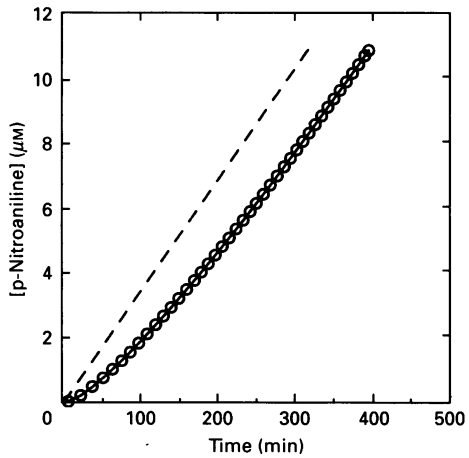



**Fig. 4. Reversal of the inhibition of PE by Ac-Ala-Ala-Pro-$CHN_2$**

PE was incubated with 5 μM-Ac-Ala-Ala-Pro-$CHN_2$ for 1 h, after which time the free inhibitor was removed and the enzyme diluted into an assay mixture containing 109 μM-Z-Gly-Pro-pNA as described in the Materials and methods section. The data were fitted to eqn. (4) by non-linear regression, and the line drawn through the data points shows the fit of the data to this equation. The broken line represents the activity found in the control sample.

position on the activity of PE was investigated. The results shown in Fig. 3 for Z-Leu-Leu-Pro-$CHN_2$ indicate the effect of this compound on the activity of PE could also be explained by slow-binding inhibition mechanism B. Similar kinetics were also observed with Z-Ala-Ala-Pro-$CHN_2$ (results not shown). Analysis of the data for Z-Leu-Leu-Pro-$CHN_2$ and Z-Ala-Ala-Pro-$CHN_2$ yielded the estimates for the kinetic parameters given in Table 1; the estimates for $K_i$ and $K_i^*$ were calculated on the basis of competitive inhibition. These results indicated that, whereas the value of $K_i$ varied greatly (1.69 μM for Z-Ala-Ala-Pro-$CHN_2$ compared with 74 nM for Z-Leu-Leu-Pro-$CHN_2$), the $K_i^*$ value was about equal for all compounds tested. The ratio $K_i/K_i^*$ was 100 for Z-Ala-Ala-Pro-$CHN_2$, whereas this ratio was only 3 for Z-Leu-Leu-Pro-$CHN_2$ (Table 1).

**Reversal of the inhibition of PE by Ac-Ala-Ala-Pro-$CHN_2$**

The reversibility of the inhibition by Ac-Ala-Ala-Pro-$CHN_2$ was tested by incubating the enzyme for 1 h with 5 μM-inhibitor. After this time the enzyme was separated from the inhibitor by spinning the sample through a Sephadex G-10 column (Salvesen & Nagase, 1989). The enzyme was then diluted $10^4$-fold into an assay mixture and the recovery of the enzyme activity monitored. The concentration of Ac-Ala-Ala-Pro-$CHN_2$ was 200-fold higher than the value of $K_i^*$ such that the enzyme was initially completely inhibited. In addition, the sample was treated such that the inhibitor concentration was effectively reduced to zero. Under these conditions the progress curve for the return of activity should theoretically be described by the equation for slow-binding inhibition (eqn. 1), except that $v_0$ will be equal to zero and eqn. (1) will reduce to eqn. (3). Moreover, the value of $k'$ should equal $k_6$ (Morrison & Walsh, 1988):

$$P = v_s t - \frac{v_s}{k'}[1 - \exp(-k' t)] \quad (3)$$

The reversal of the inhibition caused by Ac-Ala-Ala-Pro-$CHN_2$ did not, however, fit well to this equation. The recovery of activity was better described by a double-exponential function of the form of eqn. (4):

$$P = v_{s1} t - \frac{v_{s1}}{k_1'}(1 - \exp(-k_1' t) + v_{s2} t - \frac{v_{s2}}{k_2'}[1 - \exp(-k_2' t)] \quad (4)$$

The fit of one set of data to this equation is shown in Fig. 4. The first-order rate constants ($k_1'$ and $k_2'$) for the two reactions were $(7.9 \pm 0.1) \times 10^{-2}$ and $(6.1 \pm 0.1) \times 10^{-3}$ $min^{-1}$ respectively; these values represent the weighted means of ten determinations. The weighted average of total amount of activity recovered ($v_{s1} + v_{s2}$) was 91 % of the control, with the values ranging between 81 and 103 %. The values determined for $v_{s1}$ and $v_{s2}$ were about equal; the average value of $v_{s1}$ was $0.86 \times v_{s2}$. The relative amount of activity recovered did not depend on the time for which the PE was incubated with Ac-Ala-Ala-Pro-$CHN_2$ before dilution; the amount of activity recovered was between 85 and 100 % when the time of incubation was varied between 45 min and 6 h. Reversal of the inhibition by Z-Leu-Leu-Pro-$CHN_2$ also did not conform to the expected single exponential (results not shown). In this case, however, a detailed kinetic analysis was not performed. The results obtained with Ac-Ala-Ala-Pro-$CHN_2$ indicate that the reversal of inhibition proceeds by more than one pathway. Thus the mechanism of inhibition of PE by Ac-Ala-Ala-Pro-$CHN_2$ appears to be more complex than that presented in Scheme 1.

It has been reported that, in some cases, serine proteinases are capable of hydrolysing peptidyldiazomethanes with the release of the peptide portion of the inhibitor and diazomethane (Zumbrunn *et al.*, 1988). In addition, it is known that poor substrates can act as enzyme inhibitors (Fromm, 1975). Therefore it seemed possible that the peptidyldiazomethanes were inhibiting PE by acting as poor substrates. This possibility was examined by incubating [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ with a high concentration of PE (1.25 μM) and using h.p.l.c. to examine whether any breakdown of the inhibitor had occurred as described in the Materials and methods section. After incubation for 4 h at 37 °C, all the radioactive material was found to be eluted at the same position as the standard of Ac-Ala-Ala-Pro-$CHN_2$; no radioactivity was found to be eluted at the position of the free peptide (Ac-Ala-Ala-Pro-OH). Thus it can be concluded that there was no cleavage of Ac-Ala-Ala-Pro-$CHN_2$ by PE and that the

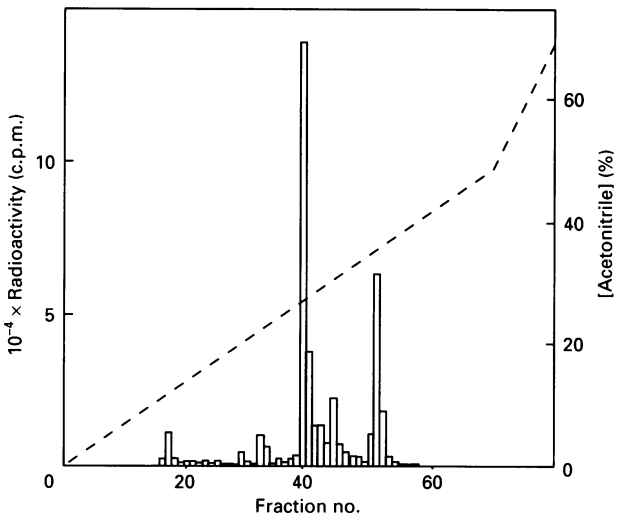



**Fig. 5. Isolation of the peptide labelled with [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$**

A chymotryptic digest of PE labelled with [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ was fractionated by reverse-phase h.p.l.c. on a $C_{18}$ column using the indicated linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid (----). The amount of radioactivity found in each fraction is plotted (bars).

inhibition of PE by Ac-Ala-Ala-Pro-$CHN_2$ is not due to this compound acting as a poor substrate.

### Identification of the site of modification of PE by [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$

In order to further characterize the nature of the complex between PE and Ac-Ala-Ala-Pro-$CHN_2$, PE (6 $\mu$M) was incubated with a 10-fold excess (62 $\mu$M) of [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ and the complex examined by electrophoresis after denaturation by heating in SDS. The results indicated that, under these conditions, the labelled inhibitor became incorporated into the enzyme (results not shown). After 120 min, the labelled protein was dialysed exhaustively and its concentration determined by amino acid analysis. A stoichiometry of incorporation of 0.7 mol of inhibitor/mol of protein was determined on the basis of this analysis. The labelled protein was denatured by heating and cleaved with chymotrypsin. The digest was fractionated by reverse-phase h.p.l.c., and the radioactivity contained in each fraction is shown in Fig. 5. The recovery of radioactivity that was loaded on to the column was about 100%, and it was distributed over two major peaks. The smaller of these comprised fractions 50–52 and contained 22% of the radioactivity that was recovered from the column. When these fractions were re-purified by reverse-phase h.p.l.c. using a shallower gradient of acetonitrile, two separate radioactive peptides were obtained (results not shown). These peptides did not contain enough material for further analysis. The larger of the two initial peaks comprised fractions 39–41 and contained 46% of the radioactivity that was recovered from the column. These fractions were rechromatographed using a shallower gradient of acetonitrile, and one radioactive peak was identified. However, this peak contained more than one peptide and therefore it was further purified by digestion with thermolysin, followed by reverse-phase h.p.l.c. One radioactive peptide was identified and characterized by sequence determination and amino acid analysis. The partial sequence of the radioactive peptide, with the yields shown in parentheses, was Thr (40 pmol), Ile (54 pmol), Asp (53 pmol), Gly (45 pmol), Gly (44 pmol), Ser (3 pmol) and Asp (1 pmol). The same sequence for the major labelled peptide was obtained in three other labelling experiments that used different chromatographic steps for the purification of the peptide. The repetitive yield decreased greatly at the serine residue at position 6 of the sequence, which suggests that the peptidyldiazomethane may be covalently bound to this residue. The partial sequence of this peptide corresponded to residues 549–555 of the deduced amino acid sequence of porcine brain PE (Rennex *et al.*, 1991). The amino acid composition of the labelled peptide indicated that the peptide consisted of residues 549–559 of PE (shown below), together with the amino acids from the peptide portion of the diazomethane (results not shown):

$^{549}$Thr-Ile-Asn-Gly-Gly-Ser-Asn-Gly-Gly- Leu-Leu$^{559}$

On the basis of the amino acid composition, the stoichiometry of incorporation of the labelled inhibitor into the peptide was 0.9 mol/mol. The peptide contains two Asn–Gly bonds, and therefore a number of isoforms of this peptide are expected. It seems possible that the minor labelled peptides were such isoforms.

## DISCUSSION

Peptidyldiazomethanes with proline in the $P_1$ position were shown to be slow-binding reversible inhibitors of PE. Kinetic studies in which the decrease in enzyme activity was monitored (Figs. 1 and 3) indicated that the mechanism of inhibition was consistent with slow-binding mechanism B (Cha, 1976; Morrison, 1982; Morrison & Walsh, 1988). In this mechanism an initial complex slowly isomerizes to form a tighter complex. The final complexes were reasonably tight for the three inhibitors tested, with dissociation constants of about 20 nM for each of the diazomethanes (Table 1). The peptide portion of the diazomethanes would be expected to direct the inhibitor to the active site of PE, and this expectation was confirmed by demonstrating that Ac-Ala-Ala-Pro-$CHN_2$ was a competitive inhibitor (Fig. 2). The reversibility of the complex between PE and Ac-Ala-Ala-Pro-$CHN_2$ was demonstrated in experiments in which the complex was formed and free inhibitor removed before monitoring the regain of activity in the presence of competing substrate. Under these conditions, almost full activity of the enzyme was regained over a period of 6–8 h (Fig. 4). The kinetics of the reversal of inhibition indicated, however, that the mechanism of inhibition was more complicated than that presented in Scheme 1 (slow-binding mechanism B) and probably involved more than one pathway for the conversion of the tight complex into the free enzyme. The nature of these pathways is not known.

The reversible inhibition observed with peptidyldiazomethanes in the present study seems at variance with the previous report of irreversible inactivation of macrophage PE by Z-Ala-Ala-Pro-$CHN_2$ (Green & Shaw, 1983). The kinetics of inactivation observed by Green & Shaw (1983), however, deviated from those expected for a simple inactivation process, with some residual activity remaining even after long periods of incubation with inhibitor. Therefore, in view of the low rate constants for the reversal of the inhibition observed in the present study, it seems possible that reversible inhibition could have been mistaken for inactivation. The microbial serine proteinases thermitase and subtilisin have also been reported to be inactivated by peptidyldiazomethanes (Ermer *et al.*, 1990). The kinetics of inactivation of thermitase and subtilisin were similar to those observed with macrophage PE. Thus it seems possible that a tight reversible complex could also have been mistaken for an irreversible complex in the case of thermitase and subtilisin.

Considering the fact that serine proteinases are in some cases capable of slowly cleaving peptidyldiazomethanes (Watanabe *et al.*, 1979; Zumbrunn *et al.*, 1988) and that poor substrates are

able to act as inhibitors (Fromm, 1975), the ability of PE to cleave Ac-Ala-Ala-Pro-$CHN_2$ was tested. No cleavage was detected, and thus an inhibitory mechanism involving the peptidyldiazomethanes acting as poor substrates could be excluded.

Denaturation of the complex between [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ and PE led to the incorporation of inhibitor into the protein. The major peptide labelled by [$^3$H]Ac-Ala-Ala-Pro-$CHN_2$ was isolated and found to consist of residues 549–559 of porcine PE. Peptidyldiazomethanes react specifically with the active cysteine residue in cysteine proteinases (Shaw, 1990). The labelled peptide did not, however, contain any cysteine. During sequencing of this peptide, the repetitive yield decreased markedly at $Ser^{554}$, which suggests that this residue was the site of modification by the diazomethane. This residue was previously shown to be the active-site serine residue in PE (Rennex *et al.*, 1991). Inhibition of thermitase and subtilisin by peptidyldiazomethanes, followed by denaturation of the complexes, results in the labelling of the active-site histidine residue (Ermer *et al.*, 1990). Thus the nature of the active-site residue labelled by diazomethanes appears to vary with the class of proteinase.

The inactivation of cysteine proteinases by diazomethanes is thought to occur by the attack of the active-site cysteine on the carbonyl carbon of the inhibitor to form a hemimercaptal. A rearrangement subsequently takes place with the formation of a thioether bond between the methylene carbon of the inhibitor and the active-site cysteine with the displacement of molecular nitrogen (Brocklehurst & Malthouse, 1978; Shaw, 1990). A similar mechanism can be envisaged for the interaction of diazomethanes with PE. Under non-denaturing conditions the complex between the enzyme and the inhibitor could contain a hemiketal formed between the active-site serine residue and the carbonyl carbon of the inhibitor. This complex would be reversible. Denaturing conditions, however, may favour the rearrangement to form an ether bond between the active-site serine residue and the methylene carbon of the inhibitor, with the resultant formation of a covalent complex and release of molecular nitrogen.

The formation of a covalent bond between a diazomethane inhibitor and a serine residue is not without precedent. When 5-diazo-4-oxonorvaline was incubated with asparaginase from *Escherichia coli*, inhibition was accompanied by enzymic hydrolysis of the inhibitor to a hydroxymethyl ketone (Jackson & Handschumacher, 1970). In the presence of increasing concentrations of dimethyl sulphoxide, the rate of inactivation increased and the rate of hydrolysis of the inhibitor decreased (Lachman & Handschumacher, 1976). A labelled peptide could be purified from the enzyme that had been incubated with inhibitor in the presence of dimethyl sulphoxide, and the labelled amino acid was identified as a serine residue (Peterson *et al.*, 1977).

We thank Renate Matthies for assistance with amino acid sequence and composition analysis.

## REFERENCES

Brocklehurst, K. & Malthouse, J. (1978) Biochem. J. **175**, 761–764
Cha, S. (1976) Biochem. Pharmacol. **25**, 2695–2702
Chamberlain, J. P. (1979) Anal. Biochem. **98**, 132–135
Dresdner, K., Barker, L. A., Orlowski, M. & Wilk, S. (1982) J. Neurochem. **38**, 1151–1154
Ermer, A., Baumann, H., Steude, G., Peters, K., Fittkau, S., Dolaschka, P. & Genov, N. C. (1990) J. Enzyme Inhibition **4**, 35–42
Fromm, H. J. (1975) Initial Rate Enzyme Kinetics, pp. 83–120, Springer-Verlag, Berlin
Green, G. D. J. & Shaw, E. (1981) J. Biol. Chem. **256**, 1923–1928
Green, G. D. J. & Shaw, E. (1983) Arch. Biochem. Biophys. **225**, 331–337
Jackson, R. C. & Handschumacher, R. E. (1970) Biochemistry **9**, 3585–3590
Knecht, R. & Chang, J.-Y. (1987) Anal. Chem. **58**, 2375–2379
Lachman, L. B. & Handschumacher, R. E. (1976) Biochem. Biophys. Res. Commun. **73**, 1094–1100
Laemmli, U. K. (1970) Nature (London) **227**, 680–684
Morrison, J. F. (1982) Trends Biochem. Sci. **7**, 102–105
Morrison, J. F. & Stone, S. R. (1985) Comments Mol. Cell. Biophys. **6**, 347–368
Morrison, J. F. & Walsh, C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. **61**, 201–301
Peterson, R. G., Richards, F. F. & Handschumacher, R. E. (1977) J. Biol. Chem. **252**, 2072–2076
Rennex, D. A., Hemmings, B. A., Hofsteenge, J. & Stone, S. R. (1991) Biochemistry **30**, 2195–2203
Salvesen, G. & Nagase, H. (1989) in Proteolytic Enzymes: A Practical Approach (Beynon, R. J. & Bond, J. S., eds.), pp. 83–104, IRL Press, Oxford
Shaw, E. (1990) Adv. Enzymol Relat. Areas Mol. Biol. **63**, 271–347
Stone, S. R., Rennex, D., Wikstrom, P., Shaw, E. & Hofsteenge, J. (1991) Biochem. J. **276**, 837–840
Watanabe, H., Green, G. D. J. & Shaw, E. (1979) Biochem. Biophys. Res. Commun. **89**, 1354–1360
Yoshimoto, T., Walter, R. & Tsuru, D. (1980) J. Biol. Chem. **255**, 4786–4792
Zumbrunn, A., Stone, S. & Shaw, E. (1988) Biochem. J. **250**, 621–623

Received 24 September 1991; accepted 30 October 1991