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A study of the specificity of barley chymotrypsin inhibitor 2 by cysteine engineering of the P1 residue

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Abstract

A combination of oligonucleotide-directed mutagenesis and chemical modification was used to produce reactive site (P1) variants of chymotrypsin inhibitor II (CI2) in an attempt to create more potent inhibitors and examine inhibitory specificity. Mutagenesis to introduce a unique cysteine (CI2M59C) followed by modification to *S*-carboxamidocysteine with iodoacetamide produced a 7-fold more potent inhibitor of subtilisin BPN′ than the wild type inhibitor. Modification with iodoacetic acid, which gives a negatively charged P1 residue (*S*-carboxymethylcysteine), generates a weaker inhibitor of subtilisin BPN′ and chymotrypsin. Further chemical modification experiments of CI2M59C with a series of iodoalkanes of increasing chain lengths was used to determine the optimal P1 side chain length required for potent inhibition of porcine pancreatic elastase. A trend was observed which implies that for CI2 the original methionine residue or its isostere *S*-ethylcysteine are most effective residues at this position and not alanine as might have been expected from the substrate specificity. The mutant CI2M59C did not inhibit human neutrophil elastase. The iodoalkane modifications not only resulted in recovery of inhibitory activity but also proved to be substantially more potent inhibitors of human neutrophil elastase than wild-type CI2.

Keywords: Protease inhibitor; Chymotrypsin; Subtilisin; Elastase; Cysteine engineering; Protein engineering

1. Introduction

With the advent of oligonucleotide-directed mutagenesis [1], it has become possible to make defined amino acid substitutions at specific positions in proteins. This method of perturbing protein structure has become popular for enzyme structure–function studies as it overcomes the many limitations associated with chemical modification studies, which have been used classically to identify functionally important amino acid residues in proteins [2]. However, only natural amino acids residues can be introduced by oligonucleotide-directed mutagenesis. Methods to incorporate non-natural amino acids into proteins have been developed involving in vitro misacylation of suppressor tRNAs [3–5], but this allows only small amounts of mutant protein to be produced. Thus, a combination of oligonucleotide-directed mutagenesis and chemical modification remains a favored way of introducing non-standard amino acids. One of the most popular combinations is the introduction of a cysteine residue by oligonucleotide directed mutagenesis and its subsequent modification using one of the many available modifying reagents to residues such

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as *S*-aminoethylcysteine [6,7], and *S*-carboxymethylcysteine [8]. This 'cysteine engineering' results in the specific introduction of a non-natural amino acid. We describe here the cysteine engineering of a serine protease inhibitor, chymotrypsin inhibitor II (CI2). Modification of the P1 residue, the primary determinant of inhibitory specificity [9], is used to create novel and more potent inhibitors of a variety of serine proteases and to further our understanding of inhibitory specificity.

CI2 belongs to the Potato Inhibitor I family of inhibitors (lacking cysteine residues) and potently inhibits serine proteases such as subtilisin BPN′, moderately inhibits chymotrypsin, porcine pancreatic elastase (PPE) and human neutrophil elastase (HNE), but does not inhibit trypsin [10]. It is a small 83-residue globular protein of molecular weight 9250 Da. The three-dimensional X-ray crystal structures have been determined for the free inhibitor [11] and for the complex with subtilisin BPN′ [12]. The CI2 molecule is a wedge-shaped disk with the reactive site loop on the narrow edge, which encompasses the Met 59–Glu 60 scissile (reactive site) bond [13]. The hydrophobic core of the protein is formed by the packing of the four strands of β-sheet against an α-helix [11]. The gene for CI2 has been cloned, sequenced [14] and the recombinant protein has been overexpressed [10]. In the current study, a truncated form of CI2 lacking the first 19 N-terminal amino acids was used [15]. This section lacks secondary or tertiary structure as indicated by NMR spectroscopy [16,17], is frequently missing in CI2 isolated from barley, presumably due to proteolysis, and apparently serves no purpose in the protein function.

In the present study, oligonucleotide-directed mutagenesis was performed to substitute the P1 methionine residue by a unique cysteine residue (CI2M59C). This cysteine residue was then chemically derivatized to *S*-carboxamidocysteine and *S*-carboxymethylcysteine by iodoacetamide and iodoacetic acid to produce a neutral and negatively charged P1 residue respectively. The mutant was also modified to *S*-methylcysteine, *S*-ethylcysteine and *S*-propylcysteine by a series of iodoalkanes varying in chain length using iodomethane, iodoethane and iodopropane, respectively. The inhibition properties of these chemical mutants were determined against subtilisin BPN′, chymotrypsin, porcine pancreatic elastase and human neutrophil elastase to examine the effects on K_i. Where possible, the individual rate constants for complex association (k_{on}) and dissociation (k_{off}) were also calculated.

2. Materials and methods

2.1. Site-directed mutagenesis

The site-directed mutant CI2M59C was constructed by the method of Kunkel [18]. To achieve this, an *Xba*I–*Hin*dIII fragment containing the truncated CI2 gene [15] was subcloned into M13mp18. Single-stranded uracil-containing phage DNA was isolated and used as a template for hybridization with the following 5′-phosphorylated oligonucleotide: 5′-CGATATTCGCAGGTCACA-3′. The mismatched nucleotides (underlined) correspond to mutation of the P1 methionine residue at position 59 to cysteine (ATG → TGC). Molecules were rendered double-stranded by the action of *Escherichia coli* DNA polymerase I (Klenow fragment) and T4 DNA ligase and transformed into JM101. Mutant clones were identified by differential hybridization with the 5′ – γ^{32}P labeled mutagenic oligonucleotide and the presence of the correct DNA sequence verified using the dideoxynucleotide chain termination method [19]. The mutant gene was then subcloned as a *Nde*I–*Hin*d III fragment into the expression vector pT7-7 [20].

2.2. Expression and purification

The inhibitor was expressed in *E. coli* K38 (*Hfr* C(λ)) [21] containing the heat shock inducible plasmid pGP1-2 [22], following transformation of the plasmid pT7CI2M59C. A single colony was picked from a Petri dish containing H-agar supplemented with ampicillin (100 μg ml^{-1}) and kanamycin (70 μg ml^{-1}). This was inoculated into 5 ml of 2 × YT containing the appropriate antibiotics and grown at 30°C for 6 h. A 50 μl aliquot of this culture was used to inoculate 500 ml of 2 × YT (containing antibiotics as before) in a 2 l baffled flask and grown at 30°C for a further 14 h. Following heat shock at 42°C for 45 min, the culture was grown for a further 3 h at 37°C to allow expression of the recombinant protein. Cells were then harvested by centrifugation at 4°C (8000

rpm, 20 min) and then resuspended in 10 ml TE buffer (0.1 M Tris–HCl, 10 mM EDTA, pH 8.0). The inhibitor was purified essentially as described by Longstaff et al. [10] and was lyophilized before use. The purity of the inhibitor was greater than 95%, as judged by SDS-PAGE.

2.3. Titration of the sulfhydryl content with 5,5′-dithiobis 2-nitrobenzoic acid (DTNB)

Titration of the sulfhydryl content of CI2M59C was carried out by the method of Ellman [23]. 1 mg of protein was dissolved in 1 ml of TE buffer and 100 μl of this solution was placed in a cuvette with 980 μl of the same buffer. The absorbance at 280 nm was recorded to determine the inhibitor concentration ($\varepsilon = 6965\ M^{-1}\ cm^{-1}$). 10 μl of DTNB (4 mg ml^{-1}) was added to the cuvette and the reaction incubated at room temperature for 15 min. The increase in absorbance at 412 nm due to 5-thio-2-nitrobenzoate was recorded to determine the concentration of free thiol groups ($\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$).

2.4. Reduction of intermolecular disulfide bonds

The mutant protein CI2M59C (2 mg) was dissolved in 1 ml 0.1 M Tris–HCl (pH 8.2) and dithiothreitol (DTT) added to a final concentration of 10 mM. Nitrogen gas was bubbled through the solution to purge the system of air and the mixture incubated overnight in the dark at 37°C.

2.5. Chemical modification

Iodoacetamide or iodoacetic acid was added to reduced CI2M59C protein (typically 10 mg) in 0.1 M Tris–HCl (pH 8.2), 10 mM DTT under nitrogen, to a final concentration of 25 mM and incubated for various times at 37°C in the dark. Excess modification and reducing agents were removed by extensive dialysis against water and the protein subsequently lyophilized. Iodomethane, iodoethane and iodopropane (1 M solutions in 20% 2,2-dimethylformamide (DMF)) were added to reduced CI2M59C protein (10 mg) to a final concentration of 35 mM. Other reaction conditions were as before. Excess modification and reducing agents were removed by desalting on a 10 ml Sephadex G-15 gel filtration column equilibrated in water. The protein was then dialyzed exhaustively against water and subsequently lyophilized.

2.6. Isoelectric focusing

Analysis of modification reactions with iodoacetic acid was carried out using the Pharmacia Phast System™, according to the manufacturers instruction, using Phastgel™ (Pharmacia-LKB) covering the pH range 3–9. For calibration, the Pharmacia Broad p*I* Calibration Kit (pH 3–10) was used.

2.7. Kinetic methods

CI2 is a competitive inhibitor of serine proteases [10]. The equilibrium constant for dissociation of the *EI* complex, K_i, is related to the individual rate constants and the concentration of enzyme, *E*, and inhibitor, *I*, as in Eq. (1).

$$K_i = \frac{k_{off}}{k_{on}} = \frac{[E][I]}{[EI]} \quad (1)$$

Characterization of k_{on}, k_{off} and K_i for the inhibitors was performed essentially as described by Longstaff et al. [10]. Additionally, values for K_i were also determined by the equilibrium method [24]. Assays for inhibition of subtilisin BPN′ (Sigma) were performed in 0.1 M Tris–HCl (pH 8.6) at 25°C. The chromogenic substrate was succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide (Sigma). Values of k_{cat} and K_m under these conditions are 55 s^{-1} and 0.15 mM [25]. Bovine pancreatic α-chymotrypsin (Sigma) was assayed in 0.144 M Tris–HCl (pH 7.78) at 25°C with the substrate succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide. The values of k_{cat} and K_m are 45 s^{-1} and 0.04 mM, respectively [26]. Where indicated in the text, buffers also contained 0.01% Tween 80. Porcine pancreatic elastase (PPE, Sigma) was assayed in 0.2 M Tris–HCl (pH 8.0) at 25°C with succinyl–Ala–Ala–Ala–*p*-nitroanilide (Sigma) as a substrate. The values for k_{cat} and K_m are 16.6 s^{-1} and 1.15 mM respectively [27]. Human neutrophil elastase (HNE, Elastin Products, MO) was assayed in 0.1 M Tris–HCl (pH 7.5) with the chromogenic substrate methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide

(Calbiochem). The values of k_{cat} and K_m are 17 s^{-1} and 0.14 mM, respectively [28]. The residual enzyme activity of all serine proteases was monitored as an increase in absorbance at 412 nm, due to the release of the *p*-nitroanilide group. The determination of K_i value for the mutant CI2M59C was carried out similarly except for addition of 10-fold excess of the disulfide reducing agent DTT to prevent dimerization of the inhibitor. Kinetic measurements were carried out using an Ultrospec III spectrophotometer (Pharmacia). All kinetic constants were confirmed by multiple (at least three) independent experiments. Parameter values and estimates for their standard errors were obtained by fitting data to the appropriate equation, as described below, using non-linear regression [29].

2.8. Measurement of k_{on} and K_i by slow-binding kinetics

The progress of a reaction inhibited by a slow-binding inhibitor can be followed spectrophotometrically by adding enzyme to a solution of inhibitor and a chromogenic substrate and plotting product formation as a function of time. This allows k_{on} and K_i values to be determined from a single experiment [10,30–32], using the relationship given in Eq. (2).

$$P = v_s t + (v_o - v_s)\frac{(1 - e^{-k't})}{k'} \quad (2)$$

This equation relates the amount of product, P, formed as a function of time, t. The parameters v_o and v_s are the initial and final steady-state rates, respectively, and k' is the apparent first-order rate constant for the transition to the steady-state rates. The variation of k' with the inhibitor concentration allows the value of k_{on} to be calculated by applying Eq. (3).

$$k' = k_{off} + \frac{k_{on}[I]}{(1 + [S]/K_m)} \quad (3)$$

A plot of k' vs. $[I]$ allows k_{on} to be calculated from the slope. Theoretically, k_{off} can be determined from the intercept on the *Y*-axis. However, in practice, k_{off} is frequently too small to allow accurate determination from such plots.

A value for K_i can be obtained from the variation of v_s with $[I]$, using Eq. (4).

$$\frac{v_o - v_s}{v_s} = \frac{[I]}{K_i(1 + [S]/K_m)} \quad (4)$$

A plot of $(v_o - v_s)/v_s$ vs. $[I]$ produces a straight line through the origin, from which a value for K_i can be determined using Eq. (5), in which m is the gradient of the line.

$$K_i = \frac{1}{m(1 + [S]/K_m)} \quad (5)$$

When values for k_{on} and K_i are both known, k_{off} can be calculated from Eq. (1).

Slow binding inhibition studies were performed using pseudo-first order conditions ($[I]_0 \gg [E]_0$). Varying concentrations of inhibitor (2.5 to 14 nM) were pre-equilibrated with substrate solution (containing 0.01% Tween 80) at a final concentration of 1 mM. The reaction was initiated by the addition of enzyme (final concentration 0.25 nM).

2.9. Measurement of K_i by the equilibrium method

The values for K_i were primarily measured after allowing binding to reach equilibrium [24]. A fixed concentration of enzyme (typically 10 nM) was incubated with varying concentrations of inhibitor and allowed to reach equilibrium (15 min). Substrate was added to a final concentration of 1 mM and the reaction followed spectrophotometrically for 30 min. For a tight-binding inhibitor, the K_i value can be determined by fitting the data to Eq. (6).

$$\text{Rate} \propto [E]_0 - \frac{1}{2}\left\{[E]_0 + [I]_0 + K_i\sqrt{([E]_0 + [I]_0 + K_i)^2 - 4.[E]_0[I]_0}\right\} \quad (6)$$

For classical (non tight-binding) inhibitors, Eq. (7) is used to derive a value for K_i.

$$V_i = V_0\left(\frac{K_i}{K_i + [I]}\right) \quad (7)$$

A plot of residual rate (V_i/V_o) vs. $[I]$ can be fitted directly by non-linear regression to obtain a value for

K_i. K_i values measured for both tight binding and classical inhibitors must be corrected by applying Eq. (9) to yield a true value for K_i at zero substrate concentration.

$$K_{i(apparent)} = K_{i(actual)}(1 + [S]/K_m) \quad (8)$$

2.10. Dissociation kinetics

Direct measurement of the dissociation rate constant, k_{off}, was performed according to the method of Laskowski and Sealock [33]. Preformed enzyme-inhibitor complex was diluted 1000-fold into 2 mM substrate solution to give a final $[E]_0$ of 1–2 nM. Release of free enzyme was monitored spectrophotometrically at 412 nm for 20 min and a value for k_{off} determined directly by fitting progress curves of product formation (P) against time using Eq. (9).

$$P = \frac{k_{cat}[E_0]\left(k_{off}t + e^{-k_{off}t}\right)}{k_{off}} \quad (9)$$

3. Results

3.1. Mutagenesis and expression

The mutant protein CI2M59C was successfully overexpressed using the expression vector pT7-7 in combination with the *E. coli* K38/pGP1-2 system. After initial isolation of the protein, the thiol content assayed by titration with Ellman's reagent was 0.24 thiol groups per molecule. Gel filtration chromatography showed that this resulted from dimer formation (not shown), and confirmed that upon reduction the peak corresponding to the monomeric form increased with a concomitant decrease in the peak corresponding to the dimeric protein. Complete reduction of dimeric form to the monomeric form was achieved after overnight incubation of the inhibitor with the reducing agent DTT (results not shown). The dimeric protein was inactive as an inhibitor, but when reduced was found to inhibit subtilisin with a stoichiometry of 1:1. In all subsequent kinetic assays involving CI2M59C, DTT was included to prevent dimerization. Investigation was carried out on the effect of DTT upon the activities of the various serine proteases used. Subtilisin BPN′ was not affected by the addition of DTT due to an absence of disulfide bridges in this enzyme. The enzymatic activity of the other proteases used was observed only to be slightly diminished by the addition of DTT ($<5\%$ loss of activity). This is consistent with the presence of several disulfide bridges in each of these enzymes. As DTT was included to the same final concentration in all assays, the effect of DTT on substrate hydrolysis could be ignored as inhibited rates are expressed as a percentage of the uninhibited rate ($[I] = 0$).

3.2. Carboxyamidation and carboxymethylation of CI2M59C

The progress of the modification of CI2M59C with iodoacetic acid was conveniently followed by IEF (data not shown). This showed the shift in pI of the protein as it is modified, and indicated that the modification reaction reached completion after 120 min. The pI of CI2M59*S*-carboxymethylcysteine was found to be 4.8, whereas CI2M59C and wild-type CI2 were found to have pI values of 6.2 and 6.25, respectively. Electrospray MS showed that the modified protein had the expected molecular weight, confirming the presence of a single modification site.

As *S*-carboxamidocysteine is not charged, the pI of the protein did not change during modification with iodoacetamide. Thus, the progress of the reaction could not be followed by IEF and an alternative procedure was required. It was possible to make use of the fact that the modified protein was a far better inhibitor of subtilisin BPN′ than the CI2M59C starting material (results shown later). After 120 min reaction, the product was found to give a 1:1 inhibition stoichiometry in titration with the protease under conditions where the CI2M59C protein inhibited poorly. Electrospray MS performed on the modified inhibitor gave the expected molecular weight, and again confirmed that only one modification occurred per molecule of protein.

3.3. Kinetic characterization of wild-type (truncated) CI2 and CI2M59C

The values of kinetic constants for the inhibition of subtilisin BPN′, chymotrypsin, PPE and HNE by wild-type (truncated) CI2 and the mutant CI2M59C

Table 1
Kinetic constants for inhibition of subtilisin BPN′, chymotrypsin, porcine pancreatic elastase and human neutrophil elastase by CI2, CI2M59C, CI2M59*S*-carboxamidocysteine and CI2M59*S*-carboxymethylcysteine

	Parameter	Full length wild type CI2[a]	Truncated wild type CI2	CI2M59C	CI2M59 *S*-carboxamido cysteine	CI2M59 *S*-carboxymethyl cysteine
Subtilisin BPN′	K_i (M)[b]	–	ND[e]	3.8×10^{-11}	–	–
	K_i (M)[c]	2.9×10^{-12}	7.2×10^{-12}	2.1×10^{-11}	1.0×10^{-12}	4.8×10^{-11}
	k_{on} (M^{-1} s^{-1})	1.8×10^{6}	1.4×10^{6}	6.9×10^{5}	1.0×10^{6}	3.0×10^{5}
	k_{off} (s^{-1})[d]	5.2×10^{-6}	9.8×10^{-6}	1.5×10^{-5}	1.0×10^{-6}	1.4×10^{-5}
Chymotrypsin	K_i (M)[b]	1.6×10^{-9}	2.2×10^{-9}	5.2×10^{-8}	4.1×10^{-8}	1.1×10^{-7}
	k_{off} (s^{-1})[f]	6.1×10^{-3}	1.2×10^{-2}	4.1×10^{-3}	ND	ND
	k_{on} (M^{-1} s^{-1})[g]	3.8×10^{6}	8.3×10^{6}	7.9×10^{4}	ND	ND
Porcine pancreatic elastase	K_i (M)[b]	30×10^{-9}[a]	12×10^{-9}	270×10^{-9}	No inhibition	No inhibition
Human neutrophil elastase	K_i (M)[b]	2.7×10^{-6}[h]	3.3×10^{-6}	No inhibition	No inhibition	No inhibition

The standard errors of the fitted parameter values, as estimated by the non-linear fitting procedure, are in each case $< 10\%$. ND, not determined.
[a]Values of kinetic constants quoted for wild-type (non-truncated) CI2 are published values from Longstaff et al. [10].
[b]K_i values were determined by the equilibrium method [24]. Each value listed represents the mean of 3–5 independent experiments.
[c]K_i and k_{on} values were determined from slow-binding kinetics. Each value listed represents the mean value from 3 independent experiments.
[d]Values for k_{off} were determined by application of the relationship $K_i = k_{off}/k_{on}$ (Eq. (2)), using K_i and k_{on} values determined from the fitting of slow-binding inhibition data.
[e]K_i values could not be determined for wild-type (truncated) CI2 by the equilibrium method as the interaction was essentially too tight to allow accurate fitting of the data.
[f]Values for k_{off} were determined by the method off Laskowski and Sealock [33]. Each value listed represents the mean value from 3 independent experiments.
[g]Values for k_{on} were determined by application of the relationship $K_i = k_{off}/k_{on}$ (Eq. (2)) using experimentally determined values for K_i and k_{off}.
[h]From Greagg et al. [35].

were determined, and are presented in Table 1. Wild-type (non-truncated) CI2 has previously been shown to exhibit characteristics of a slow, tight-binding inhibitor in its interaction with subtilisin BPN′ and those of a classical inhibitor in its interaction with chymotrypsin and PPE [10]. The studies described in this paper were carried out on CI2 lacking the unstructured amino terminal region, and so we include measurements on this truncated version to show that loss of the N-terminal region does not significantly affect activity. Both wild-type CI2 and CI2M59C were observed to exhibit tight stoichiometric inhibition and slow-binding behavior. Classical inhibition kinetics was observed for inhibition of chymotrypsin and PPE. Inhibition assays with HNE were complicated by the degradation of this enzyme due to autolysis in the absence of the inhibitor. Thus, the initial concentration of enzyme was estimated by back-extrapolation of the rate versus inhibitor concentration plots to $[I] = 0$. For all the enzymes studied, the truncated CI2 was found to give inhibition parameters that are essentially the same as the previously published values for full length CI2, and any differences fall within experimental error.

The introduction of a cysteine residue at the P1 position is found to result in a generally poorer inhibitor of all the enzymes studied. The K_i value for subtilisin BPN′ is 2.9-fold higher, for chymotrypsin it is 23.6-fold higher, and for PPE the factor is 22.5. HNE, which is inhibited most weakly by CI2, shows no inhibition in our assays using CI2M59C. Given the concentration range used in our assays, we estimate that this implies the K_i value has risen by at least a factor of 25 compared to wild type.

3.4. Kinetic characterization of carboxyamidated and carboxymethylated CI2M59C

The K_i, k_{on} and k_{off} values obtained for inhibition of subtilisin BPN′ and K_i values for inhibition of

chymotrypsin, PPE and HNE by CI2M59*S*-carboxamidocysteine and CI2M59*S*-carboxymethylcysteine are also given in Table 1. Detailed kinetic analyses confirmed that modification of CI2M59C to CI2M59*S*-carboxamidocysteine resulted in the production of a substantially improved subtilisin BPN′ inhibitor, with a K_i value of 1.0×10^{-12} M. This is 20-fold lower than the CI2M59C starting material, and a better inhibitor than the wild type protein. On examination of the individual rate constants (k_{on} and k_{off}), it is found that the improvement arises from a reduced dissociation rate. No inhibition of PPE or HNE was observed by CI2M59*S*-carboxamidocysteine.

Slow-binding kinetics revealed CI2M59*S*-carboxymethylcysteine to be less potent inhibitor of subtilisin BPN′, with a K_i value of 4.8×10^{-11} M. The K_i value for chymotrypsin was 1.1×10^{-7} M, $\approx$ 2-fold lower than CI2M59C. No inhibition of PPE or HNE was observed by CI2M59*S*-carboxymethylcysteine.

3.5. Characterization of CI2M59C modified with iodoalkanes of increasing chain lengths

3.5.1. Alkylation of CI2M59C

Modification of CI2M59C with iodomethane, iodoethane and iodopropane increased the length of the P1 side chain in increments of 1 carbon unit and these modifications were primarily made with the elastases as their target protease. The K_i values for the inhibition of PPE by CI2M59*S*-methylcysteine, CI2M59*S*-ethylcysteine and CI2M59*S*-propylcysteine are listed in Table 2. Of the 3 modifications carried out, reaction with iodoethane produced the most potent inhibitor, with a K_i value of 17.4×10^{-9} M. This value is very similar to that obtained with wild-type CI2 (12×10^{-9} M), reflecting the fact that *S*-ethylcysteine is an isostere of methionine. Reaction with iodomethane and iodopropane produced PPE inhibitors with K_i values of 127×10^{-9} M and 37.8×10^{-9} M, respectively.

Inhibition of HNE by CI2 was lost on introduction of the mutation Met 59 → Cys. This inhibition was not only regained by chemical modification with iodoalkanes, but more potent inhibitors were produced. The K_i values obtained for the inhibition of HNE by CI2M59*S*-methylcysteine, CI2M59*S*-ethylcysteine and CI2M59*S*-propylcysteine are listed in Table 2. Addition of a methyl group to the thiol moiety (CI2M59*S*-methylcysteine) produced an inhibitor with a K_i value of 2.0×10^{-7} M. This represents a 16-fold increase in potency compared to wild-type CI2. Surprisingly, modification with iodoethane (CI2M59*S*-ethylcysteine), which produced a P1 side chain isosteric with methionine, also produced a more potent (9-fold) inhibitor than wild-type CI2, with a K_i value of 3.73×10^{-7} M. Modification with iodopropane introduced the longest side chain and produced the most potent inhibitor (CI2M59*S*-propylcysteine) with a K_i value of 1.3×10^{-7} M, representing a 26-fold increase in potency compared to wild-type CI2.

Table 2
Kinetic constants for inhibition of PPE and HNE by CI2M59*S*-methylcysteine, CI2M59*S*-ethylcysteine and CI2M59*S*-propylcysteine

	P1 side chain	K_i (M)[a]	
		PPE	HNE[b]
Wild type CI2	$-CH_2CH_2SCH_3$	12.0×10^{-9}	33.0×10^{-7}
CI2M59C	$-CH_2SH$	270×10^{-9}	No Inhibition
CI2M59*S*-methylcysteine	$-CH_2SCH_3$	127×10^{-9}	2.0×10^{-7}
CI2M59*S*-ethylcysteine	$-CH_2SCH_2CH_3$	17.4×10^{-9}	3.7×10^{-7}
CI2M59*S*-propylcysteine	$-CH_2SCH_2CH_2CH_3$	37.8×10^{-9}	1.3×10^{-7}

The standard errors of the fitted parameter values, as estimated by the non-linear fitting procedure, are in each case $< 10\%$.
[a]The K_i values were determined experimentally by the equilibrium method [24]. Each value listed represents the mean value from 3–5 independent experiments.
[b]In assays with HNE, the uninhibited rate was corrected for enzyme inactivation by back-extrapolation of the rate to $[I] = 0$.

4. Discussion

Comparison of the experimentally determined inhibition constants for wild-type (truncated) CI2 with the published values for the parental (non-truncated) inhibitor [10] reveals that both proteins possess very similar inhibition properties. The slight differences in inhibitory potency observed between the two proteins (at most 2.5-fold) are unlikely to be significant. It is more probable they reflect small differences in experimental conditions, together with the inherent variation expected from the two independent studies performed several years apart. Thus, it can be confirmed that the first 19 N-terminal amino acids in the non-truncated inhibitor do not contribute to the inhibitory properties of CI2.

The mutant CI2M59C was observed to be less potent than wild-type CI2 in its inhibition of subtilisin BPN′, chymotrypsin and PPE, by 2.9, 23.6, and 22.5-fold, respectively. The inhibition of subtilisin BPN′ and chymotrypsin can be explained by the P1–S1 preferences of these enzymes. Subtilisin BPN′ exhibits broad substrate specificity with a preference for aromatic and large aliphatic side chains, whereas the specificity of chymotrypsin is limited to bulky aromatic side chains. This explains the small reduction in inhibitory potency towards subtilisin BPN′ and the larger decrease towards chymotrypsin.

Because of the construction of the S1 specificity pocket in PPE, a substrate preference for small aliphatic groups such as alanine has been predicted [27]. Hence, it is surprising that the mutant CI2M59C is a substantially weaker inhibitor of PPE than wild-type CI2. This suggests that for the CI2/PPE interaction, the predicted P1–S1 preferences may not be applicable or alternatively, the interactions of other subsites may be important. Weak inhibition of HNE was observed with wild-type CI2, but no inhibition was detected with CI2M59C. This can be explained by the substrate preference of HNE (Val $\gg$ Met $>$ Ala [28]). The modification to *S*-carboxamidocysteine results in a larger P1 side chain volume without altering the charge. As subtilisin BPN′ has a P1–S1 preference for large hydrophobic moieties this modification was designed to generate a better subtilisin BPN′ inhibitor. Modification of CI2M59C with iodoacetamide produced a 21-fold more potent inhibitor of subtilisin BPN′ than CI2M59C. Indeed, it is found that this modified inhibitor was substantially more potent (7-fold) than even wild-type CI2. As the *S*-carboxamidocysteine residue has a larger side chain ($-CH_2SCH_2CH_2CONH$) than the original methionine ($-CH_2CH_2SCH_3$), the increase in inhibitory potency may reflect the ability to form more favorable interactions within the S1 pocket. This is supported by the observation that the increase in inhibitory potency results largely from a slower rate of complex dissociation (k_{off}).

The inhibitory potency of CI2M59*S*-carboxamidocysteine towards chymotrypsin remained almost as high as the mutant CI2M59C, but did not show the improvement found with subtilisin BPN′. Chymotrypsin also has a substrate preference for large hydrophobic groups at the P1 position, but the P1–S1 interactions differ in detail and so give different specificities. A value for k_{off} could not be determined due to extremely rapid rate of complex dissociation. Inhibition of PPE and HNE was not observed following this modification, suggesting that the new P1 residue is too large to be accommodated within their respective S1 sites.

The inhibition of subtilisin BPN′ and chymotrypsin by CI2 containing a negatively-charged P1 residue resulting from reaction with iodoacetic acid was surprisingly better than might have been expected. It is known that subtilisin BPN′ has broad substrate specificity and can accommodate P1 Phe or P1 Lys residues [34]. Inhibition by *S*-carboxymethylcysteine, although weaker than wild-type CI2, exemplifies this broad specificity. Inhibition of chymotrypsin (preference for large hydrophobic residues) by this inhibitor is more surprising, and suggests that interactions other than P1–S1 may be important for inhibition of this protease. As expected, a P1 residue with a carboxyl group does not fulfil the preference for small hydrophobic and aliphatic residues by PPE and HNE, and neither of these enzymes are inhibited by this variant.

The generally accepted substrate specificity of PPE is for small aliphatic residues, such as Ala at the P1 position [27], and that of HNE for slightly larger hydrophobic groups (Val $\gg$ Met $>$ Ala [28]). This specificity results from the presence of relatively bulky amino acid side chains occluding the entrance of the S1 pocket, together with the hydrophobic nature of the cavity. Longstaff et al. [10] reported the

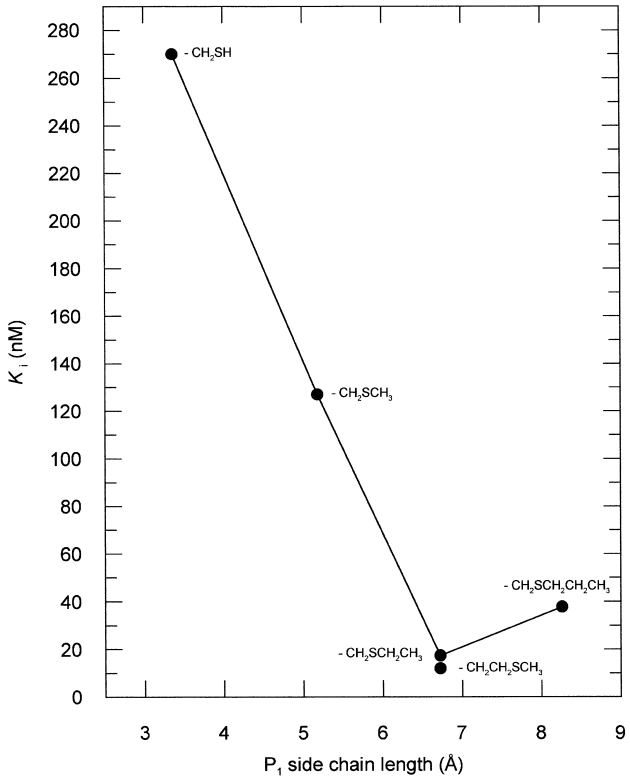

Fig. 1. The effect of increasing P1 side chain length on the inhibitory potency for interaction of CI2 variants with PPE. P1 side chain lengths were quoted as the sum of the individual bond lengths using 1.54 Å and 1.82 Å for C–C and C–S bonds respectively. Terminal C–H and S–H bond lengths are not included.

mutant CI2M59A to be a less potent (20-fold) inhibitor of PPE than wild-type CI2, suggesting that more potent PPE inhibitors cannot be designed solely by tailoring the P1 residue to the known substrate specificity of the protease. Iodoalkylation of CI2M59C allowed us to examine this specificity further using a series of inhibitor variants with aliphatic side chains of increasing length. Each variant differed from the preceding member in the series by the addition of one methylene group. The relationship between P1 side chain length and inhibitory potency towards PPE is shown in Fig. 1. It can be seen that CI2M59*S*-ethylcysteine possesses the optimum length of P1 side chain for inhibition of PPE, with the inhibitory potency decreasing if the side chain length is increased (as in CI2M59*S*-propylcysteine) or decreased (as in CI2M59*S*-methylcysteine). This is supported by the observation that wild type CI2, in which the P1 residue is isosteric with *S*-ethylcysteine, is of similar potency. It can thus be concluded that potent inhibition of PPE is best achieved by variants of CI2 possessing large, and not small, hydrophobic P1 side chains.

The K_i value obtained for the inhibition of HNE by wild-type (truncated) CI2 was found to be in close agreement with the published value for wild-type (non-truncated) CI2 [35]. Inhibition of HNE was lost in the M59C mutant but the alkylated inhibitors regained activity, indicating that larger hydrophobic P1 aliphatic side chains are preferred for HNE inhibition. Iodomethylation, iodoethylation and iodopropylation of CI2M59C produced inhibitors 16-fold, 9-fold and 26-fold more potent than wild-type CI2, respectively. However, unlike PPE, no systematic trend was observed between P1 side chain length and inhibitory potency. Surprisingly, CI2M59*S*-ethylcysteine was 9-fold more potent as an inhibitor of HNE than wild-type CI2 although the only difference between the two inhibitors is the position of the sulfur in the P1 side chain. As HNE has an extended substrate binding region on both sides of the scissile bond, covering the range P5 to at least P3′ [36], further increases in inhibitory potency might be achieved by targeting these other residues.

In summary, cysteine engineering is a useful method for modulating the inhibitory specificity of CI2. Inhibitors more potent than wild-type CI2 were constructed against subtilisin BPN′ and human neutrophil elastase, and the results provide some insights into the specificity of the proteases studied.

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