Modification of the tandem reactive centres of human inter- α -trypsin inhibitor with butanedione and *cis*-dichlorodiammineplatinum(II)

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Human inter- α -trypsin inhibitor (I α I) is a plasma proteinase inhibitor active against cathepsin G, leucocyte elastase, trypsin and chymotrypsin. It owes its broad inhibitory specificity to tandem Kunitz-type inhibitory domains within an *N*-terminal region. Sequence studies suggest that the reactive-centre residues critical for inhibition are methionine and arginine. Reaction of I α I with the arginine-modifying reagent butane-2,3-dione afforded partial loss of inhibitory activity against both cathepsin G and elastase but complete loss of activity against trypsin and chymotrypsin. Reaction of I α I with the methionine-modifying reagent *cis*-dichlorodiammineplatinum(II) resulted in partial loss of activity against cathepsin G and elastase but did not affect inhibition of either trypsin or chymotrypsin. Employment of both reagents eliminated inhibition of cathepsin G and elastase. These findings suggest that both cathepsin G and elastase are inhibited at either of the reactive centres of I α I. Trypsin and chymotrypsin, however, appear to be inhibited exclusively at the arginine reactive centre.

INTRODUCTION

Inter- α -trypsin inhibitor (I α I) is a serum glycoprotein of M_r approx. 180 000 (Steinbuch, 1976) found in mammalian species (Albrecht *et al.*, 1983*a,b*). Besides trypsin and chymotrypsin (Heide *et al.*, 1965; Heimbürger *et al.*, 1971), human I α I also inhibits leucocyte (but not pancreatic) elastase (Albrecht *et al.*, 1983*b*; Jochum & Bittner, 1983), plasmin (Lambin *et al.*, 1978) and acrosin (Fritz *et al.*, 1972). Although the role of I α I is unclear, I α I appears to be a proinhibitor that releases smaller physiological inhibitors upon proteolysis (Gebhard & Hochstrasser, 1986). I α I may also act as a shuttle that transfers proteinases to other inhibitors for clearance from the circulation (Pratt & Pizzo, 1986). A fragment with inhibitory activity identical with that of native I α I is obtained upon cleavage of I α I into two fragments with a metalloproteinase (Morii & Travis, 1985). Its sequence is identical with an *N*-terminal region of I α I (Reisinger *et al.*, 1985), where, accordingly, the inhibitory activity resides exclusively.

The broad proteinase inhibitory specificity of human I α I may be the consequence of tandem Kunitz-type inhibitory domains within the *N*-terminal region of the

proinhibitor (Reisinger *et al.*, 1985). I α I is a double-headed inhibitor; the domains have considerable sequence homology with each other (Fig. 1) and with aprotinin, or bovine pancreatic trypsin inhibitor (Kassell *et al.*, 1965; Kassell & Laskowski, 1965). In particular, cysteine residues are conserved. Sequence data (Hochstrasser & Wachter, 1979; Wachter *et al.*, 1979) suggest that the first Kunitz-type domain, closer to the *N*-terminus, has methionine in its reactive centre at position P₁; the second domain has arginine at position P₁. By analogy with α_1 -proteinase inhibitor, with a P₁ methionine residue (Johnson & Travis, 1978), and aprotinin, with a P₁ lysine residue (Wilson & Laskowski, 1971), these I α I domains might be expected to give rise to inhibition of leucocyte elastase and trypsin respectively.

That trypsin inhibition occurs exclusively at the arginine reactive centre was suggested by Hochstrasser & Wachter (1979). This hypothesis received support from sequence studies, since only peptides containing the arginine centre inhibited trypsin. Peptides containing the methionine centre, however, inhibited neither trypsin nor other proteinases (Hochstrasser & Wachter, 1979). Conversely, inhibition of elastase exclusively at the methionine reactive centre was suggested (Albrecht *et al.*,

Fig. 1. Amino acid sequences of human I α I inhibitory domains and aprotinin

(1) I α I *N*-terminal methionine-containing inhibitory domain; (2) aprotinin; (3) I α I arginine-containing inhibitory domain. Asterisks mark critical reactive centre residues. The sequences are aligned to show homology between the two I α I domains (horizontal boxes) and with aprotinin (vertical boxes). The sequence data were compiled from Hochstrasser *et al.* (1976), Hochstrasser & Wachter (1979), Wachter *et al.* (1979), Wachter & Hochstrasser (1979) and Wachter *et al.* (1980).

Abbreviations used: I α I, inter- α -trypsin inhibitor; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); CHD, cyclohexane-1,2-dione; pNpGB, *p*-nitrophenyl *p*'-guanidinobenzoate; Tos-, tosyl-; Bz-, benzoyl-; MeSuc-, 3-methoxycarbonylpropionyl-; -NH-Np, *p*-nitroanilide.

1983a,b) from incubation of trypsin-released inhibitory fragments with elastase. Incubation of such fragments with trypsin before incubation with elastase did not affect inhibition of elastase. Assuming inhibition of trypsin to be exclusively at the arginine centre, this observation was consistent with inhibition of elastase at the methionine centre but not the arginine centre. Because trypsin may be inhibited at methionine reactive centres (Johnson & Travis, 1978), however, additional studies have been needed to clarify the elastase-inhibitory activity of the domains.

Inhibition of chymotrypsin by aprotinin (Kunitz & Northrop, 1936) is well-characterized (Lazdunski *et al.*, 1974) and is a precedent for chymotrypsin inhibition by the IαI arginine reactive centre. A trypsin-complexed IαI fragment containing both domains failed to inhibit chymotrypsin (Albrecht *et al.*, 1983b). In contrast, however, an aprotinin derivative obtained by semi-synthetic replacement of its critical lysine residue with methionine afforded a potent inhibitor of chymotrypsin (Wenzel & Tschesche, 1980, 1981).

In the present study we examine proteinase inhibition at the IαI reactive centres by chemical modification of critical residues. Both cyclohexane-1,2-dione (CHD) (Toi *et al.*, 1965, 1967) and butane-2,3-dione (Riordan, 1973) are well-characterized reagents for selective arginine modification. *cis*-Dichlorodiammine-platinum(II) (*cis*-DDP) is a square-planar complex that inactivates many proteins by reacting selectively with nucleophilic sulphur-containing residues (Aull *et al.*, 1979; Gonias *et al.*, 1984, 1988; Pizzo *et al.*, 1986). Because IαI has no free cysteine residues in its inhibitory region (Hochstrasser *et al.*, 1981), *cis*-DDP is a useful reagent to probe the IαI methionine domain.

EXPERIMENTAL

Materials

Fresh frozen human plasma was obtained from the Duke University Medical Center Transfusion Service. Human leucocyte elastase and cathepsin G were purchased from Elastin Products. Bovine Tos-Phe-CH₂Cl-treated trypsin, bovine Tos-Lys-CH₂Cl-treated chymotrypsin, *p*-nitrophenyl *p*'-guanidinobenzoate (pNpGB), butane-2,3-dione, CHD, Bz-DL-Arg-NH-Np, HCl, MeSuc-Ala-Pro-Val-NH-Np and Hepes were purchased from Sigma Chemical Co. MeSuc-Arg-Pro-Tyr-NH-Np was purchased from KabiVitrum. *cis*-DDP was from Aldrich Chemical Co. Electrophoresis reagents were from Bio-Rad Laboratories, and Triton X-100 was from Research Products International. All other reagents were of the best commercially available quality.

Protein purification

IαI was purified from fresh frozen human plasma by the method of Salier *et al.* (1980) as modified by Morii & Travis (1985), and was stored in 20 mM-Hepes buffer, pH 7.4, containing 150 mM-NaCl at -20 °C. IαI was at least 95% pure on the basis of SDS/polyacrylamide-gel electrophoresis and scanning densitometry. The inhibitory activity of IαI was evaluated by incubating a fixed concentration of active-site-titrated trypsin with increasing amounts of IαI; residual trypsin activity was assayed with Bz-Arg-NH-Np as substrate. For some experiments, IαI concentration was determined by

absorbance at 280 nm by using $A_{1\text{cm}}^{1\%} = 7.1$ (Heide *et al.*, 1965).

Amino acid analysis

The amino acid composition of IαI was determined with a Beckman system 6300 Amino Acid Analyzer after 24, 48 or 96 h of hydrolysis in 6 M-HCl at 110 °C *in vacuo*. Cysteic acid content was determined after performic acid oxidation.

Proteinases and inhibition assays

Trypsin and chymotrypsin were stored in 1 mM-HCl, pH 3.0; leucocyte elastase and cathepsin G were stored in 200 mM-sodium acetate buffer, pH 5.0. Molarities of trypsin and chymotrypsin solutions were determined by active-site titration with pNpGB (Chase & Shaw, 1967). In preliminary experiments incubation of elastase with active-site-titrated α₁-proteinase inhibitor was employed to verify elastase activity reported by the supplier. Cathepsin G activity was also given by the supplier.

Proteinase inhibition assays were performed by incubation of a fixed concentration of proteinase (2.5–20 nM) with increasing concentrations of IαI at 37 °C. Unless noted otherwise, all incubations were performed in 20 mM-Hepes buffer, pH 7.4, containing 150 mM-NaCl and 0.02% Triton X-100. Triton was employed to minimize protein aggregation and surface denaturation as previously described (Pratt & Pizzo, 1987). Proteinase-catalysed substrate cleavage was halted by the addition of acetic acid (50%, v/v). Substrate cleavage was monitored by absorbance at 405 nm. Controls in which either IαI or both IαI and proteinase were omitted were designated 100% and 0% active, respectively.

Trypsin inhibition was evaluated by 10 min incubation with IαI followed by the addition of 1.0 mM-Bz-Arg-NH-Np. Chymotrypsin inhibition was evaluated by 30 min incubation with IαI followed by addition of 40 μM-MeSuc-Arg-Pro-Tyr-NH-Np. Elastase inhibition was evaluated by 12 min incubation with IαI followed by the addition of 240 μM-MeSuc-Ala-Ala-Pro-Val-NH-Np. Sufficiency of incubation times for completeness of proteinase-inhibitor interaction is documented (Pratt & Pizzo, 1987).

Because cathepsin G is chymotrypsin-like in many respects (Barrett, 1981), MeSuc-Arg-Pro-Tyr-NH-Np was examined as a cathepsin G substrate. Proteinase-catalysed substrate cleavage was observed, and K_m for the interaction was determined by combining 100 nM-cathepsin G with increasing concentrations of MeSuc-Arg-Pro-Tyr-NH-Np; the rate of reaction was monitored for several minutes at 405 nm. Cathepsin G inhibition was evaluated by 30 min incubation with IαI followed by the addition of 60 μM-MeSuc-Arg-Pro-Tyr-NH-Np.

Reaction of IαI with CHD

IαI arginine modification was performed with CHD by the methods of Patthy & Smith (1975a,b) and of Liu *et al.* (1968). CHD was added to a final concentration of 200 mM to 200 mM-boric acid/200 mM-NaCl, pH 7.4. After complete solution of solid CHD at room temperature, the pH was re-adjusted to 7.4, at which it was maintained for 30 min. A volume of 150–200 μl of this solution was combined with an equal volume of 2–4 μM-IαI at room temperature for 3 h. Free CHD was removed by dialysis versus 100 mM-boric acid/100 mM-NaCl, pH 7.4, at 4 °C. For reversal of arginine

modification, CHD-I α I was dialysed against 80 mM-hydroxylamine in 50 mM-Tris/HCl buffer, pH 7.4, containing 100 mM-NaCl at 4 °C.

Reaction of I α I with butanedione

I α I arginine modification was performed with butane-2,3-dione by the method of Riordan (1973). A 100 μ l volume of butanedione stock solution was combined with 5 ml of 50 mM-boric acid/100 mM-NaCl. The pH, which initially decreased markedly, was maintained at 7.4 for 30 min at room temperature by the addition of NaOH solution. Thereafter, 150–250 μ l of 2–4 μ M-I α I was combined with a 3-fold greater volume of butanedione solution for 3 h at room temperature. Excess reagent was removed by dialysis versus 50 mM-boric acid/100 mM-NaCl, pH 7.4 at 4 °C for 2 h, followed by exhaustive dialysis against 20 mM-Hepes buffer, pH 7.4, containing 150 mM-NaCl at 4 °C.

Reaction of I α I with *cis*-DDP

Crystalline *cis*-DDP was dissolved in 20 mM-Hepes buffer, pH 7.4, containing 150 mM-NaCl at room temperature in the dark less than 1 h before use. I α I (1–2 μ M) was incubated with 1.67 μ M-*cis*-DDP at 37 °C in the dark for 4 h in the same buffer. The reaction volume was 400 μ l. Free *cis*-DDP was removed by exhaustive dialysis against the same buffer at 4 °C.

Platinum assays

Platinum binding to I α I was assayed by the method of Ayres & Meyer (1951) as applied to proteins by Gonias & Pizzo (1983). SnCl₂·2H₂O was dissolved in conc. HCl (2.2 g in 3 ml) about 6 h before use. Upon solution clarification, 7 ml of water was added.

cis-DDP-treated I α I (450 μ l) was combined with 100 μ l of conc. HCl at 110 °C for 18 h. After cooling, these reagents were added with agitation: 20% (w/v) NH₄Cl (250 μ l) and SnCl₂ solution (200 μ l). Maximal colour developed within 1 h. The absorbance was measured at 403 nm.

A standard curve was generated with increasing concentrations of *cis*-DDP in 450 μ l to which 100 μ l of conc. HCl was added. These portions were treated as described above; their absorbance at 403 nm was a linear function of platinum concentration from 6 to 90 μ M-platinum. Platinum concentration in protein samples was compared with protein concentration.

Proteolysis of I α I

Proteinases inhibited by I α I also cleave I α I into fragments that retain inhibitory activity (Pratt & Pizzo, 1987). Native and butanedione-modified I α I (80–120 pmol) were incubated with either trypsin or chymotrypsin for 1 h at 37 °C. The ratios of proteinase to inhibitor were 1:1 and 0.5:1 respectively. Proteinase was inactivated by addition of excess pNpGB. Samples were denatured in 1% SDS at 100 °C and subjected to electrophoresis.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed according to the method of Neville (1971) with 7.5% polyacrylamide slabs. Proteins were stained either with Coomassie Brilliant Blue R-250 or with silver by a modification of the method of Morrissey (1981).

Fig. 2. Eadie-Hofstee plot of cathepsin G-catalysed cleavage of MeSuc-Arg-Pro-Tyr-NH-Np

Rates of cleavage were determined for increasing concentrations (2.0–160 μ M) of substrate incubated at room temperature with a fixed concentration (100 nM) of proteinase. The reaction buffer was 20 mM-Hepes buffer, pH 7.4, containing 150 mM-NaCl and 0.02% Triton X-100. The slope, defined as $-K_m$, afforded a K_m of 22 μ M for MeSuc-Arg-Pro-Tyr-NH-Np as a cathepsin G substrate.

Inhibition equilibrium constants

Characterization of proteinase inhibition was based on the scheme:

in which E, S and I are proteinase, substrate and inhibitor respectively; K_i is the inhibition equilibrium constant and K_m the Michaelis constant for enzyme-catalysed substrate cleavage.

Because proteinase inhibition assays were performed by addition of increasing amounts of I α I to a fixed concentration of proteinase, the method of Henderson (1972) and Bieth (1980) was employed to approximate K_i values. Graphic treatment of inhibition data afforded an apparent inhibition constant based on the equation:

$$[I]_0/(1-a) = K_{i(\text{app.})}(1/a) + [E]_0$$

in which $[I]_0$ and $[E]_0$ are initial concentrations of I α I and proteinase, and a is the fractional residual proteinase activity. The $K_{i(\text{app.})}$ so obtained was refined by accounting for the extent to which substrate may, in the scheme above, promote reaction progress to the right:

$$K_i = \frac{K_{i(\text{app.})}}{1 + ([S]_0/K_m)}$$

RESULTS

Studies with cathepsin G

Although inhibition of cathepsin G by I α I has been reported (Morii & Travis, 1985), the interaction has not been extensively characterized. This prompted a study of the suitability of MeSuc-Arg-Pro-Tyr-NH-Np as a cathepsin G substrate. Rates of catalytic cleavage were determined for increasing concentrations (2–160 μ M) of MeSuc-Arg-Pro-Tyr-NH-Np. Eadie-Hofstee graphical analysis (Eadie, 1947; Hofstee, 1959) according to the equation:

$$V = V_{\text{max.}} - (K_m \cdot V/[S]_0)$$

Fig. 3. Inhibition of cathepsin G by I α I

○, 20 nM-cathepsin G and native I α I; ▲, 20 nM-cathepsin G and *cis*-DDP-modified I α I; ■, 10 nM-cathepsin G and butanedione-modified I α I; ●, 10 nM-cathepsin G and doubly modified I α I. Modification procedures and inhibition assays are described in the Experimental section.

Fig. 4. Inhibition of leucocyte elastase by I α I

○, 2.5 nM-elastase and native I α I; ▲, 5 nM-elastase and *cis*-DDP-modified I α I; ■, 5 nM-elastase and butanedione-modified I α I; ●, 5 nM-elastase and doubly modified I α I. Modification procedures and inhibition assays are described in the Experimental section.

afforded a K_m of 22 μ M for MeSuc-Arg-Pro-Tyr-NH-Np at room temperature (Fig. 2).

Analysis of kinetic data recorded with substrate concentrations below K_m was performed to determine k_{cat} for MeSuc-Arg-Pro-Tyr-NH-Np with cathepsin G. By using the proteinase concentration supplied by the manufacturer, the equation:

$$V = [E][S]_0 k_{cat} / K_m$$

afforded an average k_{cat} of 0.5 s⁻¹.

Table 1. Inhibition equilibrium constants for I α I

Proteinase	K_i (nM)		
	I α I	Butanedione-modified I α I	<i>cis</i> -DDP-modified I α I
Trypsin	0.078*	—	0.078
Chymotrypsin	1.1*	—	1.1
Elastase	61*	~ 130	~ 78
Cathepsin G	18	~ 270	~ 270

* Taken from Pratt & Pizzo (1987).

All proteinase inhibition assays were based on the ability of I α I to bind proteinase and thus prevent proteinase-catalysed cleavage of a chromogenic substrate. I α I inhibited cathepsin G (Fig. 3) almost as effectively as it inhibited elastase (Fig. 4). We observed inhibition of elastase (Table 1) identical with a previous report (Pratt & Pizzo, 1987). As inhibitor concentration increased to 10 times that of cathepsin G, MeSuc-Arg-Pro-Tyr-NH-Np cleavage declined markedly. Henderson-Bieth analysis (see the Experimental section) of inhibition data yielded an equilibrium inhibition constant of 18 nM, comparable with that reported for elastase (see Table 1). No increase in the extent of cathepsin G inhibition by I α I was observed in incubations of proteinase with inhibitor for longer than 30 min.

Inhibition studies with CHD-modified I α I

In preliminary experiments I α I was treated with CHD for arginine modification. Borate anion stabilizes CHD-modified arginine residues (Smith, 1977); removal of borate is the basis for reversal, which is facilitated by treatment with nucleophiles such as Tris or hydroxylamine (Patthy & Smith, 1975a).

CHD-modified I α I showed loss of proteinase inhibitory activity against trypsin (Fig. 5) and elastase identical with that described below for butanedione-modified I α I; the activity of trypsin and elastase in borate buffer was indistinguishable from that in Hepes buffer. Reactions of I α I with less reagent or for less time afforded incomplete modification on the basis of residual trypsin activity. Treatment of CHD-modified I α I with 80 mM-hydroxylamine afforded substantial restoration of trypsin inhibitory activity (Fig. 5); elastase inhibition was similarly restored (results not shown).

Because cathepsin G was nearly 50% less active in borate than in Hepes buffer, butanedione modification was employed in subsequent experiments. Inhibition studies with butanedione-modified I α I were performed in Hepes buffer.

Studies with butanedione-modified I α I

Butanedione-modified I α I showed substantial loss of inhibitory activity against cathepsin G (Fig. 3) and elastase (Fig. 4). The inhibition data were analysed by the Henderson-Bieth method to estimate inhibition equilibrium constants for the modified inhibitor (Table 1). Butanedione-modified I α I was completely inactive against both trypsin (Fig. 6) and chymotrypsin (Fig. 7). Butanedione modification of proteins is not generally reversible (Riordan, 1973), and no evidence of restored

Fig. 5. Reversible elimination of I α I trypsin-inhibitory activity by CHD

●, 20 nM-trypsin and I α I modified by 3 h reaction with CHD; ■, 20 nM-trypsin and CHD-modified I α I after 13 h hydroxylamine treatment; ▲, 20 nM-trypsin and CHD-modified I α I after 34 h hydroxylamine treatment. Procedures for I α I modification and reversal are described in the Experimental section.

Fig. 6. Inhibition of trypsin by I α I

○, 50 nM-trypsin and native I α I; ▲, 20 nM-trypsin and *cis*-DDP-modified I α I; ■, 20 nM-trypsin and butanedione-modified I α I; ●, 20 nM-trypsin and doubly modified I α I. Modification procedures and inhibition assays are described in the Experimental section.

inhibitory activity was observed on removal of borate buffer or on hydroxylamine treatment. Modification reactions performed with less reagent or for less time resulted in inhibitor modification that was incomplete on the basis of residual trypsin inhibition. Extent of arginine modification by butane-2,3-dione was quantified by amino acid analysis as described previously (Riordan, 1973). Comparison of amino acid compositions of native and modified I α I suggested 66% arginine modification, or reaction of butanedione with about 41 of 62 total arginine residues per molecule. Dione reagents typically modify at least two-thirds of arginine residues (Liu *et al.*, 1968; Patthy & Smith, 1975b). Lysine content (78 residues per molecule) was largely unaffected.

Butanedione-modified I α I was treated with trypsin and chymotrypsin under conditions that result in proteolysis of native I α I. These reactions provided patterns of fragments on SDS/polyacrylamide-gel electrophoresis that were strictly analogous to those observed with trypsin- or chymotrypsin-mediated proteolysis of native I α I (results not shown). Butanedione-modified I α I had electrophoretic mobility identical with that of native I α I on SDS/polyacrylamide gels.

Because I α I is highly susceptible to proteolysis (Pratt & Pizzo, 1987), chemical modification procedures were performed only in the absence of proteinases. Although proteinases might protect inhibitor reactive centres from chemical modification, the resulting proteolytic fragmentation would, in the case of I α I, make results difficult to interpret.

Studies with *cis*-DDP-modified I α I

cis-DDP-modified I α I showed loss of inhibitory activity against cathepsin G (Fig. 3) and elastase (Fig. 4). This effect was dependent on both time of incubation with and concentration of *cis*-DDP used. Under the final conditions employed for studies with proteinases, loss of inhibitory activity was maximal. Modification with either butanedione or *cis*-DDP impaired I α I inhibition of cathepsin G to an equal extent (Fig. 3). The *cis*-DDP-modified inhibitor, however, was far less active against elastase than was butanedione-modified I α I (Fig. 4). Estimates of inhibition equilibrium constants are presented in Table 1. The inhibitory activity of I α I against trypsin and chymotrypsin was virtually unaffected by treatment of I α I with *cis*-DDP (Figs. 6 and 7). On the basis of the SnCl₂ assay for platinum incorporation, a portion with 4.48 nmol of *cis*-DDP-treated I α I contained 22.5 nmol of platinum. This suggests binding of 5.0 mol of *cis*-DDP/mol of I α I.

Studies with doubly-modified I α I

Doubly modified I α I was prepared by application of the above procedures for *cis*-DDP modification and for butanedione modification in succession. This material inhibited neither cathepsin G (Fig. 3) nor elastase (Fig. 4). As expected, the doubly modified I α I was completely inactive against trypsin (Fig. 6) and chymotrypsin (Fig. 7), as was butanedione-modified I α I.

Amino acid composition

cis-DDP reacts almost exclusively with sulphur-containing amino acids in proteins (Gonias *et al.*, 1984).

Fig. 7. Inhibition of chymotrypsin by I α I

○, 5 nM-chymotrypsin and native I α I; ▲, 5 nM-chymotrypsin and *cis*-DDP-modified I α I; ■, 5 nM-chymotrypsin and butanedione-modified I α I; ●, 5 nM-chymotrypsin and doubly modified I α I. Modification procedures and inhibition assays are described in the Experimental section.

Amino acid analysis showed I α I to have 30 methionine and 21 cysteine residues per protein molecule. As only 5 mol of platinum was incorporated per mol of I α I, these results suggest that *cis*-DDP modifies only a limited number of potential target residues. A similar result has been obtained with a number of other proteins, including several proteinase inhibitors (Gonias & Pizzo, 1983; Gonias *et al.*, 1984, 1988; Pizzo *et al.*, 1986; Swaim & Pizzo, 1988).

DISCUSSION

Localization of proteinase inhibition to either of two reactive centres of I α I has remained equivocal. We have examined proteinase interaction with I α I to which we applied selective chemical modification procedures aimed at sterically hindering critical inhibitory residues.

I α I inhibited cathepsin G, confirming the preliminary report by Morii & Travis (1985). Characterization showed the inhibition effectiveness to be comparable with that of leucocyte elastase (Table 1). Inhibition of elastase, trypsin and chymotrypsin was identical with a previous report (Pratt & Pizzo, 1987). Both CHD and butanedione eliminated the trypsin-inhibitory activity of I α I. The reagents were equally effective in partially eliminating leucocyte elastase inhibition by I α I. Reaction with butanedione eliminated chymotrypsin inhibition and diminished cathepsin G inhibition.

Both dione reagents react highly selectively with arginyl guanidinium moieties; they are particularly useful in determining functional arginine residues in native proteins (Malinowski & Fridovich, 1979; Marshall &

Cohen, 1980). Elimination of trypsin-inhibitory activity by blocking ovomucoid arginine residues is well-characterized (Liu *et al.*, 1968).

Arginine residues are not equally reactive with these reagents (Marshall & Cohen, 1980) and thus are not indiscriminately modified. Functional exposed arginine residues react most readily. I α I contains arginine residues highly susceptible to proteolysis at which inhibition does not occur (Pratt & Pizzo, 1987). Arginine-blocked I α I failed to inhibit trypsin. Trypsin proteolysis of the modified I α I, however, afforded the same fragments as native I α I. This suggests that sites of proteolysis were not modified by the dione reagents, and mitigates against denaturation by the reagent. Moreover, we restored both trypsin- and elastase-inhibitory activity to CHD-modified I α I with hydroxylamine. Unlike butanedione, CHD reacts reversibly with arginine side chains (Riordan, 1973; Smith, 1977).

Our observations strongly suggest that inhibition of both trypsin and chymotrypsin occurs exclusively at the arginine reactive centre. Arginine-blocked I α I, however, retained partial ability to inhibit both cathepsin G and elastase. This finding was probed by reaction of I α I with *cis*-DDP, a selective modifier of methionine residues. *cis*-DDP-treated I α I showed partial loss of inhibitory activity against cathepsin G and slight loss of inhibitory activity against elastase. Its capacity to inhibit trypsin and chymotrypsin, however, was unaffected. Complete elimination of activity against cathepsin G or elastase was obtained only when *cis*-DDP-treated I α I was treated with butanedione.

cis-DDP is an anti-neoplastic drug that reacts with proteins in a specific and limited manner (Howe-Grant & Lippard, 1980). In nearly every study, *cis*-DDP is selectively bound at methionine and cysteine residues of proteins (Dickerson *et al.*, 1969; Aull *et al.*, 1979; Gonias & Pizzo, 1981, 1983; Bodenner *et al.*, 1986; Pizzo *et al.*, 1986). *cis*-DDP has proven invaluable for probing reactive centres of the plasma proteinase inhibitors α_2 -macroglobulin, α_1 -proteinase inhibitor and α_2 -plasmin inhibitor, where exposed methionine residues are crucial for function (Pizzo *et al.*, 1986; Gonias *et al.*, 1988; Swaim & Pizzo, 1988). These methionine residues, freely accessible to solvent, are more reactive with *cis*-DDP than are other methionine residues. We have recently demonstrated that *cis*-DDP reacts exclusively with the reactive-centre methionine of α_1 -proteinase inhibitor and inactivates the inhibitor (Gonias *et al.*, 1988). Likewise, *cis*-DDP abolishes the plasmin-inhibitory capacity of human α_2 -plasmin inhibitor (Swaim & Pizzo, 1988). This effect almost certainly owes to modification of the α_2 -proteinase inhibitor P $_1$ ' methionine residue (Shieh & Travis, 1987).

Platinum-binding assay results suggest covalent association of five molecules of *cis*-DDP with each molecule of I α I. By analogy with Kunitz-type inhibitors, the P $_1$ methionine residue of the I α I N-terminal inhibitory domain (and the nearby P $_2$ ' methionine residue) should be exposed and highly susceptible to modification by *cis*-DDP. *cis*-DDP-treated I α I strongly inhibited trypsin and chymotrypsin, and thus the integrity of the molecule was largely unaffected.

Given the binding specificity of *cis*-DDP, additional molecules may bind to other methionine residues within the methionine-containing inhibitory domain (Fig. 1). The selectivity of *cis*-DDP is underscored by the fact

that, although a total of 51 sulphur-containing residues occur per I α I molecule, only five molecules of *cis*-DDP bind. The likelihood of binding to additional methionine residues within the methionine-containing inhibitory domain (Fig. 1) is enhanced by the absence of free thiol moieties in the active portion of I α I (Hochstrasser *et al.*, 1981). The absence of methionine residues within the arginine-containing domain (Fig. 1) bears emphasis; proteinase inhibition that we ascribe to the arginine reactive centre is unaffected by *cis*-DDP.

We propose that the cathepsin G and leucocyte elastase may be inhibited at either of the Kunitz-type reactive centres of I α I. Chemical blockade of either critical reactive-centre residue alone was necessary but insufficient to eliminate inhibition of these proteinases. The failure of *cis*-DDP to eliminate completely the inhibition of elastase and cathepsin G strongly suggests that these proteinases are also bound at a second reactive centre of I α I. We cannot rule out the possibility that inadvertent chemical modification of the P₅' arginine residue near the methionine reactive centre impairs binding of these proteinases at the methionine inhibitory domain. However, CHD and butanedione modification impaired I α I inhibition of elastase to an equal extent (results not shown). If modification of the P₅' arginine residue sterically inhibited access of proteinases to the methionine reactive centre, one might expect CHD-modified I α I to be a poorer elastase inhibitor than butanedione-modified I α I, since CHD is a bulkier reagent.

Inhibition equilibrium constants for modified I α I are shown in Table 1; these express approximate extents of inhibition at either I α I domain. On the basis of the preceding discussion, the constants for butanedione-modified I α I are approximate representations of proteinase interaction with the methionine reactive centre. Likewise, constants for *cis*-DDP-modified I α I represent proteinase interaction with the arginine reactive centre. From these data, cathepsin G is inhibited with approximately equal affinity at either reactive centre. Leucocyte elastase, however, is apparently inhibited more effectively at the arginine centre. The relation, if any, between these constants and that for simultaneous inhibition at either centre remains unclear.

Arginine modification alone eliminates inhibition of trypsin and chymotrypsin, which we propose are inhibited solely at the arginine reactive centre. Chymotrypsin inhibition at a charged residue may seem surprising given its proteolytic specificity. However, inhibition of chymotrypsin by aprotinin, with a reactive-centre lysine residue, is well-characterized (Blow & Wright, 1972). Guanidination of this lysine has no effect on chymotrypsin inhibition (Schweitz *et al.*, 1973).

The inhibition constants for trypsin and chymotrypsin with aprotinin are, respectively, 6.0×10^{-5} M and 9.0 M (Lazdunski *et al.*, 1974). Constants for the same reactions with I α I are only two orders of magnitude apart (Table 1). This may owe to differences in nearby (for example, P₁', P₂') residues, which has been generally suggested (Albrecht *et al.*, 1983a,b). Vicinal residues differ in the inhibitory domains of I α I (Fig. 1) and may help account for the breadth of inhibitory specificity as well as inhibition of proteinases at either domain. This concept may provide the basis for studies on the relation of inhibitory specificity to inhibitor reactive-centre sequence.

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