The structural basis for neutrophil inactivation of C $\bar{1}$ inhibitor

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Limited proteolysis of C $\bar{1}$ inhibitor (C $\bar{1}$ -INH) by neutrophil elastase, *Pseudomonas* elastase and snake venoms resulted in initial cleavage within the molecule's *N*-terminus followed by further cleavage within the molecule's *C*-terminally placed reactive centre. *N*-Terminal proteolysis occurred at peptide bonds 14–15, 36–37 and 40–41. This had no effect on either the inhibitory activity or the heat-stability of C $\bar{1}$ -INH. Proteolysis within the reactive centre occurred at peptide bonds 439–440, 440–441, 441–442 and 442–443. Cleavage at any one of these sites inactivated C $\bar{1}$ -INH and conferred enhanced heat-stability upon a previously heat-labile molecule. Released neutrophil proteinases also cleaved and inactivated C $\bar{1}$ -INH, suggesting that they may physiologically regulate C $\bar{1}$ -INH during inflammatory episodes.

INTRODUCTION

Antitrypsin, antithrombin III and C $\bar{1}$ inhibitor (C $\bar{1}$ -INH) are members of the **serine-proteinase inhibitor** superfamily (serpins) (Carrell & Travis, 1985). These inhibitors are involved in the physiological regulation of elastolysis, coagulation, fibrinolysis, kinin formation and complement activation, forming stable complexes with a range of activated serine proteinases (Travis & Salvesen, 1983; Carrell & Boswell, 1986). The distribution of each inhibitor in these complexes in plasma and a knowledge of their kinetic parameters determined *in vitro* (Beatty *et al.*, 1980; Schapira *et al.*, 1985) have helped to define the target proteinase specificity of each. For antitrypsin the target proteinase is neutrophil elastase, and complex-formation is accompanied by peptide-bond cleavage of the inhibitor at the Met³⁵⁸–Ser³⁵⁹ peptide bond within the reactive centre (Carrell *et al.*, 1979). Antithrombin III, as its name implies, is a physiological regulator of thrombin, and C $\bar{1}$ -INH is the sole regulator of the classical-pathway components C1r and C1s, the respective cleavages occurring at the Arg³⁸⁴–Ser³⁸⁵ peptide bond in antithrombin III (Jörnvald *et al.*, 1979) and at the Arg⁴⁴⁴–Thr⁴⁴⁵ peptide bond in C $\bar{1}$ -INH (Salvesen *et al.*, 1985).

Other proteinases can catalytically inactivate these inhibitors by cleaving between residues that are believed to lie on an exposed peptide loop and include the reactive site (Johnson & Travis, 1977; Kress *et al.*, 1979; Kress & Catanese, 1981; Virca *et al.*, 1982; Morihara *et al.*, 1984; Carrell & Owen, 1985; Potempa *et al.*, 1986; Banda *et al.*, 1987). When such cleavage occurs both antitrypsin and antithrombin III undergo a marked increase in antigenic heat-stability (Carrell & Owen, 1985). A study of C $\bar{1}$ -INH denaturation has demonstrated a single transition for the native inhibitor at 60 °C but no major detectable transition for the C $\bar{1}$ -INH–C1s complex (Lennick *et al.*, 1985).

The present paper describes the action of several proteinases and snake venoms on native C $\bar{1}$ -INH and investigates the effect that limited proteolysis has upon the stability of this molecule.

MATERIALS AND METHODS

Materials

Benzoylarginyl ethyl ester, cytochalasin b, *N*-formyl-methionyl-leucylphenylalanine, pig pancreatic trypsin and snake venoms from *Bungarus fasciatus* and *Bitis arietans* were supplied by Sigma Chemical Co. Mucus heparin (25000 i.u./ml) was from Weddel Pharmaceuticals. S-2302 (D-prolylphenylalanylarginine *p*-nitro-anilide) was from Kabi Vitrum. Anti-C $\bar{1}$ -INH sera were supplied by Atlantic Antibodies. Purified neutrophil elastase (0.6 mg/ml) was a gift from P. George (Department of Pathology, Christchurch Clinical School, University of Otago, Christchurch, New Zealand), and *Pseudomonas aeruginosa* elastase (19.6 mg/ml) was a gift from Kazuyuki Morihara (Kyoto Research Laboratories, Tokyo, Japan). Fresh plasma in 10 mM-EDTA/10 mM-benzamidinium was used for all protein purifications and stored frozen at –70 °C before fractionation. For all other assays plasma was either freshly drawn into 10 mM-EDTA/10 mM-benzamidinium or had been stored at –70 °C for up to 18 months.

Protein purifications

C $\bar{1}$ -INH was isolated as previously described (Harrison, 1983). Plasmin was purified, activated and active-site-titrated as previously described (Jackson *et al.*, 1981). C $\bar{1}$ -INH was titrated against plasmin to calculate its activity and analysed by SDS/polyacrylamide-gel electrophoresis for purity.

Human neutrophil isolation

Human neutrophils were isolated on the day of use from whole blood by Ficoll/Hypaque fractionation (Boyum, 1968). Neutrophil purity exceeded 95%, with eosinophil contamination of 0–5%. The concentration was adjusted to 5 × 10⁶ cells/ml in phosphate-buffered saline (3 mM-KCl/140 mM-NaCl/1.5 mM-KH₂PO₄/15 mM-Na₂HPO₄ buffer, pH 7.4) containing 1 mM-MgCl₂, 1 mM-CaCl₂ and 5 mM-glucose.

Abbreviation used: C $\bar{1}$ -INH, C $\bar{1}$ inhibitor.

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Proteinase and snake-venom cleavage of C $\bar$ I-INH

Pseudomonas aeruginosa-elastase cleavage of C $\bar$ I-INH was carried out as previously described (Salvesen *et al.*, 1985) at an enzyme/inhibitor ratio of 1:800 (w/w) and the reaction mixture was incubated at 37 °C for 5 h. The reaction was stopped by the addition of 0.2 M-EDTA to a final concentration of 5 mM. Cleavage and inactivation were assessed by SDS/polyacrylamide-gel electrophoresis, plasmin inhibition and heat-stability.

Neutrophil-elastase cleavage of C $\bar$ I-INH was carried out under the same conditions as the *Pseudomonas aeruginosa*-elastase cleavage but the reaction was stopped by the addition of an equimolar amount of antitrypsin.

Snake-venom cleavages were carried out as previously described (Kress *et al.*, 1983). A 23 μ l portion of *Bungarus fasciatus* venom (10 mg/ml in 50 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0) or 12 μ l of *Bitis arietans* venom (1 mg/ml in 50 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0) was added to 1 ml of C $\bar$ I-INH (1 mg/ml in the same buffer). The mixtures were incubated for 4 h (*Bungarus fasciatus* venom) or 1 h (*Bitis arietans* venom) at 37 °C and assayed as above. *Bungarus fasciatus* venom was removed by gel filtration on Sephadex G-200 (medium grade).

Purification of C $\bar$ I-INH cleavage fragments

Fragments from the neutrophil-elastase, *Pseudomonas aeruginosa*-elastase and snake-venom digests of C $\bar$ I-INH were separated by reverse-phase h.p.l.c. on a PLRP-S 30 nm-pore-size 8 μ m-particle-size column (250 mm $\times$ 4.6 mm; Polymer Laboratories) coupled to Waters instrumentation. Samples were injected and the column was washed at 1 ml/min for 10 min with 5% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. C $\bar$ I-INH fragments were eluted with a 5–80% (v/v) acetonitrile/0.1% trifluoroacetic acid gradient developed over 40 min. The eluate was monitored at 214 nm, and pools containing either single peaks or several peaks (insufficiently resolved) were collected into 5 ml chronic acid-washed glass tubes. Separate portions from each pool were either dried down under vacuum for amino acid analysis or for analysis by SDS/5–20%-polyacrylamide-gel electrophoresis. Purified fragments were partially sequenced on an Applied Biosystems gas-phase sequencer.

Heat-stability assays

Heat-stability assays were carried out by a modification of the method of Lieberman (1973). Samples (200 μ l) of native, *Pseudomonas aeruginosa*-elastase-cleaved, neutrophil-elastase-cleaved or snake-venom-cleaved C $\bar$ I-INH (0.2 mg/ml in 75 mM-NaH₂PO₄/75 mM-glycine/75 mM-Tris/HCl buffer, pH 7.5) were heated at 60 °C for 2 h. Samples were taken every 20 min, centrifuged (in an Eppendorf Microfuge for 15 min) and filtered (0.45 μ m-pore-size filters), and 3 μ l portions were taken for single-dimension crossed immunoelectrophoresis in agarose (Laurell, 1966).

Neutrophil cleavage of C $\bar$ I-INH

For neutrophil activation, 1 μ l of cytochalasin b (10 mM) was added to 1 ml of isolated human neutrophils and the cells were incubated at 37 °C for 2 min. A 1 μ l portion of the tripeptide formylmethionyl-leucylphenyl-

alanine (100 μ M) was then added and the cells were incubated for a further 10 min.

Two assays were performed. The first examined the effect of released neutrophil proteinase on C $\bar$ I-INH; the second examined the involvement of other neutrophil functions in inactivating C $\bar$ I-INH. For the first assay a 10 mg/ml solution of C $\bar$ I-INH was diluted to 1 mg/ml with the supernatant from stimulated neutrophils and the mixture was incubated at 37 °C for 4 h. Samples were taken at 30 min intervals and assayed as described above for the purified-proteinase cleavages. For the second assay the cells were stimulated in the presence of 1 mg of C $\bar$ I-INH/ml, then removed by centrifugation (300 *g* for 5 min at room temperature) before further analysis of C $\bar$ I-INH in the supernatant.

RESULTS

Protein isolation and activity

Native C $\bar$ I-INH gave one band on SDS/polyacrylamide-gel electrophoresis of molecular mass 105 kDa and was > 95% active as a plasmin inhibitor.

C $\bar$ I-INH cleavage patterns

Human neutrophil elastase. Neutrophil-elastase cleavage of C $\bar$ I-INH generated an SDS/polyacrylamide-gel-electrophoresis pattern similar to that described by Brower & Harpel (1982) (Fig. 1a). After 15 min incubation fragments of 100 kDa and 60 kDa appeared. At this time C $\bar$ I-INH retained 90% of its plasmin-inhibitory capacity (Fig. 2). Complete inactivation of C $\bar$ I-INH occurred after 5 h, when only four bands, of 89 kDa, 86 kDa, 55 kDa and 5 kDa, were visible. H.p.l.c. followed by amino acid and sequence analyses resolved and identified four protein-containing pools (Fig. 3 and Table 1).

Pool 1 gave a sequence corresponding exactly to the published *N*-terminus of C $\bar$ I-INH (Harrison, 1983; Bock *et al.*, 1986).

Pool 2 contained two peptides in differing amounts. One, representing 20% of the pool, possessed an *N*-terminal sequence identical with that of the peptide on pool 1. The second, representing 80% of the pool and comprising only three residues, had the sequence Leu-Glu-Val. This sequence occurs only once in C $\bar$ I-INH (residues 38–40), thus mapping two cleavage sites within the *N*-terminal region of the native inhibitor, i.e. at the Ile³⁷–Leu³⁸ and Val⁴⁰–Ser⁴¹ peptide bonds (Fig. 4).

Sequencing of pool 3 showed it to consist of two overlapping peptides in equivalent amounts derived from the *C*-terminus of the native inhibitor and differing by the residues Ser-Val. This mapped two further cleavage sites to within the reactive centre of C $\bar$ I-INH, i.e. at the Ile⁴⁴⁰–Ser⁴⁴¹ and Val⁴⁴²–Ala⁴⁴³ peptide bonds (Fig. 4).

Bitis arietans venom. Inactivation of C $\bar$ I-INH by *Bitis arietans* venom was complete within 1 h (Fig. 2) and generated three bands in common with the neutrophil-elastase-cleavage pattern (Fig. 1b). The first band to appear was the transient 100 kDa species, and the second the inactive 89 kDa species, which appeared concomitantly with the third, the 5 kDa species.

H.p.l.c. followed by amino acid and sequence analyses resolved and identified four protein-containing pools (Table 1 and Fig. 4).

Pool 1 contained two *N*-terminal peptides in equivalent

Fig. 1. Elastase and snake-venom cleavage of C1-INH

Time-point samples were taken from each incubation mixture, non-reducing electrophoresis sample buffer was added and the samples were heated in a boiling-water bath before electrophoresis in either SDS/10%-polyacrylamide gels or SDS/5–20%-polyacrylamide gradient gels. Gels were run overnight at 8 mA. (a) Neutrophil-elastase cleavage of C1-INH. Lane 1, native C1-INH; lanes 2–9, consecutive 15 min time-point samples taken up to 120 min; lane 10, sample taken at 210 min; lane 11, sample taken at 300 min; lane 12, markers [C1-INH (104 kDa), antithrombin III (61 kDa), ovalbumin (45 kDa) and myoglobin digest (14 kDa, 6 kDa and 2.5 kDa)]. (b) *Bitis arietans*-venom and *Bungarus fasciatus*-venom cleavage of C1-INH. Lane 1, native C1-INH; lanes 2–5, consecutive 15 min time-point samples taken from the *Bitis arietans* venom incubation up to 60 min; lane 6, *Bungarus fasciatus* venom; lane 7, native C1-INH; lanes 8–11, consecutive 60 min time-point samples taken from the *Bungarus fasciatus* venom incubation up to 240 min. (c) *Pseudomonas aeruginosa*-elastase cleavage of C1-INH. Lane 1, Native C1-INH; lanes 2–9, consecutive 15 min time-point samples taken up to 120 min; lane 10, markers [plasminogen (94 kDa), antithrombin III (61 kDa), soya-bean trypsin inhibitor (24 kDa) and myoglobin (18 kDa)].

amounts, one identical with the native protein's *N*-terminus, the other corresponding to a sequence starting at residue 15.

Pools 2 and 3 each gave single sequences corresponding to 5 kDa species derived by cleavage at the Val⁴⁴²–Ala⁴⁴³ and Ala⁴³⁹–Ile⁴⁴⁰ peptide bonds respectively.

Pool 4 gave an *N*-terminal sequence identical with one starting at residue 17 (Ile) in the native protein.

***Bungarus fasciatus* venom.** *Bungarus fasciatus*-venom cleavage of C1-INH did not inactivate the native molecule (Fig. 2) but did generate the 100 kDa band described previously (Fig. 1b). Sequence analysis revealed a new *N*-terminal derived by cleavage at the Pro³⁶–Ile³⁷ peptide bond of the native protein (Table 1 and Fig. 4).

***Pseudomonas aeruginosa* elastase.** *Pseudomonas aeruginosa* elastase generated a cleavage pattern of C1-INH similar to that obtained with *Bitis arietans* venom

(Fig. 1c). The rate of inactivation was comparable with that obtained with neutrophil elastase, and after 5 h incubation only the 89 kDa and 5 kDa species were present. The 5 kDa species has previously been shown to arise by cleavage at the Ser⁴⁴¹–Val⁴⁴² peptide bond within the reactive site loop (Salvesen *et al.*, 1985), and our sequence data confirm this (Table 1 and Fig. 4).

Neutrophils. When C1-INH was incubated either with neutrophils undergoing stimulation or with the supernatant retained from stimulated neutrophils, the resultant cleavage pattern resembled that generated by purified neutrophil elastase (Fig. 5a). However, C1-INH inactivation was faster in the presence of stimulated neutrophils than was achieved by the supernatant retained from stimulated neutrophils (Fig. 5b). C1-INH in the presence of non-stimulated neutrophils was fully converted into the 100 kDa fragment (with visible amounts of the 89 kDa and 86 kDa bands) and retained 86% of the native molecule's ability to inhibit plasmin.

Fig. 2. Elastase and snake-venom inactivation of C1-INH

Time-point samples were taken from each of the incubations and treated with an equimolar amount of active-site-titrated plasmin. Residual plasmin activity was monitored with the chromogenic substrate S-2302 and the results are expressed as percentages of the inhibition obtained with native C1-INH. ●, *Bungarus fasciatus* venom; □, *Bitis arietans* venom; ▲, *Pseudomonas aeruginosa* elastase; ○, human neutrophil elastase.

Antigenic heat-stability

Purified C1-INH denatured fully within 60 min at 60 °C. This denatured material was unable to inhibit plasmin, but remained soluble and could be quantified by single-dimension crossed immunoelectrophoresis (Fig. 6). This Figure shows dual precipitin lines (rockets) in lanes 2–7, the smaller rockets corresponding to residual native inhibitor, the larger rockets representing the denatured form of the protein. In contrast, *Pseudomonas aeruginosa*-elastase-inactivated C1-INH was antigenically heat-stable, even for several hours.

All proteolytically inactivated C1-INH preparations showed similar stability to the *Pseudomonas aeruginosa*-elastase-inactivated preparation, and the *Bungarus fasciatus*-venom modified C1-INH retained the unstable profile characteristic of the native inhibitor.

DISCUSSION

The regulation of homeostasis, fibrinolysis, kinin generation and complement activation is critically dependent on the balance between proteinases and their respective inhibitors. This balance may be disturbed

Fig. 3. H.p.l.c. resolution of C1-INH cleavage fragments

A sample (100 µl) from each elastase digest of C1-INH was taken after 5 h incubation and injected on to the column, and the C1-INH fragments were resolved as described in the Materials and methods section. Similar samples were taken from the *Bitis arietans*-venom and *Bungarus fasciatus*-venom digests of C1-INH and analysed. *Bungarus fasciatus* venom was removed by gel filtration before analysis. (a) H.p.l.c. resolution of C1-INH fragments generated by neutrophil elastase. (b) SDS/5–20% polyacrylamide-gel electrophoresis of pools collected in (a).

Fig. 4. Identified cleavage sites in C1-INH

Key to cleavage sites: 1, *Bitis arietans* venom; 2, *Bungarus fasciatus* venom; 3, neutrophil elastase; 4, *Pseudomonas aeruginosa* elastase; 5, C1s (Salvesen *et al.*, 1985).

Table 1. Sequence analyses of h.p.l.c.-resolved C1-INH fragments

– denotes an unidentified residue. These residues are known carbohydrate-attachment sites in native C1-INH (Bock *et al.*, 1986).

C1-INH digest	Pool no./ fragment size	Sequence result	% of total pool analysis
Neutrophil elastase	1.	N P – A T S S S	100
	2.	N P – A T S S S	20
		L E V	80
	3./5 kDa	S V A R T L L V	50
		A R T L L V F E	50
	4.	Not analysed	
<i>Bitis arietans</i> venom	1.	N P – A T S S S	50
		L Q N R G E G K	50
	2./5 kDa	A R T L L V F E	100
	3./5 kDa	I S V A R T L L	100
	4./89 kDa	I L E V S – L P	100
<i>Bungarus fasciatus</i> venom	100 kDa	I L E V S – L P	100
<i>Pseudomonas</i> elastase	5 kDa	V A R T L L V F	100

Fig. 5. Neutrophil cleavage and inactivation of C1-INH

Time-point samples were taken from each incubation and treated as described in Fig. 1 legend. Further samples were taken from each incubation mixture and treated with equimolar amounts of active-site-titrated plasmin as indicated in Fig. 2 legend. (a) Neutrophil cleavage of C1-INH. Lanes 1, 6 and 8, native C1-INH; lanes 2–4, consecutive 30 min time-point samples taken from the C1-INH incubation with cells undergoing stimulation up to 90 min; lane 5, C1-INH after 90 min incubation with non-stimulated cells; lane 7, cells only. (b) Neutrophil inactivation of C1-INH. ●, Non-stimulated cells with C1-INH; □, stimulated cells with C1-INH; △, stimulated-cell supernatant with C1-INH.

either through consumption of an inhibitor by its target proteinase, e.g., antithrombin III by thrombin, or by catalytic inactivation of an inhibitor by proteinases derived from leucocytes or from a number of potentially pathogenic organisms (Kress *et al.*, 1979; Kress & Catanese, 1981; Virca *et al.*, 1982; Potempa *et al.*, 1986; Banda *et al.*, 1987).

Catalytic inactivation by cleavage within the reactive centre loops of antitrypsin and antithrombin III confers a marked resistance to denaturation by heat (Carrell & Owen, 1985). Structural evidence suggests that this change in stability is due to a significant conformational change as the molecule moves from its active stressed (S) structure to the relaxed (R) form (Loebermann *et al.*, 1984). The work reported in the present paper has shown

that the analogous 'exposed loop' in C1-INH is also susceptible to proteolytic cleavage, but this event is usually preceded by the removal of 30–40 N-terminal residues. The possibility that removal of these N-terminal residues (and not reactive-centre cleavage) could be responsible for the observed increase in stability has been considered, but the evidence obtained with the snake venoms indicated that the generation of heat-stable C1-INH is only dependent on reactive-centre cleavage. *Bungarus fasciatus* venom was observed to cleave only at the N-terminus of C1-INH, generating a molecule that was heat-labile; however, *Bitis arietans* venom also cleaved at this site and at two additional sites within the reactive centre to generate a heat-stable C1-INH.

To summarize, the inactivations described here occur

Fig. 6. Antigenic heat-stability of native and cleaved C₁-INH preparations

Lane 1, native C₁-INH; lanes 2–7, consecutive 20 min time-point samples taken during the heat denaturation of native C₁-INH; lane 8, *Pseudomonas aeruginosa*-elastase-cleaved C₁-INH. Lanes 9–14, consecutive 20 min time-point samples taken during the heat denaturation of *Pseudomonas aeruginosa*-elastase-cleaved C₁-INH; lane 15, neutrophil-elastase-cleaved C₁-INH; lane 16, as for lane 15 but heated for 120 min; lane 17, *Bitis arietans*-venom-cleaved C₁-INH; lane 18, as for lane 17 but heated for 120 min; lane 19, *Bungarus fasciatus*-venom-cleaved C₁-INH; lane 20, as for lane 19 but heated for 120 min.

as transitions from a 100 kDa species (with only *N*-terminal cleavage) to an 89 kDa species (with additional reactive-centre cleavage), which is heat-stable.

That such inactivation could occur *in vivo* is suggested by the finding that stimulated neutrophils or released neutrophil proteinases generate cleavage patterns identical with that obtained with purified neutrophil elastase.

Furthermore, neutrophils undergoing stimulation inactivate C₁-INH more rapidly than do the released proteinases, suggesting the involvement of some other neutrophil-associated function, possibly the short-lived oxidative component (Badwey & Karnovsky, 1980). Such an increased susceptibility to enzymic hydrolysis after free-radical damage has been reported elsewhere and suggested to be of biological significance (Wolff & Dean, 1986).

The occurrence of this transition should be of use in detecting inactive heat-stable C₁-INH (as well as anti-trypsin and antithrombin III) *in vivo* and may provide a physiological mechanism for the de-regulation of the complement cascade at inflammatory sites.

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