Inactivation of prolyl endopeptidase by a peptidylchloromethane

Kinetics of inactivation and identification of sites of modification

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The kinetics of inactivation of prolyl endopeptidase by acetyl-Ala-Ala-Pro-CH₂Cl were studied by progress-curve methods in the presence of substrate. The kinetic mechanism was found to involve the formation of an initial complex between the enzyme and the chloromethane followed by an inactivation step. The substrate was shown to compete for the formation of the initial complex, indicating that binding at the active site was a prerequisite for inactivation. After reaction of the enzyme with [³H]acetyl-Ala-Ala-Pro-CH₂Cl, it was possible to isolate five labelled peptides. Four of these peptides contained a cysteine residue as the site of modification, whereas the fifth peptide contained no cysteine and a histidine residue was identified as the site of modification. This residue (His-680) probably represents the active-site histidine of prolyl endopeptidase.

INTRODUCTION

Until recently it was thought that serine prote(in)ases could be divided into two families, whose archetypal members are chymotrypsin and subtilisin (Neurath, 1984). More recent studies, however, suggest that additional families of serine prote(in)ases may exist. Carboxypeptidase Y (Breddam, 1986), dipeptidyl peptidase IV (Ogata *et al.*, 1989) and acyl-amino acid hydrolase (Kobayashi *et al.*, 1989; Mitta *et al.*, 1989) provide examples of enzymes that can be classified as serine proteases on the basis of their inactivation by di-isopropyl phosphorofluoridate but show no sequence similarity to other serine prote(in)ases or to each other. Prolyl endopeptidase (PE) is a cytoplasmic serine protease that cleaves peptide bonds after proline (Wilk, 1983). Its primary structure also shows no significant sequence similarity to other serine prote(in)ases (Rennex *et al.*, 1991). It is of interest to determine whether enzymes such as PE that are classified as serine prote(in)ases on the basis of their inactivation by di-isopropyl phosphorofluoridate also possess the other two members of the catalytic triad, that is the catalytically important histidine and aspartic acid residues. Active-site-directed chloromethanes have been very useful in identifying active-site histidine residues in serine prote(in)ases (Shaw, 1970), and in the present work Ac-Ala-Ala-Pro-CH₂Cl was shown to be a competitive inactivator of PE. Moreover, by using a radioactively labelled Ac-Ala-Ala-Pro-CH₂Cl, it was possible to identify the major sites of alkylation by this compound. One of these sites was a histidine residue that can tentatively be identified as the active-site histidine.

MATERIALS AND METHODS

Materials

Trypsin was from Worthington (Malvern, PA, U.S.A.). Z-Gly-Pro-NH-Np and [³H]acetic anhydride were purchased from Bachem (Bubendorf, Switzerland) and Amersham International (Amersham, Bucks., U.K.) respectively. PE was purified from pig skeletal muscle (Rennex *et al.*, 1991).

Synthesis of peptidylchloromethanes

Z-Ala-Ala-Pro-CH₂Cl. This was prepared by standard methods (Kettner & Shaw, 1981).

Fmoc-Ala-Ala-Pro-CHN₂. This was prepared by coupling Z-Ala-Ala via the mixed-anhydride method to Pro-OMe, HCl and saponification of the tripeptidyl ester to the tripeptide acid, which was deprotected with concentrated trifluoroacetic acid. Ala-Ala-Pro trifluoroacetate (1.43 g, 3.9 mmol) was acylated with Fmoc-Cl and converted into Fmoc-Ala-Ala-Pro-CHN₂ as described by Crawford *et al.* (1988). This was purified by standard procedures and crystallized from ethyl acetate to give 147 mg (10% yield) of a pure pale-yellow solid product (m.p. 128–130 °C). The structure of this product (C₂₇H₂₉N₅O₆) was confirmed by f.a.b.-m.s. One major peak was observed at (M+H)⁺ 476, corresponding to that expected for Fmoc-Ala-Ala-Pro-CHN₂ minus N₂.

Ac-Ala-Ala-Pro-CHN₂. The above product was deprotected with piperidine as described by Crawford *et al.* (1988), and the resultant Ala-Ala-Pro-CHN₂ (16 mg, 57 μmol) was dissolved in 1 ml of acetonitrile containing 20 μl of piperidine and acetylated by the addition of 57 μmol in tetrahydrofuran (2 ml). F.a.b.-m.s. of the purified product (7.3 mg; 50% yield) yielded the expected mass, (M+H)⁺ 296, for Ac-Ala-Ala-Pro-CHN₂ (C₁₄H₂₁N₅O₄, 323.4) minus N₂. [³H]Ac-Ala-Ala-Pro-CHN₂ was prepared by the same method with tritiated acetic anhydride. The tritiated product was homogeneous by t.l.c. and h.p.l.c. and co-migrated with the unlabelled product in both chromatographic procedures.

Ac-Ala-Ala-Pro-CH₂Cl. Ac-Ala-Ala-Pro-CHN₂ was converted into Ac-Ala-Ala-Pro-CH₂Cl with HCl in ethyl acetate (Wikstrom *et al.*, 1989).

Inactivation of PE by peptidylchloromethanes

Kinetic parameters for inactivation of PE by chloromethanes were determined by analysing progress-curve data for the hydrolysis of the substrate Z-Gly-Pro-NH-Np in the presence of different concentrations of inhibitor. The assays were performed at 37 °C in 50 mM-Hepes buffer, pH 7.4, containing 0.1 M-NaCl, 1.0 mM-EDTA, 0.02% (v/v) Tween 20 and 2% (v/v) methanol

Abbreviations used: PE, prolyl endopeptidase; Ac-, acetyl-; -NH-Np, *p*-nitroanilide; Z-, benzyloxycarbonyl-; Fmoc-, fluoren-9-ylmethoxycarbonyl-; f.a.b.-m.s., fast-atom-bombardment m.s.

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as previously described (Rennex *et al.*, 1991). No dithiothreitol was included in the assay mixture in order to avoid its reaction with the peptidylchloromethane. The enzyme was preincubated for 1 h in the assay buffer containing 5.0 mM-dithiothreitol to ensure that the enzyme was maximally active. The assays were started by adding 10 μ l of enzyme stock solution to 1.0 ml of assay mixture; the resultant concentration of dithiothreitol in the assay was 50 μ M. Under the conditions of the assay, the enzyme was stable for more than 30 min. Data were obtained at a number of different substrate concentrations ranging from 24 to 142 μ M. Each progress-curve experiment consisted of one reaction without chloromethane and five with different concentrations of the inhibitor. Data analysis was performed as described by Gray & Duggleby (1989) to yield estimates for the apparent dissociation constant of the initial complex formed between the chloromethane and PE (K_i') and the inactivation rate constant (k_i).

Labelling of PE with [3 H]Ac-Ala-Ala-Pro-CH $_2$ Cl

PE (250 μ g, 3.1 nmol) was incubated for 30 min at 37 °C in 150 μ l of assay buffer containing 5.0 mM-dithiothreitol in order to ensure that the enzyme was maximally active. This buffer was exchanged for 50 mM-ammonium bicarbonate by spinning the sample through a 1.0 ml column of Sephadex G-25 that had previously been equilibrated with the bicarbonate buffer (Salvesen & Nagase, 1989). Results of a control experiment in which a solution of dithiothreitol was spun through the column indicated that about 1% of this reagent would be carried over with the enzyme. The sample was incubated at 37 °C, and a 10-fold molar excess (31 nmol) of [3 H]Ac-Ala-Ala-Pro-CH $_2$ Cl was added to give a final concentration of about 200 μ M. After 10 min, a further 15 nmol of the chloromethane was added and the incubation was continued for an additional 90 min. Unbound [3 H]Ac-Ala-Ala-Pro-CH $_2$ Cl was removed by spinning through a Sephadex G-25 column. The results from a control experiment with [3 H]Ac-Ala-Ala-Pro-CH $_2$ Cl indicated that 2% of the unbound inhibitor would be carried over with the enzyme. The radioactivity of the enzyme sample was determined and corrected for carry-over of unbound inhibitor. A sample was also taken to determine the protein concentration by amino acid analysis. The labelled protein was denatured by heating at 80 °C for 10 min and digested with 5% (w/w) trypsin for 2 h at 37 °C.

The digest was diluted 1:10 in 25 mM-potassium phosphate buffer, pH 6.5, and chromatographed on a reverse-phase C $_{18}$ h.p.l.c. column (Vydac, Hesperia, CA, U.S.A.) equilibrated with the same buffer. A flow rate of 1.0 ml/min was used in all h.p.l.c. procedures. The column was washed for 10 min with the phosphate buffer before the peptides were eluted with a linear gradient of 0–50% (v/v) acetonitrile in the same buffer over 180 min. Fractions (1.0 ml) were collected and the radioactivity was determined. The five radioactive peaks were further purified on the same column with a linear gradient of 14–56% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 60 min. One peptide (A) was subjected to a third purification step on the same column with a shallower gradient, namely 10–17% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid over 60 min.

Protein chemistry

Amino acid sequencing and analysis were performed as previously described (Rennex *et al.*, 1991).

RESULTS AND DISCUSSION

The inactivation of PE by Ac-Ala-Ala-Pro-CH $_2$ Cl was studied in the presence of the five different concentrations of the substrate Z-Gly-Pro-NH-Np ranging from 24 to 142 μ M. A typical set of data is shown in Fig. 1(a). The mechanism of inactivation was

found to involve the formation of a complex between the enzyme and the chloromethane before the inactivation step. The data were analysed according to the equation that describes this mechanism, and estimates of the apparent dissociation constant for the initial complex (K_i') and of the inactivation rate constant (k_i) were obtained. The value of k_i did not vary with the substrate concentration, and a mean value of $3.72 \times 10^{-3} \pm 0.29 \times 10^{-3}$ s $^{-1}$ was obtained. In contrast, the estimate for K_i' displayed a linear dependence on the substrate concentration, as shown in Fig. 1(b). Such a dependence is expected for a competitive mechanism, and in this case the value of K_i' will be related to the true dissociation constant (K_i), the substrate concentration ([S]) and the Michaelis constant for the substrate (K_m) by eqn. (1):

$$K_i' = K_i(1 + [S]/K_m) \quad (1)$$

The data of Fig. 1(b) were fitted to eqn. (1) by weighted linear regression, and estimates of 0.55 ± 0.04 μ M and 63 ± 8 μ M were obtained for K_i and K_m respectively. The estimate of K_m compares reasonably well with that of 48 μ M obtained from initial-velocity studies (Rennex *et al.*, 1991), and the agreement between the two estimates confirms the competitive mechanism. Similar results have been obtained for the inactivation of PE by Z-Ala-Ala-Pro-CH $_2$ Cl; in this case, however, estimates of 0.36 ± 0.02 μ M and 38 ± 9 μ M were obtained for K_i and K_m respectively (results not shown). In summary, the results of the kinetic studies indicate that the inactivation of PE by Ac-Ala-Ala-Pro-CH $_2$ Cl is dependent on the binding of this compound at the active site of the enzyme.

In order to identify residues modified by the chloromethane, PE was labelled with [3 H]Ac-Ala-Ala-Pro-CH $_2$ Cl as described in the Materials and methods section. Under these conditions 3.3 mol of chloromethane was incorporated per mol of protein. The labelled protein was denatured and digested with trypsin and subjected to reverse-phase h.p.l.c. Five major radioactive peaks were obtained, as shown in Fig. 2. Each of these peaks was purified as described in the Materials and methods section and sequence data were obtained (Table 1). These data allowed the positions of the peptides in the primary structure of PE to be established. One of these peptides (A) contained a histidine residue as the probable site of modification; no amino acid phenylthiohydantoin derivative could be identified in the position where a histidine residue was expected. In the four other peptides the modified amino acid residue appeared to be a cysteine; no amino acid phenylthiohydantoin derivative could be identified at the position where a cysteine residue occurred in the deduced amino acid sequence, and the yield of amino acid phenylthiohydantoin derivative fell markedly. With peptide C, for example, the yield of amino acid phenylthiohydantoin derivative declined from 107 pmol for the valine residue preceding the cysteine residue to 2 pmol for the aspartic acid residue after the cysteine residue. Amino acid analysis was also performed on peptides A, B and C (results not shown), and these findings established that these peptides corresponded respectively to residues 678–688, 252–260 and 49–60 of pig PE. The amounts of peptides D and E recovered after the second purification step were insufficient to permit an accurate amino acid analysis to be performed. No histidine was found in the amino acid composition of peptide A, which provides good evidence that this residue has been modified (Shaw, 1970). Lower than stoichiometric amounts of cysteine (approx. 0.3 mol/mol) were obtained in peptides B and C, and these results are consistent with cysteine being the site of modification in these peptides. The stoichiometry of incorporation of radioactive label was also calculated on the basis of the amino acid analysis and found to be 1.2, 1.1 and 1.2 mol of inhibitor per mol of peptide for A, B and C respectively.

Fig. 1. Inactivation of PE by Ac-Ala-Ala-Pro-CH₂Cl

(a) Progress curves for the hydrolysis of substrate by PE in the presence of Ac-Ala-Ala-Pro-CH₂Cl. Assays were performed as described in the Materials and methods section with 0.62 nM-PE and 47 μM-Z-Gly-Pro-NH-Np. The assays contained 0 μM- (○), 0.62 μM- (●), 1.24 μM- (▽), 1.85 μM- (▼), 2.47 μM- (■) and 3.09 μM-Ac-Ala-Ala-Pro-CH₂Cl (■). Data points at times less than 3 min are not shown and only each second point at times thereafter is shown. The data were analysed by non-linear regression to yield estimates for the apparent dissociation constant of initial complex between the chloromethane and PE (K_1') and the inactivation rate constant (k_i). The curves drawn in the Figure represent the fit of the data obtained from the analysis. (b) Variation of the estimates of K_1' with the concentration of the substrate Z-Gly-Pro-NH-Np. Estimates of K_1' were obtained from data similar to those presented in (a). The data in (b) were fitted to eqn. (1) by weighted linear regression and the line drawn shows the fit of the data to this equation.

Fig. 2. Separation by h.p.l.c. of radioactive peptides obtained by tryptic digestion of PE that had been labelled with [³H]Ac-Ala-Ala-Pro-CH₂Cl

A tryptic digest of PE that had been labelled with [³H]Ac-Ala-Ala-Pro-CH₂Cl was fractionated by reverse-phase h.p.l.c. on a C₁₈ column with the indicated gradient (----) of acetonitrile in 25 mM-potassium phosphate buffer, pH 6.5. Fractions (1.0 ml) were collected and the radioactivity found in each fraction is plotted. The radioactive peaks labelled A-E were purified further as described in the Materials and methods section.

On the basis of the stoichiometric incorporation of inhibitor into peptide A, the lack of an unmodified histidine in the amino acid sequence and composition of this peptide, His-680 can be identified as the site of modification in peptide A. The alkylation of His-680 represents a remarkably selective reaction, since PE contains 23 histidine residues (Rennex *et al.*, 1991). In comparison, four out of 16 cysteine residues were modified. Although cysteine residues are readily alkylated by chloromethanes, the

Table 1. Amino acid sequences derived from the purified radioactive peptides obtained by tryptic digestion of PE that had been labelled with [³H]Ac-Ala-Ala-Pro-CH₂Cl

The peptides were purified and their sequences determined as described in the Materials and methods section. Numbers are given above the first and last residues to indicate the position of the peptide in the deduced amino acid sequence of PE (Rennex *et al.*, 1991). Cys* and His* denote positions where no amino acid phenylthiohydantoin derivative could be identified and the deduced amino acid sequence indicated respectively cysteine and histidine. The symbol '→' indicates that the peptide could not be sequenced to completion.

Peptide	Sequence
A	678 Ala-Gly-His*-Gly-Ala-Gly-Lys-Pro-Thr-Ala-Lys 688
B	253 Glu-Gly-Cys*-Asp-Pro-Val-Asn-Arg 260
C	24 Val-Cys*-Asp-Pro-Tyr-Ala-Trp-Leu-Glu-Asp-Pro-Asp→ 35
D	593 His-Ala-Trp-Thr-Thr-Asp-Tyr-Gly-Cys*→ 601
E	48 Ile-Thr-Val-Pro-Phe-Leu-Glu-Gln-Cys*→ 57

only histidine residues in serine prote(in)ases that react at a readily detectable rate with these compounds are those involved in catalysis (Shaw, 1970). Thus the increased reactivity of His-680 is consistent with the proposal that this residue represents the active-site histidine residue of PE. Whether the modification of His-680 is solely responsible for the inactivation of PE is not known. PE is also inhibited by thiol-modifying reagents (Wilk, 1983), and it seems possible that modification of one of the cysteine residues by the chloromethane is at least partially responsible for the observed inactivation. The modified cysteine residue would have to be located within the active site since the kinetics of inactivation indicate that a complex with the active site is a necessary prerequisite for the inactivation. In this

Ala	Gly	His*	Gly	Ala	PE
Asn	Gly	His*	Gly	Thr	Subtilisin Carlsberg
Ala	Gly	His*	Glu	Val	Wheat serine carboxypeptidase II
Ala	Ala	His*	Lys	Leu	Chymotrypsin

Fig. 3. Comparison of the sequence around the putative active-site histidine residues of PE with those surrounding active-site histidine residues of other serine prote(in)ases

The active-site histidine residues are denoted by His*. Conserved residues are boxed. The sequences of subtilisin Carlsberg, wheat serine carboxypeptidase II and chymotrypsin were taken from Smith *et al.* (1968), Liao & Remington (1990) and Brown & Hartley (1966) respectively.

respect PE would be similar to certain members of the subtilisin serine prote(in)ase family that are inactivated by modification of a non-essential cysteine residue within the active site (Betzel *et al.*, 1988).

Comparison of the sequence around His-680 with those around the active-site histidine residues of other serine prote(in)ases indicates a limited sequence similarity to members of the subtilisin family and a member of the carboxypeptidase Y family, as shown in Fig. 3. The active-site histidine residue of serine prote(in)ases of the subtilisin and chymotrypsin families occurs in a position in the sequence that is *N*-terminal to that of the active-site serine residue. In contrast, the position His-680 is *C*-terminal to that of the active-site serine residue (Ser-554) of PE (Rennex *et al.*, 1991). In this respect PE is similar to wheat serine carboxypeptidase II, which is a member of the carboxypeptidase Y family. The recently determined crystal structure of this enzyme

indicates that the active-site histidine residue (His-397) is found *C*-terminal to the active-site serine residue (Ser-146; Liao & Remington, 1990). In addition, the sequence surrounding the active-site histidine residue in wheat serine carboxypeptidase II shows some sequence similarity to that around His-680 (Fig. 3).

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