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The Human Protease Inhibitor Cystatin C Is an Activating Cofactor for the Streptococcal Cysteine Protease IdeS

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SUMMARY

Human cystatin C is considered the physiologically most important inhibitor of endogenous papain-like cysteine proteases. We present here an unexpected function of cystatin C. Instead of acting as an inhibitor, cystatin C acts as a facultative, endogenous cofactor for the papain-like IgG-cleaving enzyme IdeS of the human pathogen *Streptococcus pyogenes*. IdeS activity is not dependent on cystatin C, but is significantly enhanced in the presence of cystatin C. We report a protease inhibitor that accelerates the activity of its putative target protease and a unique example of how a host protease inhibitor is "hijacked" by a bacterial protease to increase its activity. This finding has important implications for the view on protease-inhibitor interactions, and is relevant to consider in the therapeutic use of protease inhibitors.

INTRODUCTION

Proteases play significant roles in all aspects of cellular life, including cell division, metabolic and catabolic processes, protein translocation, immune defense, and apoptosis (Rawlings et al., 2004). To prevent proteolytic activity that might be harmful for the living organism, the activity of different proteases has to be tightly controlled, which is most commonly achieved by endogenous protease inhibitors (Rawlings et al., 2004). The cystatins are natural inhibitors of the papain-like cysteine proteases of family C1 (MEROPS; Rawlings et al., 2004), including the lysosomal enzymes cathepsin B, H, L, K, and S. Thus far, 12 human cystatins (A, B, C, D, E/M, F, G, S, SN, SA, and H- and L-kininogen) have been identified (Abrahamson et al., 2003), all of which are evolutionary-related and belong to the protease inhibitor family I25 (MEROPS; Rawlings et al., 2004). Cystatin C is the most widespread cystatin, detected in all body fluids and tissues investigated and present at concentrations sufficiently high to effectively inhibit any papain-like cysteine protease (Abrahamson et al., 1986). Cystatin C functions as an emergency inhibitor for cysteine protease activity during inflammation and tissue remodeling, e.g., during vascular injury and in bone resorption (Koenig et al., 2005; Leung-Tack et al., 1990; Turk et al., 2002). In addition, cystatin C is considered to take part in the innate immune response by acting as a scavenger of cysteine proteases from pathogenic microorganisms (Turk et al., 2002; Scharfstein, 2006; Björck et al., 1990) and has also been implicated in various diseases such as cerebral amyloid angiopathy (Levy et al., 2006) and metastasing cancer, and may in this context serve as a putative biomarker (Kos et al., 2000).

The common and important human bacterial pathogen *Streptococcus pyogenes* (or group A streptococcus) has evolved a broad variety of molecular mechanisms to adapt to the dynamic environment in its human host (Cunningham, 2000). In particular, two papain-like cysteine proteases are of importance: the classical cysteine protease, SpeB, and the immunoglobulin G (IgG) degrading protease, IdeS. SpeB has broad proteolytic activity and degrades or activates numerous human and bacterial proteins (Rasmussen and Björck, 2002; von Pawel-Rammingen and Björck, 2003), while IdeS is highly specific in its proteolytic activity recognizing only IgG as substrate (von Pawel-Rammingen et al., 2002; Vincents et al., 2004). IdeS, also described as Mac-1 (Lei et al., 2001), constitutes the first member of a family of cysteine proteases (family C66) (Rawlings et al., 2004), but despite little sequence similarity, the enzyme adopts a typical papain-like structural fold (Wenig et al., 2004; Agniswamy et al., 2006). The papain-like structure of IdeS opened the possibility that cystatins, as natural inhibitors of papain-like proteases, might inhibit the activity of this important virulence factor. In fact, synthetic peptide derivatives mimicking the N-terminal of cystatin C have previously been shown to efficiently inhibit streptococcal cysteine proteases (Björck et al., 1989; von Pawel-Rammingen et al., 2002), indicating that the protease inhibitor might be of importance for the innate immune defense against streptococcal proteases. Because no natural inhibitors of streptococcal cysteine proteases have yet been identified, we screened human cystatins for inhibitory activity toward the IgG endopeptidase IdeS. Unexpectedly, IdeS activity was not inhibited by host protease inhibitors of the cystatin family; instead, protease activity was markedly stimulated in the presence of human cystatin C. Kinetic studies revealed that the human protease inhibitor cystatin C efficiently accelerated the enzymatic velocity of the streptococcal cysteine protease IdeS and thus functions as a facultative cofactor for this bacterial

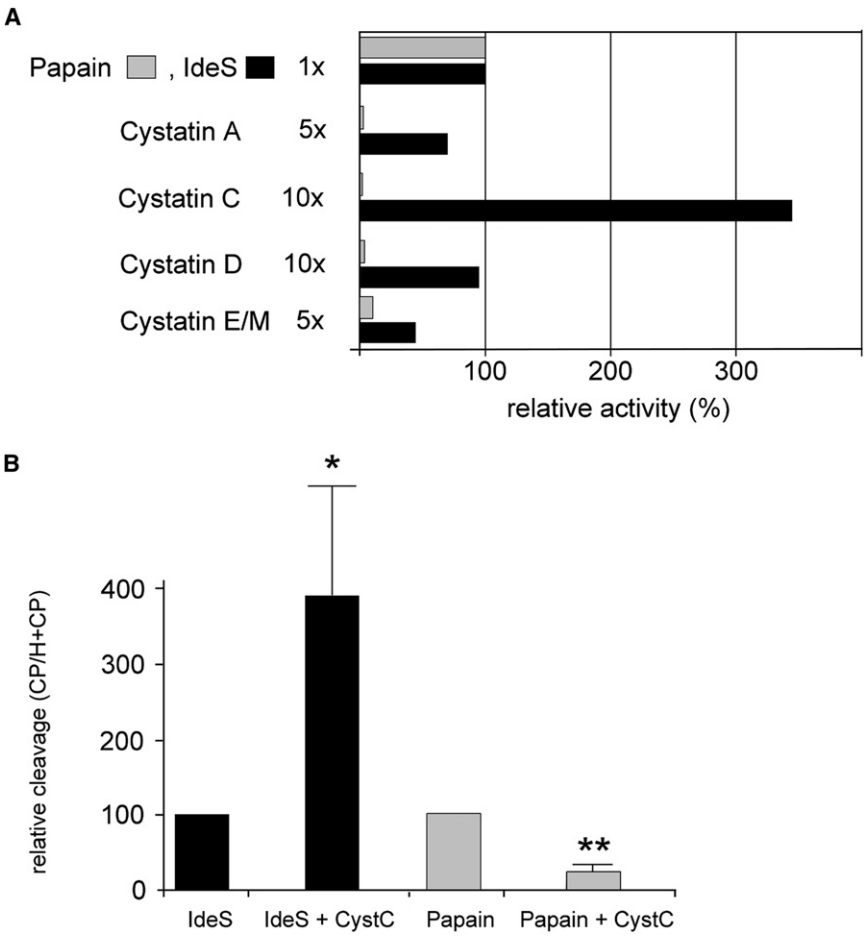

Figure 1. Stimulation of IdeS Activity by Cystatin C

(A) Human cystatins A (5 μM), C (10 μM), D (10 μM), and E/M (5 μM) were tested for inhibitory action on IdeS by preincubation in molar excess to the enzyme as indicated. The inhibitory capacity of the cystatins was assayed using papain as a control (gray bars). The results are related to the activities of IdeS or papain in the absence of potential inhibitors, which were arbitrarily set to 100%. Experiments were repeated with several different IdeS preparations. The experimental variation for the IdeS assay was up to 20% for cystatin A, D, and E/M and up to 30% for cystatin C. For the papain assay, utilizing a chromogenic substrate, the experimental variation was less than 5%. One representative experiment out of four is shown.

(B) IgG-endopeptidase activity (black bars) and papain activity (gray bars) in the presence of equimolar amounts of cystatin C. For determination of IdeS activity, purified proteins were incubated with ^{125}I- myeloma IgG$_1$ as substrate and enzyme activity was calculated as the ratio of radioactivity in the cleavage product to total heavy chain radioactivity. IdeS activity in the absence of cystatin C was set to 100. All values are ± SD (n = 3). *$p < 0.012$, Student's t test. The inhibitory capacity of cystatin C was assayed using papain as a control and the chromogenic BAPA as a substrate. Papain activity in the absence of cystatin C was set to 100. All values are ± SD (n = 3). **$p < 0.001$, Student's t test.

protease. This finding is an example of a protease inhibitor having an activating effect on a proteolytic enzyme and has important implications for the current view on protease-inhibitor interactions.

RESULTS

Screening of Cystatins for Inhibitory Activity Against IdeS

The papain-like structure fold of IdeS (Wenig et al., 2004; Agniswamy et al., 2006) suggests that cystatins might act as natural inhibitors of this important streptococcal virulence factor. Therefore a panel of purified cystatins, human cystatin A, C, D, E/M, as well as chicken cystatin and human kininogen were screened for an effect on the IgG-endopeptidase activity of IdeS. Prior to the experiment the inhibitory capacity of the protease inhibitors was assessed by determining the capacity of the inhibitors to block papain activity toward the synthetic substrate Nα-benzoyl-L-arginine 4-nitroanilide (BAPA). All proteins efficiently inhibited papain activity at the concentrations used (Figure 1A). Putative effects of the protease inhibitors on IdeS activity were investigated in an FPLC-based assay using human IgG as substrate as previously described (Vincents et al., 2004). Neither kininogen nor cystatins A and D were found to have an effect on the activity of IdeS, even when present in large excess, while cystatin E/M showed some minor inhibition (Figure 1A) (Vincents et al., 2004). In sharp contrast, human cystatin C exhibited a surprising activating effect in that IdeS activity increased to more than 300% in the presence of cystatin C. This activating effect seems to be restricted to human cystatin C, because chicken cystatin was unable to stimulate (or inhibit) IdeS (Vincents et al., 2004). To confirm the activating effect of cystatin C, a different standard assay for determination of Ig-endopeptidase activity was employed (Gilbert et al., 1983). Subsequent to digestion of radiolabeled IgG by IdeS, IgG cleavage products were separated by SDS-PAGE, and relative IgG endopeptidase activity was determined as the ratio of radioactivity in the cleavage product to total heavy chain radioactivity as previously described (Åkesson et al., 2006). Again, in the presence of cystatin C, a significant increase in IdeS activity was observed (Figure 1B), confirming a stimulating effect of cystatin C on IdeS.

Enzymatic Characterization of Cystatin C Effects on IdeS Hydrolysis of IgG

To clarify the specific action of human cystatin C on IdeS, enzyme kinetic studies were performed using isothermal titration calorimetry (ITC). ITC methodology is based on the measurement of heat from interacting molecules e.g., binding and conformational changes. In addition, the technique has proved useful as an assay for enzymatic reactions by measurement of heat

Table 1. Enzyme Kinetic Parameters for IdeS Activity in the Absence or Presence of Cystatin C

IdeS (μM)	Cystatin C (μM)	V_{max} (nM s^{-1})	Km (μM)	k_{cat} (s^{-1})	k_{cat}/Km (s^{-1} mM^{-1})	Km/V_{max} (s)
0.1	—	26[a]	45[a]	0.26	5.8	1731
	10	43	73	0.43	5.9	1714
	20	80	65	0.8	12.3	789
0.3	—	39	34	0.13	3.8	868
	20	86	47	0.29	6.1	552
1	—	63	11	0.06	5.7	178
	20	85	21	0.09	4.0	246

IdeS activity was determined via an ITC-based assay. V_{max} and K_m were estimated from plots of [S]/v against [S]. k_{cat} was estimated from the relationship of $V_{max} = k_{cat} \times [E]$ assuming 100% activity of the enzyme. The specificity time, K_m/V_{max}, specifies the time needed to hydrolyse all the substrate if the enzyme acts under first-order conditions and sustain the initial rate indefinitely (Cornish-Bowden, 1987).

[a] Under conditions when obvious non-Michaelis-Menten kinetics occurred, V_{max} and K_m were estimated directly from the velocity plot of *v* versus [S].

development during hydrolysis, which is proportional to the amount product formed (Todd and Gomez, .2001). For the characterization of enzyme activity V_{max}, K_m, k_{cat}, the specificity constant, k_{cat}/K_m, and the specificity time, K_m/V_{max}, were determined at various concentrations of IdeS and cystatin C (Table 1). For IdeS at low concentration (0.1 μM), V_{max} and pseudo-K_m values were estimated directly from the velocity curve to ~26 nM/s and 45 μM, respectively (Table 1). Interestingly, at this concentration IdeS displayed non-Michaelis-Menten kinetics illustrated by the weakly sigmoidally shaped velocity curve (Figure 2A), similar to results obtained from direct IgG hydrolysis assays (Vincents et al., 2004). However, in the presence of 10 μM or 20 μM cystatin C, an accelerated reaction was observed with a more than 3- fold increased V_{max} in the presence of 20 μM cystatin C (Table 1). Interestingly, the K_m values also increased, to 73 μM at 10 μM cystatin C and to 65 μM at 20 μM cystatin C, respectively. When the IdeS concentration was raised to 0.3 μM (Figure 2B) and 1 μM (Figure 2C), the presence of cystatin C also resulted in an increased V_{max} value, though to a lesser extent (2.2-fold and 1.3-fold, respectively) (Table 1). Again, K_m values at these concentrations were higher in the presence of cystatin C when compared with K_m values obtained with IdeS alone (Table 1). Notably, K_m values were found to decrease with increasing enzyme concentration, independent of the presence or absence of cystatin C (Table 1). Determination of the specificity constants (k_{cat}/K_m) for IdeS under the different conditions clearly show an improved enzyme performance in the presence of cystatin C at lower IdeS concentrations, which is also reflected by considerably shorter specificity times (K_m/V_{max}) at these concentrations. At the higher IdeS concentration, however, where a smaller increase in V_{max} (1.3-fold) was observed, neither the specificity constant nor the calculated specificity time indicated any stimulatory effect of cystatin C on IdeS activity (Table 1).

Cystatin C Binding to IdeS

To further strengthen the idea that cystatin C functions as a cofactor for IdeS, we sought experimental evidence for an

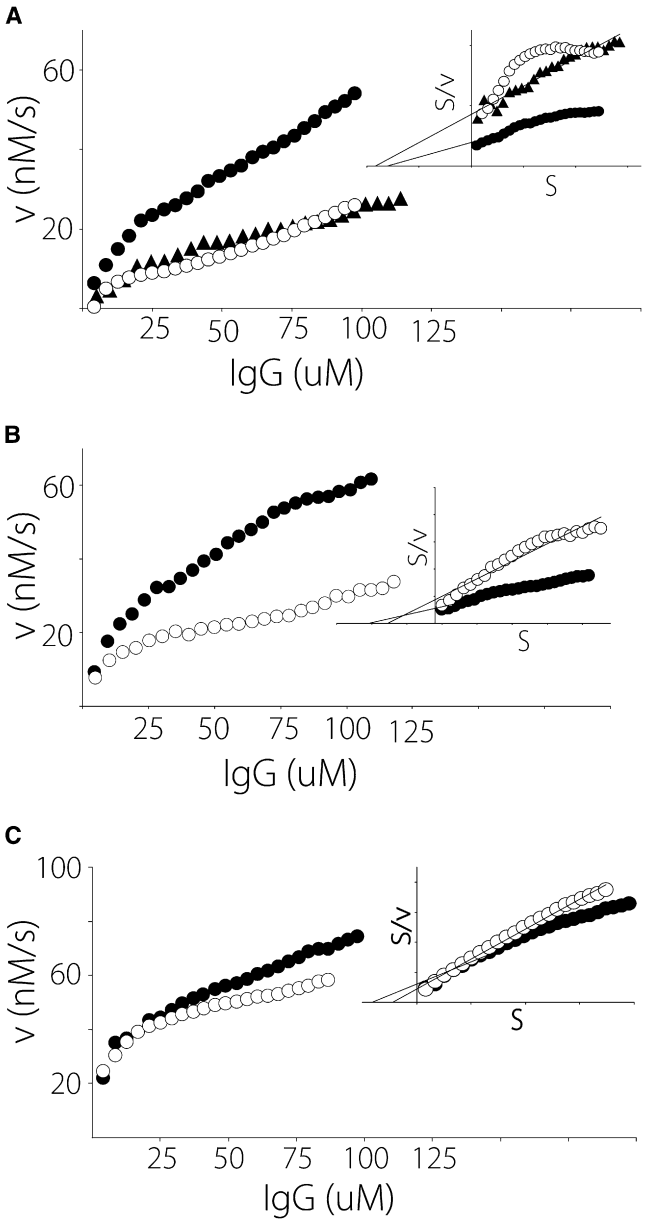

Figure 2. ITC-Kinetic Experiments of Cystatin C Effects on IdeS Hydrolysis of IgG

Graphs show the enzymatic behavior of IdeS visualized by plots of *v* versus [S].

(A) Velocity plot of IdeS at 0.1 μM (○) and supplemented with cystatin C at 10 μM (▲), and 20 μM (●) concentration. Inset shows the corresponding [S]/v versus [S] plot, where 0.1 μM IdeS in the absence of cystatin C does not follow Michaelis-Menten kinetics, but where Michaelis-Menten-like kinetics are achieved by addition of cystatin C allowing estimation of K_m and V_{max}.values.

(B) Velocity plot of IdeS at 0.3 μM (○) and supplemented with cystatin C at 20 μM (●) with inset of the corresponding [S]/*v* versus [S] plot from which K_m and V_{max} were estimated.

(C) Velocity plot of IdeS at 1.0 μM (○) and supplemented with cystatin C at 20 μM (●), with inset of the corresponding [S]/*v* versus [S] plot from which K_m and V_{max} values were estimated. Note that the shape of the velocity and Hanes plots most likely reflects an average of several reaction pathways, resulting in a nonideal linear fit.

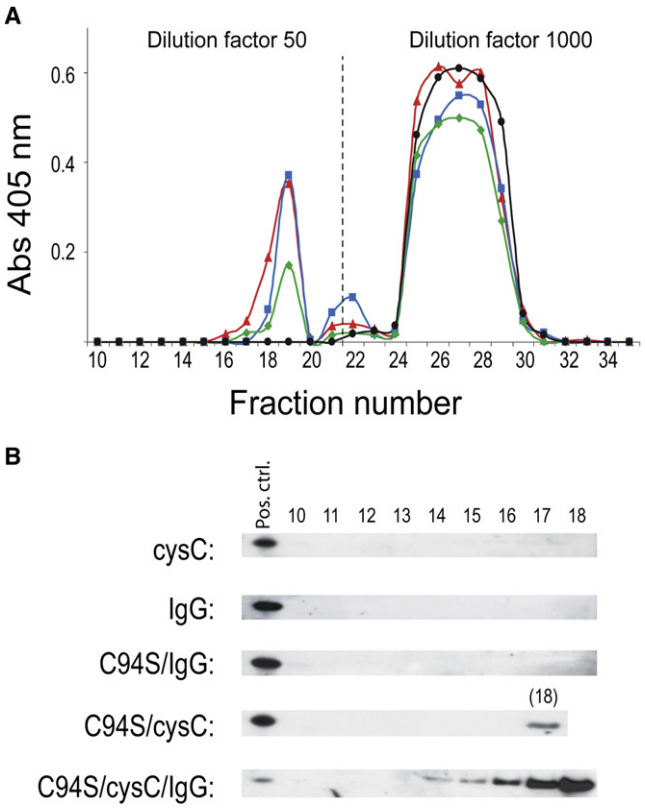

Figure 3. Interaction between IdeS and Cystatin C

(A) Diagnostic ELISA was used to detect cystatin C complex formation with IdeS^{C94S} and IgG. Samples from ITC analysis were separated and fractionated via SEC. Cystatin C alone (black curve) was seen to peak in fraction 27, but elutes in complex with IdeS^{C94S} (blue curve) or IgG and IdeS^{C94S} (red curve) in fraction 19 and fraction 14, respectively. A minor interaction of cystatin C and IgG could also be observed (green curve).

(B) Western blot analysis of relevant SEC fractions using rabbit anti-cystatin C antiserum. Note that fraction 17 in IdeS^{C94S}/cystatin C is missing.

interaction between the protease inhibitor and IdeS. Experimental ITC samples were analyzed by size exclusion chromatography (SEC) followed by immunochemical (enzyme-linked immunosorbent assay [ELISA]) identification of cystatin C in the collected fractions (Figure 3). To be able to detect possible binding of cystatin C to intact IgG or IgG bound to IdeS, the enzymatically inactive IdeS^{C94S} variant (Wenig et al., 2004) was used in these experiments. Cystatin C alone eluted in fraction 27, in accordance with its molecular weight, when compared to the elution profiles of a set of standard proteins. However, when cystatin C was applied in a mixture with IdeS^{C94S}, it could be detected already in fraction 19 (Figure 3, blue curve), which demonstrates complex formation of cystatin C with IdeS^{C94S}. Complex formation was not affected by the presence of IgG (Figure 3, red curve), although some interaction between cystatin C and IgG could be observed (Figure 3, green curve), implying different binding sites on cystatin C for IdeS and IgG. To confirm these results, relevant samples were analyzed via western blotting using a human cystatin C specific polyclonal antiserum (Figure 3B).

Interaction of Cystatin C and IdeS

Complex formation between IdeS and cystatin C was further studied via surface plasmon resonance (SPR) measurements in an attempt to gain insight into the mechanisms of stimulation of IdeS activity by cystatin C. Cystatin C bound to immobilized IdeS with high affinity, reflected in an equilibrium constant for dissociation (K_D) of approximately 10^{-7} to 5×10^{-8} M (Figure 4A), whereas in contrast to gel filtration experiments (Figure 3, green curve), no binding of cystatin C to immobilized IgG could be detected (Figure 4C). Importantly, binding of IdeS to immobilized IgG was not affected by the presence of cystatin C, indicating that the stimulatory effect of cystatin C on IdeS activity is not at the level of substrate recognition (Figure 4C). Interestingly, the best fit to the experimental sensorgrams was achieved with a model assuming a heterogeneous immobilized ligand. IdeS has previously been suggested to form homodimers (Agniswamy et al., 2006). IdeS was found to interact with immobilized IdeS with a K_D of ~1.7 μM, confirming homodimer formation of IdeS (Figure 4B) and suggesting that both monomeric and dimeric forms of IdeS are immobilized on the sensorchip surface. To analyze whether the presence of cystatin C could affect dimerization of IdeS, a series of SPR experiments were performed using mixed analyte solutions containing varying concentrations and different molar ratios of IdeS and cystatin C. The SPR experiments revealed that cystatin C binding to IdeS does not interfere with IdeS dimerization; instead, at a 1:1 molar ratio of IdeS and cystatin C, the affinity for IdeS binding to immobilized IdeS increased to ~4×10^{-8} M (Table 2). Taken together, these data demonstrate that cystatin C does not affect substrate recognition, but instead acts on the conformational state or stoichometry of the enzyme. This finding is confirmed by the fact that the sensorgram obtained with equimolar amounts of IdeS and cystatin C are best fitted to a two-state binding model, implying a conformational change. However, due to the complexity of interactions, additional experiments are certainly required to elucidate the precise mechanism of cystatin C action on IdeS.

Stimulation of IdeS Activity Ex Vivo

The unexpected finding of a dedicated cysteine protease inhibitor acting as a facultative cofactor for a protease raised the question of whether cystatin C cofactor activity might be of relevance in a physiological environment. The majority of streptococcal infections are localized to the mucosal epithelium of the pharynx, and saliva is all-prevalent in the mouth and throat region. Cystatin C is present in saliva at concentrations of approximately 0.1 μM (Abrahamson et al., 1986), and IdeS is readily secreted during bacterial growth in saliva (Shelburne et al., 2005). Therefore, a putative accelerated activity of IdeS in saliva samples was investigated by time-course experiments using radiolabeled IgG as a substrate. However, although the assay was clearly suitable for detection of IdeS activity in a complex physiological environment, it appeared that the method reached its limitations when employed for measurements of differences of initial enzyme velocities. Nevertheless, despite large interexperimental variations, IgG endopeptidase activity in fractionated human saliva, depleted in low-molecular-weight proteins such as cystatins, appeared clearly lower compared with IdeS activity in native saliva (Figure 5A–5C, open and filled squares). This was also true when saliva was treated by incubation with solid phase

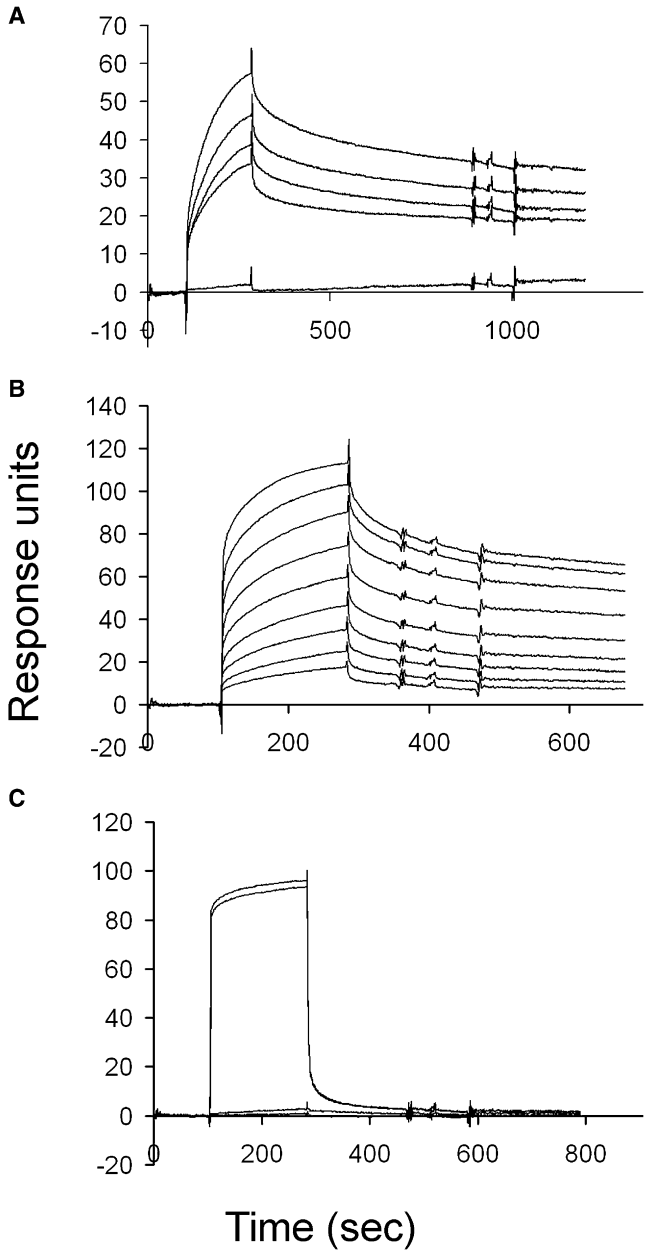

Figure 4. SPR Analysis of Cystatin C-IdeS Interactions and IdeS Homodimer Formation

(A) Cystatin C binding to immobilized IdeS^{C94S}. Serial dilutions of cystatin C from 6 to 0.75 μM were used. A representative sensorgram is shown.

(B) IdeS^{C94S} binding to immobilized IdeS^{C94S}. Serial dilutions of IdeS^{C94S} from 20 to 0.15 μM were used. A representative sensorgram is shown.

(C) IdeS^{C94S} binding to immobilized IgG in the presence or absence of equimolar amounts of cystatin C. IdeS^{C94S} and cystatin C at concentrations from 0.1 to 9 μM were used. A single representative sensorgram obtained with IdeS alone and IdeS^{C94S} and cystatin C at a concentration of 5 μM is shown. No binding of cystatin C to immobilized IgG could be detected.

coupled Cm-papain, which specifically binds and thereby inactivates cystatins (Figure 5D). Incubation of IdeS with the remaining fraction showed a marked reduction of immediate IgG endopeptidase activity compared with the native saliva sample

Table 2. Dissociation Constants for Binding of IdeS and IgG

Analyte	Concentration (μM)	Ligand KD (μM) IdeS	Concentration (μM)	Ligand KD (μM) IgG
IdeS	0.15–20	3.4	0.11–9	2.5
Cystatin C	0.75–6	0.056	5	ND
IdeS + (Cystatin C)	0.75–3	0.0439	0.11–9	2.15

ND, not detectable.

(Figure 5D, open and filled squares), and similar results were obtained using saliva that was preincubated with protein G-coupled antibodies against cystatin C (Figure 5E, open and filled squares). Most importantly, when cystatin C (100 nM) was added to treated saliva samples, IdeS activity was restored to levels similar to the activity observed in native saliva (Figures 5D and 5E, filled triangles). To further extend the investigation of an accelerating effect of cystatin C in physiological samples, IdeS activity was determined in plasma samples supplemented with various amounts of cystatin C. IdeS activity in these experiments was detected by the presence of a diagnostic 31 kDa cleavage product (von Pawel-Rammingen et al., 2002) and quantified using Fc-specific antibodies. Clearly, the presence of cystatin C stimulated IdeS activity in plasma samples in a dose-dependent manner, reflected by an increase in band intensity (CP) in the presence of increasing concentrations of cystatin C (Figure 5F). Estimates of Fc product bands (CP) by densitometry indicate an approximately doubled rate of product formation in the presence of cystatin C, which is in accordance with the increased velocity rates obtained in ITC kinetic experiments (Table 1, Figure 2). The fact that cystatin C does exert its stimulatory activity also in physiological environments, such as human saliva and blood plasma, clearly suggests the relevance of this mechanism in vivo.

DISCUSSION

In the present study, the ubiquitous human protease inhibitor cystatin C was identified as a natural, facultative cofactor for the streptococcal IgG endopeptidase IdeS. Although the enzyme structurally resembles target enzymes for cystatin C, the inhibitor fails to block enzymatic activity of IdeS and instead efficiently accelerates the enzymatic velocity of this cysteine protease. This finding is an example of a protease inhibitor having an activating effect on a proteolytic enzyme and is in accordance with our previous suggestion that IdeS might be able to interact with an effector molecule (Vincents et al., 2004). The sole substrate of IdeS, IgG, is present in vast amounts in the circulation (70–90 μM). Upon secretion from invading streptococci, initially high IdeS concentrations are achieved in the vicinity of the bacteria, allowing the protease to function as an effective defense toward specific antibodies in the circulation. This view is supported by the finding of a Michaelis-Menten-like velocity curve at high IdeS concentrations (Figure 2C), demonstrating an optimally functioning enzyme. At these high IdeS concentrations cystatin C does apparently not improve the performance of IdeS according to our kinetic results (compare K_{cat}/K_m) (Table 1). However, after secretion and radial diffusion, an increasing

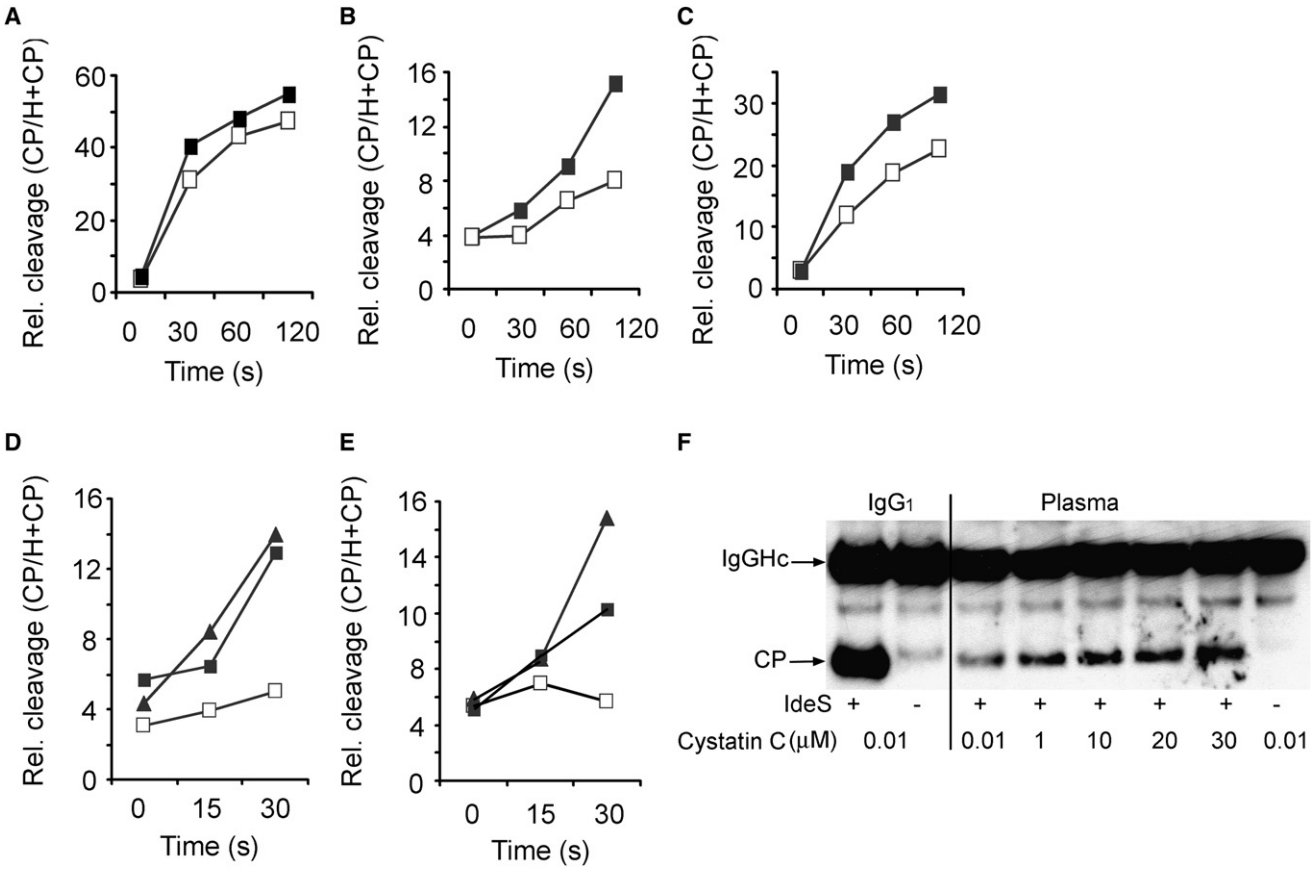

Figure 5. Acceleration of IdeS Activity by Cystatin C in Saliva and Human Plasma

(A–E) Time-course of IdeS activity in human saliva or saliva treated to deplete cystatins. Relative cleavage (cpm cleavage product [CP]/total heavy chain cpm) is shown. IdeS was incubated with ^{125}I-IgG and myeloma IgG_1 in 25% human saliva. (A–C) Time course of IdeS activity in human saliva (■) of three different donors (A–C), or the respective human saliva diminished for cystatins by ultrafiltration (□). (D) IdeS activity in native saliva samples of one donor (filled squares) and corresponding human saliva treated with Cm-papain (□), and Cm-papain treated saliva supplemented with 100 nM cystatin C (▲). (E) IdeS activity in native saliva samples of another donor (■) and corresponding human saliva depleted for cystatin C by immunopreciptiation (□), and cystatin C depleted saliva supplemented with (100 nM) cystatin C (▲). H, heavy chain; CP, cleavage product.

(F) IdeS activity in diluted human plasma, supplemented with cystatin C. Aliquots of the reaction were separated via SDS-PAGE and analyzed via western blotting using Fc specific secondary antibody. Final cystatin C concentrations are indicated. CP, cleavage product; IgGHc, heavy chain. Purified IgG_1 was used as cleavage control.

dilution of IdeS in the environment will occur that will not favor an effective protection of the bacteria as the enzyme functions suboptimally. Under these conditions, cystatin C, which is abundant in host liquids and tissue, acts as a cofactor to the bacterial protease and accelerates IdeS activity to increase enzyme activity also at a distance from the pathogen. This is reflected by clearly increased V_{max} and reduced specificity times in the presence of cystatin C. Such a strategy to counteract the immune system at a distance (i.e., remote from the bacteria), is not uncommon to group A streptococci. For instance, streptococcal IgG-binding proteins, in complex with IgG, have the ability to activate and consume complement at a distance, thereby avoiding complement activation at the bacterial surface (Berge et al., 1997). Thus, as a biological consequence of the widespread distribution of cystatin C, the pathogen has the ability to maintain IdeS activity and thus, an effective cleavage of specific IgG, even at low IdeS concentrations, for instance during the early stages of an infection when bacterial numbers are low.

The molecular mechanism for IdeS activity is complex since it involves several different reactions steps leading to product formation both in the presence and absence of cystatin C. A schematic view of such possible reaction pathways has been described as the general-modifier-mechanism (Botts and Morales, 1953). When E, S, and A represent IdeS, IgG, and cystatin C, respectively, a possible reaction pathway could be described as:

$$\begin{array}{ccccc} E + SA & \leftrightarrows & ESA & \rightarrow & EA + P \\ \downarrow\uparrow & & \downarrow\uparrow & & \\ E + S & \leftrightarrows & ES & \rightarrow & E + P \\ + & & + & & \\ A & & A & & \end{array} \quad (1)$$

IdeS activity has been suggested as an allosteric enzyme (Vincents et al., 2004) and to function in a dimeric form (Agniswamy et al., 2006), notions, which are supported by data presented in

this study and that can elucidate some of the observed enzyme kinetics. The decrease of K_m with increasing concentration of IdeS appears unusual since K_m is generally meant to be independent of the enzyme concentration. However, when considering an overall hydrolysis pathway involving dimerization of IdeS,

$$E + E \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} E2 + S \underset{k_2}{\overset{k_{-2}}{\leftrightarrows}} E2S \xrightarrow{k_3} E2 + P \quad (2)$$

the equilibrium ($E + E \leftrightarrows E2$) could be a rate-limiting step of the reaction. Dimerization of IdeS (E2) should be concentration dependent and if the K_D for dimer formation is high, this could explain our results for effects on K_m, when the IdeS concentration is raised. In fact, the earlier reported K_D value for dimer formation in vitro is very high (~250 μM) (Agniswamy et al., 2006), compared to IdeS concentrations of 50 nM detected in in vitro laboratory cultures (von Pawel-Rammingen et al., 2002), and the putative presence of a dimeric form of IdeS might offer an explanation for the kinetic behavior of the enzyme observed in ITC-kinetic experiments. SPR experiments confirmed a K_D for dimer formation in the μM range, albeit approximately 100-fold lower, compared with the earlier reported values. Including cystatin C into these considerations it can be assumed that cystatin C affects the dimer equilibrium ($E + E \leftrightarrows E2$) of IdeS. This hypothesis is supported by SPR analysis, indicating an increased affinity for IdeS-IdeS interactions in the presence of cystatin C. Although SPR experiments indicate that cystatin C affects IdeS stoichometry and/or its conformational state, the data have to be interpreted with caution and further studies are required to dissect the precise molecular mechanism of cystatin C action on IdeS. However, most importantly, cystatin C does not affect substrate recognition of IdeS.

It is intriguing that a bacterial pathogen has evolved a papain-like cysteine protease that utilizes a highly abundant host protease inhibitor to accelerate its enzymatic activity. We report a protease inhibitor exhibiting an accelerating rather than inhibiting effect on its putative target protease. This finding is not only a profound example of coevolutionary processes between bacterial proteases and the human host defense, but also suggests a fine balance between inhibition and stimulation of proteolytic systems. The current finding questions that the result of protease inhibitor interactions solely has to be inhibition of the enzymatic activity of a protease. Because protease inhibitors are used as therapeutic agents (e.g., as antiviral drugs [Deeks et al., 1997]) and have been suggested as a treatment for microbial infections, the finding of an enzyme-activating rather than inhibiting protease inhibitor should have broad implications for the future evaluation of therapeutic drugs targeting proteolytic enzymes.

SIGNIFICANCE

***S. pyogenes* infections are clinically highly significant and include throat and skin infections, as well as life-threatening conditions, such as toxic shock. The survival of *S. pyogenes* in its human host depends on the ability of the bacteria to avoid the antimicrobial actions of the innate and adaptive immune systems, and to modulate the inflammatory responses induced during the course of an infection. Immunoglobulin G (IgG) plays a central role in the human immune response. To avoid the detrimental actions of specific IgG, *S. pyogenes* has developed an IgG endopeptidase, IdeS, that is specific for the hinge region of human IgG and mediates streptococcal survival in the presence of opsonizing antibodies. Proteases are highly involved in the establishment of streptococcal infection, and several proteases are important virulence factors. Endogenous proteolytic activity in humans has to be tightly controlled by protease inhibitors to prevent cellular damage by unwanted proteolytic activities. Cystatin C is considered the physiologically most important inhibitor of these cysteine proteases. Cystatin C is a biomarker for renal function, but serum cystatin C levels have been also been correlated with vascular disease. The present study reveals an unexpected role for cystatin C in stimulating, rather than counteracting, protease activity. This is the first report of an activating role of a dedicated protease inhibitor toward a putative target protease. This finding does not only change the view on the relation of protease inhibitor and target proteases, but should also initiate a re-evaluation of the role of cystatin C.**

EXPERIMENTAL PROCEDURES

Proteins and Peptides

Recombinant IdeS and IdeS^{C94S} were expressed in *Escherichia coli* and purified as described elsewhere (von Pawel-Rammingen et al., 2002; Wenig et al., 2004). Cystatins A, C, D, and E/M were produced in *E. coli* or Baculovirus expression systems as described previously (Abrahamson et al., 1988; reviewed in Abrahamson et al., 2003). Papain (Sigma, St. Louis, MO) and human gamma globulin (Kabi Pharmacia, Sweden) were repurified as described previously (Hall et al., 1995; Vincents et al., 2004), while human polyclonal IgG and myeloma IgG_1 (kappa) were purchased from Sigma.

Collection of Human Saliva

Saliva production in healthy volunteers was stimulated by chewing 1 g of paraffin for 5 min. Twenty-milliliter saliva samples were collected, cleared by centrifugation (3200*g* for 30 min) and sterilized by filtration (Millipore Stericup, 0.2 μm). Saliva samples were kept at 4°C and used within 24 h after collection. Saliva was fractionated using ultrafiltration centrifugal filter units with a nominal molecular weight limit of 30 kDa (Ultrafree-MC, Millipore). Saliva samples were depleted for cystatins either by absorption with Cm-papain (Anastasi et al., 1983; Brillard-Bourdet et al., 1998) or by immunoprecipitation with polyclonal rabbit antibodies directed against cystatin C. Antibodies were purified and coupled to protein G sepharose (Amersham Bioscience) according to the manufacturer's instructions.

Inhibitor Scan

IdeS (1 μM) and papain (0.5 μM) were preincubated with molar excess of human cystatin A, C, D, or E/M for 20 min at 37°C or room temperature, respectively. IdeS activity was assessed by addition of 100 μM human IgG and analysis by separation of hydrolysis products on a routine FPLC assay as previously described (Vincents et al., 2004). The inhibitory capacity of cystatins was assayed using papain as control, in a chromogenic assay by the addition of BAPA (Nα-benzoyl-L-arginine 4-nitroanilide, Sigma) as described (Abrahamson, 1994). The resulting enzymatic activity in the presence of cystatin was expressed as the percentage of IdeS or papain activity in absence of cystatin that was arbitrarily set to 100%.

Quantification of IdeS Activity using Radiolabeled IgG

Quantification of IdeS endopeptidase activity was determined as described previously for microbial IgA proteases (Gilbert et al., 1983). Briefly, 1 pmol of the enzyme was incubated with ~10^5 cpm of ^{125}I-IgG and 2.5 μg of unlabeled myeloma IgG1 in 20 mM Tris-HCl buffer (pH 6.8) with 20% human saliva or

cystatin-depleted saliva. Reactions were stopped by the addition of SDS sample buffer and incubation at 95°C for 5 min. IgG chains and IgG cleavage products were separated on 12% SDS-PAGE and visualized by staining with Coomassie Blue (R-250) (USB Chemicals). Protein bands corresponding to the IgG heavy chain (H), IgG light chain (L), and the IdeS cleavage product (CP) were excised from the gel for determination of radioactivity. Samples were counted in a LKB Wallac Compugamma counter, and IdeS activity was calculated as the ratio of radioactivity in the cleavage product, CP to total heavy chain radioactivity (CP + H).

Quantification of IdeS Activity in Human Plasma

Quantification of IdeS endopeptidase activity in human plasma was determined via western blotting. Briefly, IdeS (~50 μM) was diluted 1:2000 and preincubated in phosphate-buffered saline containing 1 mM dithiothreitol. The reaction mixture was added to a 1:10 dilution of human plasma supplemented with increasing amounts of cystatin C. Incubation was continued for 20 min at 37°C. Reactions were stopped by the addition of SDS sample buffer and incubation at 95°C for 5 min. IgG chains and IgG cleavage products were separated on Novex gels (Invitrogen) and subjected to western blot analysis. As a primary antibody, rabbit anti-human IgG-CH_2 (DAKO) was used, followed by incubation with HRP-conjugated swine anti-rabbit IgG (DAKO) and visualization using ECL Plus (Pierce). Protein bands corresponding to the IgG heavy chain (IgGHc) and the IdeS cleavage product (CP) are indicated. IdeS activity was quantified by densiometric determination of CP band intensity using NIH image software.

Enzyme Kinetic ITC Studies

Enzyme kinetic studies were performed using isothermal titration calorimetry (ITC), a well-documented methodology for use in enzymology (Spink and Wadsö, 1976; Watt, 1990; Morin and Freire, 1991; Lonhienne et al., 2001; Bianconi, 2007; Todd and Gomez, 2001; Willemoes and Sigurskjöld, 2002). In brief, the method is based on measuring the heat (*Q*) generated during enzymatic hydrolysis of substrate, which is proportional to the enthalpy of the reaction and thus the amount of product formed. Heat generation is measured as a function of time (*t*), where *v* and *dQ/dt* are directly proportional ($v = d[P]/dt = (1/Vol. \times \Delta H_{app})\, dQ/dt$). Measurement at different substrate concentrations was achieved by continuous injection of substrate in large excess into the reaction cell, thus creating pseudo-first order reaction (Todd and Gomez, 2001). All ITC experiments were performed using a VP-ITC microcalorimetry instrument (Microcal, Northampton, MA) equilibrated at 26°C. The reference cell was filled with degassed deionized water. All reagents were exhaustively dialyzed against 0.1 M Bis-Tris buffer (pH 6.5) at 26°C, (I = 154 μM adjusted with NaCl), degassed for 5-10 min, and kept 1–2°C below the experimental temperature. The sample cell was filled with the buffer for measurement of the dilution heat of the substrate or with IdeS (0.1–1.0 μM) alone or in the presence of cystatin C (10–20 μM). The syringe was filled with human IgG (617– 900 μM) and purged at least two times. Before starting the reaction, the calorimeter was equilibrated at 26°C with mechanical stirring (310 rpm) and a preinjection baseline of 300–600 s was monitored prior to the first injection. The enzymatic reaction was started by injection of IgG (5–10 μL) into the IdeS solution, followed by 24–39 injections increasing the substrate concentration stepwise. The time spacing between substrate injections was 60–300 s and optimized so that a stable line with thermal power at a lower level than the baseline was established during equilibrium. This period reflects the steady state of the enzymatic reaction and substantiates initial velocities. To determine enzymatic rates, the thermal power change was averaged over the 15–30 s immediately preceding an injection to reduce the noise from the dilution of substrate. Obtained values were subtracted from the thermal power of the baseline and divided by the ΔH of the reaction. To estimate $\Delta H_{apparent}$ for the hydrolysis reaction, human IgG (5–20 μM) was injected into IdeS (0.5–1 μM). Spacing time was set to 600 s between each injection to ensure the thermal power to return to the baseline level as an indication for complete hydrolysis of the substrate. From integration of the peaks, we estimated the average $\Delta H_{apparent}$ of the hydrolysis reaction to be −2285 cal/mole in absence of cystatin C and −2353 cal/mole in the presence of cystatin C (20 μM) (data not shown). All data were visually inspected and analyzed using the Origin software package (Microcal Instruments, Northampton, MA).

SPR Experiments

SPR experiments were performed using a BIAcore X-100 biosensor (GE Healthcare). Purified $IdeS^{C94S}$ was immobilized on a CM-5 sensor chip using standard amino coupling chemistry according to the manufacturer's instructions. $IdeS^{C94S}$ was injected at a concentration of 0.15 μM in 5 mM sodium acetate buffer (pH 5.5) until approximately 1200 resonance units (RU) were coupled. IgG was injected at a concentration of 0.03 μM in 10 mM sodium acetate buffer (pH 5.0) until approximately 1200 RU were coupled. After coupling, the remaining activated groups at the sensor chip surface were blocked with ethanolamine. Binding experiments were performed at flow rate of 30 μl/min and at different analyte concentrations (0.75 μM to 6 μM for cystatin C; 0.11 μM to 20 μM for $IdeS^{C94S}$). Blank values obtained from control experiments without coupled ligand were subtracted from experimental responses obtained from the active surface. Binding and kinetic data were derived from obtained data using the BIAevaluation software (GE Healthcare).

ELISA

Samples from ITC analysis of human IgG (55 μM), titrated into inactive $IdeS^{C94S}$ (5 μM) in the presence or absence of human cystatin C (20 μM) were subjected to SEC (Superdex 75 10/30, Amersham Biosciences) and fractionated in volumes of 500 μL. The fractions were analyzed via ELISA (Olafson et al., 1988) modified to utilize a SA-HRP conjugate (DAKO) for detection of the biotinylated monoclonal antibody as detecting antibody. Recombinant human cystatin C (Abrahamson et al., 1988) was used for appropriate calibration curves.

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