Enzymatic Characterization of the Streptococcal Endopeptidase, IdeS, Reveals That It Is a Cysteine Protease with Strict Specificity for IgG Cleavage Due to Exosite Binding†

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ABSTRACT: *Streptococcus pyogenes*, an important pathogen in humans, secretes an IgG specific endopeptidase named IdeS. To elucidate the mechanism that is responsible for this specificity, we have here characterized the activity of IdeS in detail. Both γ chains of human IgG or its Fc fragment were cleaved in the hinge region after Gly236 by IdeS, but other proteins or synthetic peptides containing sequences such as the P_4-P_1 segment in the IgG cleavage site, or long peptides resembling the IgG hinge, were not hydrolyzed at all. This is likely due to a second binding site interacting with the Fc part of IgG. The lack of IdeS activity on peptide substrates necessitated the development of an assay with IgG as the substrate for kinetic studies. IdeS showed a sigmoidal velocity curve at physiological IgG concentrations, and a declining enzyme rate at higher IgG concentrations. This atypical velocity curve suggests product inhibition and/or allosteric control, which again indicates the presence of an exosite involved in substrate binding. The pseudoequilibrium constant for IdeS hydrolysis of IgG was 90 μM. The enzyme exhibited activity in the pH range of 5.1−7.6, with an optimum at pH 6.6. IdeS was stable above pH 10 but not at acidic pH. It exhibited an activity maximum around 37 °C and a decreased thermal stability at 42 °C. Iodoacetate and iodoacetamide inhibited IdeS, as expected for a cysteine protease, and biochemical evidence verified this classification. E-64 and chicken cystatin, specific inhibitors of family C1 and C13 cysteine proteases, were without effect on enzyme activity, as were class specific serine, aspartic, and metallo protease inhibitors. No significant similarities were found in protein sequence comparisons with known enzyme families, suggesting that IdeS represents a novel family of cysteine proteases.

During the past decade, it has been recognized that proteases of pathogenic bacteria are important virulence factors, playing an essential role in such bacteria in colonizing and circumventing host defense systems (*1*−*3*). Direct interference with the adaptive immune system by cleaving immunoglobulins (Igs)[1] is a common mechanism utilized by many pathogenic bacterial species. Thus, *Porphyromonas* species, *Staphylococcus aureus*, *Neisseria gonorrhea*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenza*, *Prevotella intermedia*, *Prevotella nigrescens*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes* have evolved proteases apparently with the particular function of cleaving the hinge region of Ig (reviewed in ref *1*), most likely to avoid opsonization. These proteases are selective for IgA, IgG, or IgM.

The five classes of proteases (aspartate, serine, cysteine, threonine, and metallo proteases) are all found in bacteria. The latter is the most common class, whereas the former class has only rarely been observed. Also, the cysteine protease class appears to be underrepresented in prokaryotes compared to the other classes (*1*). Four bacterial species with well-characterized extracelluar cysteine proteases are *Clostridium histolyticum* (clostripain), *Porphyromonas gingivalis* (periodontain, gingipain R1, R2, and K), *S. aureus* (staphopain A and B), and *S. pyogenes* (reviewed in ref *1*).

S. pyogenes (group A *Streptococcus*) is an important human pathogen that causes a variety of diseases such as pharyngitis, impetigo, and scarlatina as well as more severe conditions such as toxic shock-like syndrome and necrotizing fasciitis. Molecular mechanisms used by *S. pyogenes* to evade the defense systems of the host are numerous, and among

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[1] Abbreviations: Ig, immunoglobulin; IdeS, immunoglobulin G-degrading enzyme of *S. pyogenes*; OpN, *p*-nitroaniline; NHMec, aminomethyl coumarin; PBS, phosphate-buffered saline [50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl]; NTCB, 2-nitro-5-thiocyanobenzoic acid; Z, benzyloxycarbonyl; CHN_2, diazomethyl; NaDOC, sodium deoxycholate; TFA, trifluoroacetic acid.

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these is protease activity. Streptopain/SpeB, which was the first cysteine protease to be isolated from a prokaryote (*4*), exhibits hydrolytic activity against many human host proteins, e.g., the extracellular matrix proteins fibronectin and vitronectin (*5*). Furthermore, streptopain/SpeB can release kinin from kininogens (*6*), activate interleukin-1β (*7*), and activate the matrix metallo protease MMP-2 (*8*) as well as hydrolyze IgG (*9*). The newly discovered protease of *S. pyogenes*, IdeS (*10*), also known as Mac (*11*), seems to be an atypical cysteine protease with selective properties that are more pronounced than those of streptopain/SpeB, being highly specific for the four IgG subclasses (*10*). When sequenced, it showed no obvious resemblance to any known protease, which suggested a new enzyme family (*10*). The aim of this study was to examine the biochemical properties of IdeS in detail, to better understand the nature of the enzyme and its role in the microbe−host relationship.

EXPERIMENTAL PROCEDURES

In Silico Studies. BLAST searches were performed with the IdeS sequence, at both the DNA and the protein level with and without the signal sequence [residues 1−29 (*10*)] via the NCBI server (www.ncbi.nlm.nih.gov/) and the MEROPS database (http://merops.sanger.ac.uk/, August 2003). Secondary structure analyses of IdeS with and without the signal sequence were performed through the Meta server, 3D-Jury system (http://Bioinfo/PL/Meta, April 2004) (*12*).

Purification of IdeS. IdeS was recombinantly expressed and purified from *Escherichia coli* as described elsewhere (*10*).

Proteins. Normal human γ globulin (Kabi Pharmacia) was repurified by ion exchange chromatography (MonoQ 10/10; Pharmacia) equilibrated with buffer A [20 mM ethanolamine (pH 9.0)]. Bound IgG was eluted with a linear gradient of buffer B [20 mM ethanolamine (pH 9.0) and 1 M NaCl] at 4 mL/min, and the peak fractions were collected by protein detection at 280 nm. The eluted IgG, neutralized by addition of 1 M Tris (pH 7.4), was concentrated using Vivaspin columns (MWCO = 100 000; Vivascience AG) to a final concentration of 0.8−1.1 mM determined from the absorption at 280 nm. Human Fc fragment was purchased from Calbiochem (La Jolla, CA). Human cystatins A and C−E, mouse cystatin C, and rat cystatin C were isolated recombinant proteins (*13*). Chicken cystatin was purified from hen egg white (*14*). Kininogen and α_2-macroglobulin were purified from fresh human plasma. Chagasin was purified recombinant protein (*15*), and bovine aprotinin was purchased from Sigma-Aldrich (St. Louis, MO).

Peptides. The H_2N-HTCPPCPAPELLGGPSVF-CO_2H and H_2N-ELLGGPSVF-CO_2H peptides, based on the IgG1 hinge region, were synthesized by solid phase peptide synthesis and HPLC purified at the Department of Clinical Chemistry, Malmö University Hospital, Malmö, Sweden. The H_2N-LKTPLGDTTHTC-CO_2H peptide was a kind gift from V. Lindström (Department of Clinical Chemistry, Lund University, Sweden), and the H_2N-IVPFLGPLLGLLT-$CONH_2$ peptide was purchased from Sigma-Aldrich. The chromogenic substrates Boc-LG-OpN, Boc-AAG-OpN, and Boc-A-OpN and a fluorogenic substrate, H-G-NHMec, were purchased from Bachem (Bubendorf, Switzerland). Boc-LLG-NHMec was synthesized using a published protocol (*16*) but using H_2N-LL-OH instead of H_2N-AA-OH. The Enzchek Protease assay kit for green fluorescence was purchased from Molecular Probes (Eugene, OR).

Exploring the Specificity of IdeS. Proteins (3−40 μM) and peptides (40−1000 μM) containing a potential cleavage site in their primary sequence were incubated with up to 0.4 mM IdeS in PBS (final volume of 40−150 μL) for up to 22 h at 37 °C, as well as in the presence of 4 M urea and after preincubation at 70 °C for 10 min. Cleavage patterns for the proteins were assayed by SDS−PAGE in 4 to 12% gradient gels (Novex, Invitrogen, Carlsbad, CA), and cleavage of peptides was analyzed by HPLC separation using reversed phase chromatography on a C18 column (Waters, Milford, MA). TFA (0.1%) was used as solvent A and 100% acetonitrile containing 0.1% TFA as solvent B. A 10 to 100% linear gradient of solvent B at 1 mL/min eluted the column, and protein in the eluate was detected by absorption at 214 nm. Mass spectrometry was performed through the Swegene Resource Center (Lund University, Lund, Sweden). Additionally, a series of chromogenic and fluorogenic substrates were tested for hydrolysis in continuous rate assays (*17*). IgG and Fc substrates (1−40 μM) were incubated for 10−20 min with IdeS (0.3−2 μM) at 37 °C under both reducing and nonreducing conditions. Cleavage patterns for the proteins were assayed by SDS−PAGE in 4 to 12% gradient gels under both reducing and nonreducing conditions.

Deglycosylation of Fc. Human IgG−Fc fragments were incubated with PNGase F (New England Biolabs, Beverly, MA) in water (1 unit/10 μg of Fc fragment) at 37 °C overnight in a final volume of 200 μL. Two hundred microliters of coupling buffer [50 mM Tris (pH 7.4) and 0.5 M NaCl] and 200 μL of protein A-coupled Sepharose beads (Amersham-Pharmacia Biosciences, Uppsala, Sweden) prewashed in coupling buffer were added to the deglycosylated Fc fragment, and the mixture was incubated for 1 h at room temperature. After centrifugation at 13600*g* for 1 min at room temperature in a microcentrifuge, the supernatant was discarded and the pelleted beads were resuspended in 50 mM glycine buffer (pH 2.2) containing 0.5 M NaCl and incubated for 15 min at room temperature. After centrifugation as described above, the supernatant was saved and 25 μL of 1 M Tris buffer (pH 7.4) and 25 μL of dH_2O were added, whereafter the sample was stored at −20 °C.

Establishment of an IdeS Enzyme Activity Assay. The routine enzyme activity assay contained 0.10−0.16 nmol of IdeS [IdeS concentrations determined spectrophotometrically at 280 nm, using a theoretical molar extinction coefficient of 48 220 M^{-1} cm^{-1} (http://www.expasy.org/tools/protparam.html)] in reaction buffer [0.1 M Bis-Tris (pH 6.6) at 37 °C, with the ionic strength adjusted with sodium chloride (I_{NaCl}) to 150 mM], in a final volume of 100 μL adjusted with dH_2O, which was judged to be the optimal conditions for reasons described in the Results and Discussion. Upon addition of repurified IgG, the enzymatic reaction mixture was incubated for 5−15 min at 37 °C, the reaction was stopped by addition of 5 μL of 5 M sodium formate (pH 3.5), and the mixture was thoroughly vortexed. Ninety-five microliters of the stopped reaction mixture was applied automatically with an autosampler (ÄKTA A900, Amersham-Pharmacia) to an SEC column (Superdex 75 10/30, Pharmacia) controlled by an FPLC system (ÄKTA purifier, Pharmacia) run by Unicorn 4.11, and separation of the hy-

drolysis products was carried out in 10 mM sodium phosphate buffer (pH 7) with an I_{NaCl} of 150 mM, at 0.3 mL/min. From the resulting chromatograms, the Fc product peak height (mAU) at 9.9 mL was baseline corrected and measured. Due to peak tailing from the IgG peak at 9.9 mL (see the text and Figure 3A), the retention volume of the Fc_{papain} hydrolysis product was background corrected using a calibration curve, which was obtained by plotting the absorbance at 9.9 mL against the peak height of the IgG peak at different concentrations. The consequential peak height of the IdeS-generated Fc product was converted to moles by a calibration curve of the peak height of the IdeS-cleaved Fc_{papain} product plotted against moles of Fc fragment passed over the column. The enzymatic activity was expressed as the velocity of the enzymatic reaction obtained by dividing the number of moles of Fc product generated by the reaction time (picomoles per minute). To ensure that initial velocities were measured, IdeS was used in catalytic amounts determined from preliminary experiments, showing the limits of linearity, i.e., by plots of v versus [E] and [P] versus time. The assay was validated with respect to absorbance calibration curves, and in every series of experiments, positive and negative controls were included in the assay in addition to duplicate or triplicate samples. Each experimental series was performed with the same batch of IdeS and IgG to ensure conditions were as equal as possible with respect to the active enzyme concentration and IgG concentration.

Determination of pH Dependence. IdeS hydrolytic activity on human IgG was analyzed in the pH range of 4.5−10.3. The enzymatic reactions were carried out at 37 °C in buffers adjusted to an I_{NaCl} of 150 mM also taking buffer temperature sensitivity into consideration, using 0.1 M sodium acetate (pH 4.6 and 5.1), 0.1 M MES (pH 5.6 and 6.1), 0.1 M Bis-Tris (pH 6.6 and 7.1), 0.1 M Hepes (pH 7.6), 0.1 M Tris (pH 8.2 and 8.8), or 0.1 M ethanolamine (pH 9.3, 9.8, 10.3, and 10.8). For the buffer calculations, a Web-based program at www.bi.umist.ac.uk/users/mjfrbn/Buffers/Makebuf.asp.uk was used (*18*). The hydrolysis products were separated as described for the routine enzyme activity assay. The pH stability of IdeS was determined by incubation of IdeS in a total volume of 20 μL for 20 min at 37 °C at pH values in the range of 3.5−10.8, prior to diluting it 5-fold into the routine assay for enzyme activity at the optimum pH. The hydrolysis products were separated, and the enzymatic activity was determined as described for the routine enzyme activity assay.

Effects of Temperature on IdeS Activity. The routine enzyme activity assay was performed at 22, 27, 32, 37, and 42 °C at the optimum pH. The hydrolysis products were separated, and the enzymatic activity was determined as described for the routine enzyme activity assay. For the heat stability studies, IdeS was preincubated at 37, 42, 47, 52, 57, and 60 °C in a total volume of 20 μL for 20 min, prior to mixing it as in the routine assay for enzyme activity at 37 °C. The hydrolysis products were separated, and the enzymatic activity was determined as described for the routine enzyme activity assay.

Enzyme Kinetics. For the determination of the K' of IdeS for human IgG, the routine enzyme activity assay was performed in the human IgG concentration range of 5−252 μM. The hydrolysis products were separated as described previously with the exception that a 32 cm long, repacked SEC column (Superdex 75 10/30) was used and the retention volume of the Fc product was measured at 10.2 mL instead of 9.9 mL. The repacked column was calibrated and validated as described above. The enzymatic activity was determined by dividing the peak height by the reaction time, giving the velocity expressed as milliabsorbance units per minute. The velocity was plotted against the concentrations of IgG, and from the velocity curve, V_{max} was determined. The Lineweaver−Burke plot was drawn within the IgG concentration range of 30−252 μM. The Hill plot analysis was based on the Hill equation, $v/V_{max} = [S]^n/(K' + [S]^n)$, rearranged to a linear form, $\log(v/V_{max} - v) = n \log[S] - \log K'$ (*19*). A line was fitted to the points within the IgG concentration range of 30−102 μM of the velocity curve. The x-intercept equals log K', and the slope of the fitted curve resembles n_H, the number of cooperative sites in an allosteric enzyme (*19*). K' includes the dissociation constant of the enzyme equilibrium as well as interaction factors of the cooperativity (*19*).

Inhibitor Scan. The routine enzyme activity assay was performed in the presence of protease class specific inhibitors: chicken cystatin (10 μM), human kininogen (10 μM), E-64 (10 mM), iodoacetate (1 mM), and iodoacetamide (1 mM) for the cysteine protease class, aprotinin (10 μM), PMSF (1 mM), and Pefabloc (1 mM) for the serine protease class, pepstatin (10 μM) for the aspartic acid protease class, and EDTA (1 mM) for the metallo protease class. The resulting enzymatic activities in the presence of inhibitors were expressed as the percentage of IdeS activity without the presence of inhibitors, which was set to 100%.

Classification of IdeS by NTCB Chemical Cleavage. NTCB cleavage of IdeS was perfomed as described previously (*20*−*22*) with a few modifications. IdeS (0.8 nmol) and 50 μL of reaction buffer (pH 6.6) were mixed with and without 2 nmol of Z-LVG-CHN_2 in a final volume of 100 μL and incubated for 30 min at 37 °C. Fifty microliters of NTCB buffer [8 M guanidinium hydrochloride, 100 mM Tricine (pH 8.6), and 100 mM NTCB (Sigma-Aldrich)] was added and incubated for 5 min at room temperature. Ten microliters of 1% NaDOC was added and incubated for 15 min, whereafter 28 μL of 50% TCA was added and the mixture further incubated for 15 min. The samples were centrifuged at 13600*g* for 5 min at room temperature, and the supernatant was discarded. Pellets were resuspended in 10 μL of 8 M urea, freshly prepared with 150 μL of 25−35% ammonium hydroxide, and incubated for 1 h at room temperature. The samples were analyzed by 4 to 12% gradient SDS−PAGE.

RESULTS AND DISCUSSION

Sequence Analysis of IdeS. A homology search in the MEROPS database of August 2003 with the sequence of IdeS without its potential signal sequence (*10*) generated an alignment with an uncharacterized protease from the soil bacterium *Pseudomonas putida* assigned to cysteine protease family C39 of clan CA. The sequences were 28% identical over 57 amino acid residues (E value = 0.12) (Figure 1A). The suggested active site residue of IdeS, Cys_{94} (*10*), aligned to another cysteine residue of the *P. putida* protease. This finding could indicate a common ancestral relationship. A PDB BLAST search gave a significant hit to the human

A

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IdeS      DSFSANQEIRYSEVTPYHVTSVWTKGVTPPANFTQ  64
                                      *
IdeS      GEDVFHAPYVANQGWYDITKTFNGKDDLLCGAATA  99
P.putida                          KTDFSCGAAAL

IdeS      GNMLHW--WFDQ-NKDQIKRYLEEHPEKQKINF-- 134
P.putida ATILRQAYWLDVDEH-QIIEGMLAHADQDLV--RT

IdeS      NGEQMFDVKEAIDTKNHQLDSKLFEYFKEKAF...  169
P.putida QGFSMLDMKRYVES
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B

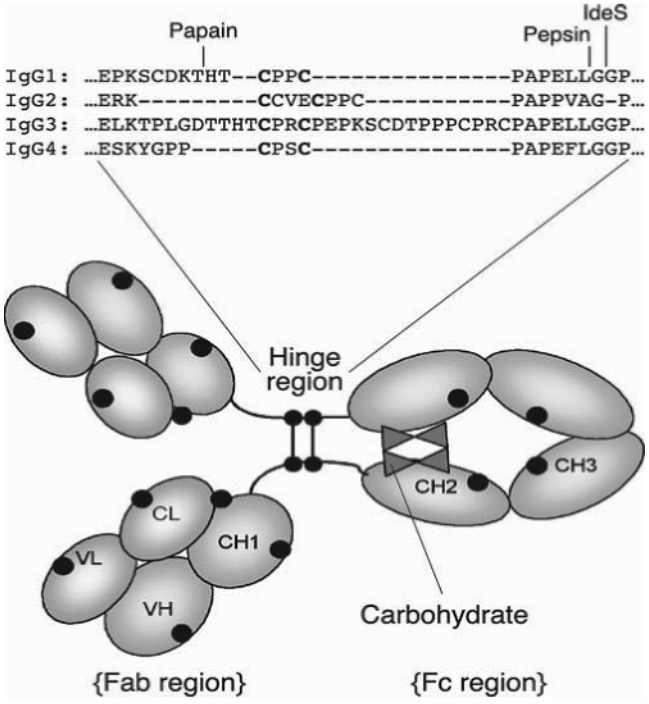

FIGURE 1: Sequence alignments for IdeS and its IgG substrates. (A) Amino acid sequence of IdeS without the signal sequence [residues 28−320 (*10*)] aligned with the MEROPS subject sequence from *P. putida.* Identical residues are marked with gray boxes, and dashes are inserted to optimize the alignment. The sole cysteine residue in IdeS, believed to be the catalytic residue which aligns with a cysteine residue in the *P. putida* sequence, is marked with an asterisk. (B) The Y-shaped IgG molecule is composed of two identical light chains and two identical heavy chains. The γ chains (50 kDa each) consist each of four domains: the variable heavy chain (V_H) and the constant heavy chain domains (C_H1-C_H3). Carbohydrate moieties at C_H2 are represented as black butterflies, and inter- and intradomain disulfides are represented as black dots. A hinge region of varying length and flexibility in the different subclasses (shown at the top) connects domains C_H1 and C_H2. The heavy chains are covalently attached in the hinge region by two disulfide bridges between residues 226 and 229 (IgG1 numbering) marked with bold in the hinge sequence alignment. The primary cleavage sites for proteases cleaving in the hinge region are indicated.

Mac-1 subunit (CD11b) with an *E* value at 0.001 as noted previously (*11*), but no other significant hits of homology were retrieved, indicating that IdeS belongs to a novel protein family.

The *P. putida* sequence aligns at IdeS residues 89−149 (Figure 1A) and the CD11b subunit sequence at residues 144−335 (*11*). Thus, the *P. putida* and CD11b sequences only overlap with five residues, which may indicate that IdeS consists of two functional domains. The N-terminal part with the single Cys residue in the IdeS sequence and *P. putida* protease resemblance could form a catalytic domain. A second domain built from the more C-terminal part of the sequence could be a ligand interacting domain, in agreement with its resemblance to the known ligand binder, Mac-1 (*23*), and data indicating that the IgG binding property of IdeS is not associated with the N-terminal part of the sequence (*24*).

Upon using Meta predictors, consensus method, and secondary fold recognition servers, additional information about the probable IdeS structure was obtained. With analysis of the entire bank of protein sequences, the 3D jury system (*12*) gave several hits from different servers which were all structurally classified as cysteine proteases (d.3.1). For IdeS without and with its signal sequence, six and seven hits were retrieved, respectively. The hits of the 3D jury system were human recombinant procathepsin B (PDB entry 1pbh), the plant cysteine protease Ervatamin C (PDB entry 1o0e), and human cathepsin F (PDB entry 1m6d) from the FFAS03 server (*25*), rat cathepsin B (PDB entry 1the) and porcine cathepsin H (PDB entry 8pch) from the 3D-PSSM server (*26*), and rat procathepsin B (PDB entry 1mir) and human procathepsin L (PDB entry 1cs8) from the INGBU server (*27*). All of the proteins are proteases assigned to family C1 of papain-like cysteine proteases in clan CA. Other Meta servers also suggested the IdeS sequence to adopt a papain-like cysteine protease fold: human cathepsin F (PDB entry 1m6d) from SAM T99 (*28*), human procathepsin K (PDB entry 7pck) and cathepsin H (PDB entry 8pch) from the Superfamily server (*29*), and caricain (PDB entry 1ppo) from the 3D PSSM server. Even if the scores were relatively low, they could be relevant with the endopeptidase activity of IdeS in mind. Three-dimensional structural similarities are seen within the cysteine class of proteases despite very little conservation of the primary sequence. A most relevant example is streptopain/SpeB, which has a canonical papain fold despite an insignificant degree of sequence similarity with papain of the protease region (*30*). In support of our conclusions, MEROPS has classified it after independent analysis of the IdeS sequence recently as a cysteine protease, in a new family C66 due to its low level of similarity to known enzyme sequences.

Exploring IdeS Substrate Specificity and a Possible Exosite. IdeS cleaves after Gly_{236} in the hinge region just before the C_H2 domain in the heavy chains of human IgG1 (*10*) (Figure 1B). To gain more insight into its specificity, we used HPLC analysis and fluorometry to investigate if IdeS could hydrolyze some common protein substrates such as insulin B-chain and casein. IdeS did not hydrolyze any of these protein substrates. We also tested whether IdeS was capable of hydrolyzing other proteins with similar segments in their primary sequence such as the P_4-P_1 residues in the cleavage site in IgG (Figure 1B). The human protease inhibitor cystatin C and its homologues from other species, as well as human cystatin D, bovine aprotinin, and chagasin from *Trypanosoma cruzi*, which all contain a putative cleavage site for IdeS (Table 1A), were incubated with IdeS for up to 22 h, and hydrolyzing activity was analyzed by SDS−PAGE, with a protein band shift expected upon cleavage. No cleavage of these putative substrates was detected; neither could cleavage products be detected when IdeS was incubated with whole human plasma or with fibrinogen, fibronectin, albumin, transferrin, lactoferrin, laminin, H-kininogen, or α_1-antitrypsin (data not shown).

Table 1: Substrate Specificity of IdeS[a]

A

Protein	*Putative Cleavage Site*	*Sequence position*	*Cleavage*
IgG1	... **E L L G** ...	**Hinge (233-236)**	**Yes**
IgG2	... **P V A G** ...	**Hinge (230-233)**	**Yes**
IgG3	... **E L L G** ...	**Hinge (233-236)**	**Yes**
IgG4	... **E F L G** ...	**Hinge (233-236)**	**Yes**
Chagasin	... **K L L G** ...	**62-65**	**No**
Cystatin (chicken)	... **R L L G** ...	**8-11**	**No**
Cystatin C (rat)	... **R L L G** ...	**8-11**	**No**
Cystatin C (mouse)	... **R M L G** ...	**8-11**	**No**
Cystatin C (human)	... **R L V G** ...	**8-11**	**No**
Cystatin D	... **T L A G** ...	**4-7**	**No**
Aprotinin seq. A	... **P Y T G** ...	**10-13**	**No**
Aprotinin seq. B	... **V Y G G** ...	**35-38**	**No**
Aprotinin seq. C	... **T C G G** ...	**55-58**	**No**

B

Peptides	*Putative Cleavage Site*	*Cleavage*
Synth. peptide ($IgG1_{233-241}$)	H_2N-**E L L G G P S V F** COOH	**No**
Synth. peptide ($IgG3_{217-228}$)	H_2N-**L K T P L G D T T H T C**-COOH	**No**
Synth. peptide ($IgG1_{224-241}$)	H_2N-**H T C P P C P A P E L L G G P S V F**-COOH	**No**
Synth. peptide	H_2N-**I V P F L G P L L G L L T**-NH_2	**No**
Fluorogenic substrate	H-**G**-NHMec	**No**
Chromogenic substrate	Boc-**L G**-OpN	**No**
Fluorogenic substrate	Boc-**L L G**-NHMec	**No**
Chromogenic substrate	H-**A A G**-OpN	**No**
Chromogenic substrate	Boc-**A**-OpN	**No**

[a] (A) Proteins containing putative IdeS cleavage sites in their primary sequences were incubated with IdeS and analyzed by SDS−PAGE. (B) Natural and synthetic peptides containing amino acid segments similar to that of the IdeS cleavage site in IgG (putative P_4−P_1 residues are underlined) were incubated with IdeS and the mixtures analyzed by HPLC or continuous rate flourometry. A 21-mer peptide corresponding to the entire IgG1 hinge region ($IgG_{221-241}$) was <98% hydrolyzed after incubation with IdeS for 22 h at 37 °C.

From these experiments, it was clear that IdeS was extremely specific for IgG. When the putative other protein substrates were predenatured by heat or chemically denatured by urea and a reducing agent (DTT), IdeS could still not hydrolyze them. In contrast, IdeS remained active toward IgG in up to 4 M urea (data not shown).

IdeS was also tested for hydrolysis of synthetic and natural peptides as well as synthetic fluorogenic peptides containing residues from the P_4−P_1 subsites at the IgG1 hinge (Table 1B), by HPLC separation, mass spectrometry, and continuous rate fluorometry. It was evident that no cleavage of any of the potential substrates occurred (data not shown). Even the peptides resembling the IgG1 hinge region were not significantly hydrolyzed (<98% cleavage after 22 h). Taken together, these results verify and further stress the high degree of substrate specificity of IdeS (*10*) and strongly indicate a highly specialized role for the enzyme.

Obviously, extended sequence homology to the segment around the IgG cleavage site is not sufficient for the enzyme to hydrolyze other proteins. This extreme specificity of IdeS raised the question about the presence of a second substrate binding site or an exosite on IdeS. We investigated whether IdeS was capable of hydrolyzing only the Fc part of IgG generated by papain cleavage (Fc_{papain}) (Figures 1 and Figure 2A, I). Analysis by SDS−PAGE under nonreducing or reducing conditions clearly showed that this was possible. Fc_{papain} (50 kDa) (Figure 2A, lane 2), incubated with IdeS under nonreducing conditions to keep the hinge disulfide bridges intact, gave two hydrolysis products at approximately 25 and 4 kDa (Figure 2A, lane 3). Reducing conditions break the disulfide bridges, and the two hinge regions would appear as free substrates (Figure 2A, II). Again, SDS−PAGE demonstrated a band shift of the reduced Fc_{papain} molecule treated with IdeS, meaning that cleavage of the reduced hinge region had occurred (Figure 2A, lanes 1, 4, and 5). From these experiments, it can be concluded that IdeS must contain an exosite for substrate binding. IdeS could not cleave the hinge region peptide in its own (Table 1B) without the presence of the remaining Fc molecule, strongly indicating that the enzyme binds to the heavy chain domain C_H2 or C_H3 or their interface. Since IdeS can cleave IgG bound at the bacterial surface through an interaction between *S. pyogenes* protein H and the C_H2−C_H3 interface region (*10*, *31*), the IdeS exosite most likely interacts with the C_H2 domain. Exosites or distant recognition sites have also been suggested for other types of Ig-cleaving bacterial proteases, i.e., IgA proteases (*32*, *33*). Furthermore, the presence of an exosite is supported by the finding that truncated variants of IdeS lacking the proposed catalytic domain still bind IgG (*24*). The exosite binding postulated by our enzyme activity results also agrees well with the activity of IdeS/Mac protein in blocking phagocytosis by interfering with the interaction between human CD16 and immune complexes (*22*).

Additionally, we tested if IdeS required "paired heavy chains" to act or if it could hydrolyze separated heavy chains of Fc_{papain}. To obtain this type of substrate, we deglycosylated the Fc_{papain} molecule since the carbohydrate moieties on the C_H2 domains interact by noncovalent forces (*34*) and the heavy chains do not separate fully merely upon reduction of the hinge region disulfide bridges. The C_H2 domains do not interact, whereas the two C_H3 domains interact strongly and do not split easily unless the ionic strength is reduced or they are in the presence of detergents (*35*). However, the two heavy chains containing the two cleavage sites would most likely be physically separated, giving a semimonomeric substrate in terms of IdeS recognition (Figure 2B). From

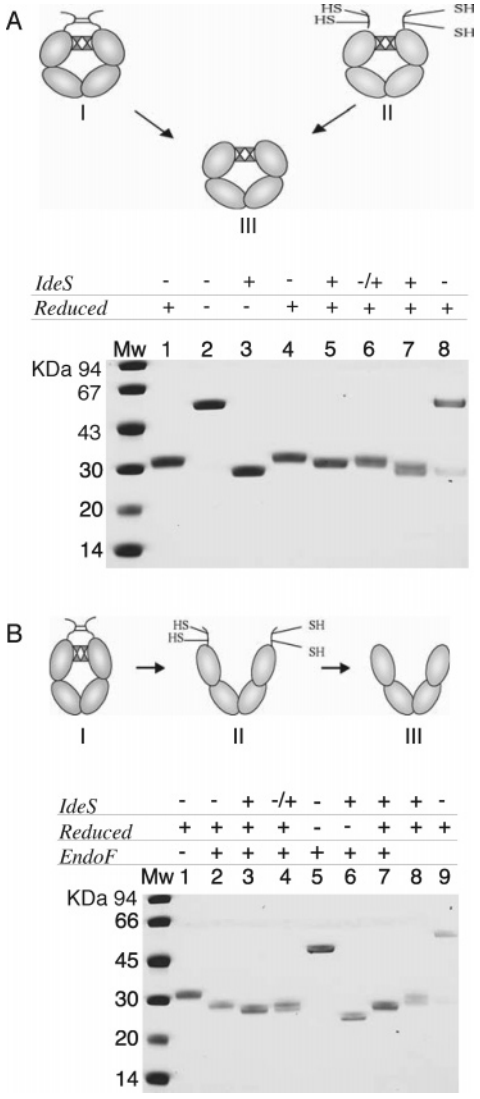

FIGURE 2: IdeS cleavage of Fc_{papain} fragments. (A) Fc_{papain} was incubated with IdeS under nonreducing (I) and reducing (II) conditions to investigate hydrolysis of the Fc_{papain} fragment (III). The incubation mixtures were analyzed by 4 to 12% gradient SDS−PAGE. Mw size markers (phosporylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin), with sizes given at the left. Lanes 1−5 show analysis of Fc_{papain} (lane 6 shows samples from lanes 4 and 5 run together) and lanes 7 and 8 analysis of IgG as a positive control. The reactions carried out under reducing conditions (1 mM DTT) are identified above the gel (Reduced). Samples in lanes 2 and 3 were analyzed in nonreducing sample buffer. (B) Fc_{papain} was deglycosylated with EndoF (I), and additionally, the hinge disulfide bridges were reduced with DTT, leading to formation a semimonomeric substrate (II), for investigating hydrolysis of the hinge peptides (III). The incubation mixtures were analyzed by SDS−PAGE as in panel A. Lanes 2−7 show analysis of deglycosylated Fc_{papain} (lane 4 shows samples from lanes 2 and 3 run together) and lanes 8 and 9 analysis of IgG as a positive control. The reactions carried out under reducing conditions (1 mM DTT) are identified above the gel (Reduced). Samples in lanes 5−7 were analyzed in nonreducing sample buffer.

SDS−PAGE analysis under reducing conditions, it was demonstrated that a band shift from 27 to 25 kDa occurred (Figure 2B, lanes 1−4). This experiment was repeated under nonreducing conditions keeping the disulfide bridges intact, also giving a band shift in SDS−PAGE (Figure 2B, lanes 6 and 7). These experiments demonstrated that IdeS is capable of cleaving the semimonomeric substrate (Figure 2B, lanes 5−7), supporting the notion that the IgG substrate is cleaved in two steps, where the heavy chains are cleaved one at a time.

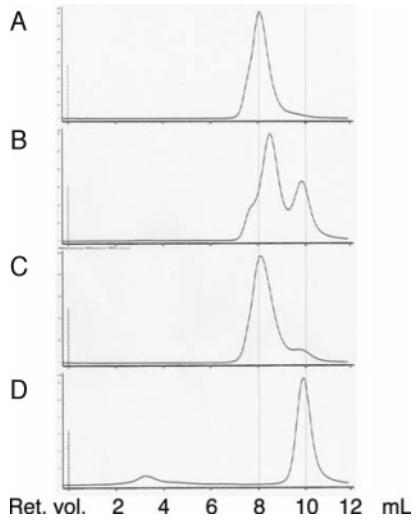

FIGURE 3: FPLC-based IdeS activity assay. A chromatography assay for monitoring the cleavage of IgG by IdeS was established as described in detail in Experimental Procedures. (A) Superdex chromatogram of IgG (150 kDa). (B) Chromatogram of IgG digested to approximately 75% with IdeS, generating two major peaks. The first peak of uncleaved IgG and $F(ab)_2$ fragments eluted with a retention volume of 8.3 mL, and the second peak containing Fc fragments eluted with a retention volume of 9.8 mL. (C) Chromatogram of IgG digested to 5% with IdeS. (D) Chromatogram of Fc_{papain} digested with IdeS. Thin vertical lines mark the retention volumes of 8.0 and 10.0 mL.

The importance of glycosylation for Ig cleavage varies among bacterial IgA-cleaving proteases (*32*, *36*). In the case of IdeS, the results above clearly demonstrated that removal of the carbohydrate moieties in the C_H2 domains of whole IgG or Fc_{papain} does not hinder IdeS from cleaving IgG, although our experiments cannot rule out an effect on the kinetic parameters for cleavage.

Establishment of an FPLC-Based Enzyme Activity Assay. Since a spectrophotometric enzyme activity assay was not available for kinetic measurements due to the lack of any chromogenic or fluorogenic substrates, we developed an activity assay based upon separation of hydrolysis products by FPLC. IgG digested by IdeS generated Fc products (50 kDa) which were separated from the $F(ab)_2$ fragments (100 kDa) and uncleaved IgG (150 kDa) by SEC. Evaluation of the assay by inspection of chromatograms (Figure 3) and SDS−PAGE analysis (not shown) established that uncleaved IgG eluted with a retention volume at 8.0 mL (Figure 3A) and enzymatically digested IgG eluted as two independent peaks at 8.0−8.1 and 9.8 mL (Figure 3B,C), resembling the uncleaved IgG together with $F(ab)_2$ fragments and Fc products. For illustrative purposes, panels B and C of Figure 3 show the chromatograms of IgG more than 75% hydrolyzed and approximately 5% hydrolyzed, respectively. Due to minor peak tailing of the IgG $F(ab)_2$ peak, the true elution volume of the Fc fragment was 9.9 mL, not 9.8 mL (Figure 3D). Consequently, the amount of Fc product was measured from this point. All calibration curves, i.e., detector sensitivity curves for IgG and Fc fragment and background correction curves, gave R^2 values of ≥ 0.98 (data not shown).

The aim when developing the IdeS assay was that it should be sufficiently simple to be reproducible in a standard

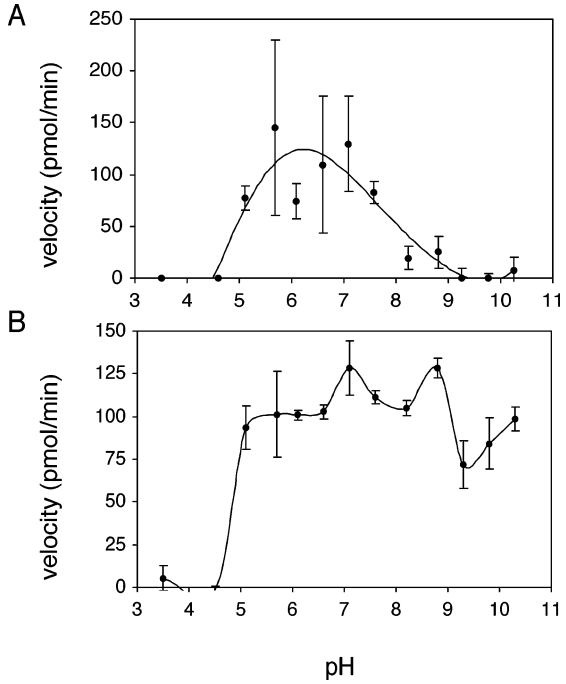

FIGURE 4: pH dependence. (A) The effect of pH on the activity of IdeS was investigated using the IgG chromatography assay. The results are shown as mean velocities at the indicated pH values. The data points were fitted to a bell-shaped curve. (B) Effect of pH on stability of IdeS shown as the mean velocity at pH 6.6 after preincubation of IdeS at the indicated pH values.

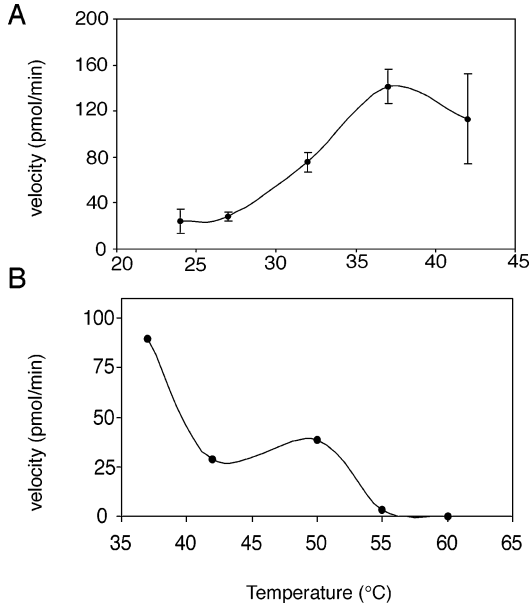

FIGURE 5: Temperature dependence. (A) The effect of temperature on the activity of IdeS was investigated using the IgG chromatography assay. The results are shown as velocities at the indicated temperatures. (B) Effect of temperature on stability of IdeS, shown as the velocity at 37 °C after preincubation of IdeS at the indicated temperatures. The curve was drawn from results of a representative experimental series.

equipped laboratory. As the substrate, we therefore used polyclonal human IgG, consisting of 70% IgG1, 20% IgG2, 8% IgG3, and 2% IgG4 (*37*). The substrate composition resembles the physiological composition, and the resulting products are identical in molecular size, giving rise to the same product peak. Measuring peak heights from the chromatograms made it possible to determine velocities of the enzymatic activity. Instead of using peak areas and direct calculation of protein concentration by extinction coefficients, we used peak heights (*38*) and calibration curves. The validations showed that this was fully acceptable, and the assay was found to be reproducible and legitimate for enzyme kinetic studies.

Effects of pH. The pH dependence of IdeS activity indicated a nearly bell-shaped curve (Figure 4A). Below pH 5.1 and above pH 7.6, the enzymatic activity declined significantly. pH 6.6 was chosen for further experiments, which was the optimum in regression analysis of all individual series of experiments. This pH optimum of IdeS in the acidic part of the pH scale is similar to those of other, unrelated cysteine proteases (*39*).

From the pH stability experiments, it was clear that incubation of IdeS at pH values below 5.1 for 20 min at 37 °C, prior to performing the enzymatic activity assay at pH 6.6 and 37 °C, resulted in the loss of enzymatic activity, suggesting an irreversible denaturation of IdeS (Figure 4B). Preincubation of IdeS above pH 5.1 for 20 min at 37 °C had no effect on the activity of IdeS then measured at pH 6.6. After preincubation above pH 7.6, where the enzyme activity declined in the pH dependence experiments, IdeS activity recovered when the enzyme was transferred to pH 6.6. Only a vague decline in activity was observed after preincubation at pH 8.8, and even after preincubation at pH 10.3, activity was seen when it was measured at pH 6.6, suggesting that very slow irreversible denaturation occurs at alkaline pH. Thus, the decline in activity measured at pH $\geq$7.6 (Figure 4A) must be due to reversible formation of an improper conformation or ionized state of the substrate and/or enzyme. From a physiological point of view, the pH optimum and stability of IdeS agree well with the *in vivo* conditions for *S. pyogenes* growth, on skin (pH 5.5−6.5) and in human plasma and saliva (pH 7.4).

Effects of Temperature. IdeS activity was found to be temperature-dependent (Figure 5A). The enzymatic activity increased ~6-fold when the temperature was increased from 24 to 42 °C, peaking at 37 °C. Additionally, temperature stability was investigated by preincubation of IdeS for 20 min at different temperatures before the enzyme activity was measured at 37 °C (Figure 5B). Irreversible heat denaturation was apparent already at temperatures of $\geq$42 °C. From these experiments, 37 °C was the temperature chosen for the activity assays as the optimum temperature. The low thermal stability noted may be a consequence of the lack of disulfide bridges in IdeS. Interestingly, it has also been observed for two other bacteria expressing IgG-cleaving proteases, *Pr. intermedia* and *Pr. nigrescens*, that their proteolytic activity is markedly reduced upon incubation at 42 °C (*40*).

Enzyme Kinetics. The IdeS hydrolysis rate of IgG did not show the hyperbolic shape of a classic Michaelis−Menten velocity curve (Figure 6A), and a Lineweaver−Burke plot did not show linearity (not shown). Rather, the velocity curve tends to be sigmoidal until it reaches a maximum at ~175 μM, and then the enzyme velocity declines at higher IgG concentrations. The shape of the curve could indicate allosteric control either by means of cooperative binding sites or by the presence or lack of an effector molecule (*19*). Moreover, the drop in velocity after V_{max} has been reached strongly points toward substrate or product inhibition of IdeS (*19*).

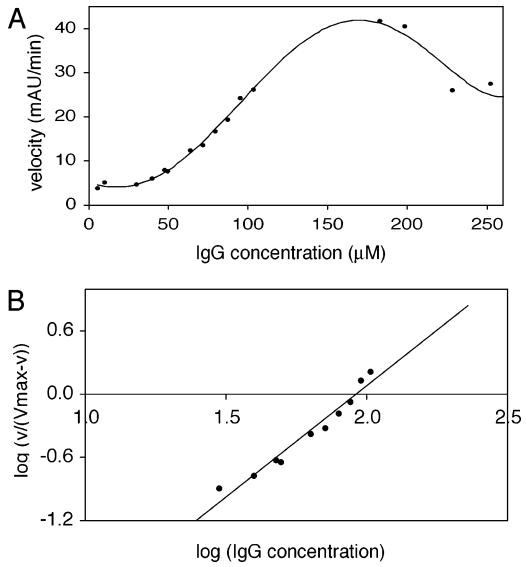

FIGURE 6: Velocity curve for IdeS cleavage of IgG. (A) The effect of substrate concentration on the IdeS activity was investigated using the IgG chromatography assay. The results are shown as velocity vs substrate concentration, and the curve is a representative experimental series of three independent ones. (B) Hill plot analysis of the velocity data shown as $\log(v/V_{max} - v)$ vs $\log[S] - \log K$ as described in detail in Experimental Procedures.

In general, multisite allosteric enzymes give rise to sigmoidal-shaped velocity curves (*19*) due to cooperative binding sites. In such a case, the numbers of cooperative sites in an allosteric enzyme can be determined from a Hill plot. A Hill plot analysis for the IdeS data (Figure 6B) predicted the enzyme to have 2.1 cooperative sites, or two sites in reality, which may indicate that IdeS is active as a dimer. Under our experimental conditions, we have not seen evidence for significant amounts of a dimeric form of IdeS by SEC experiments. However, a functional dimer of IdeS is still a possibility if its K_d happens to be very high *in vitro* as described for herpes simplex type 1 protease (*41*) and caspase-8 and -9 (*42*, *43*). Alternatively, the two binding sites suggested by the Hill plot analysis could indicate that two sites in monomeric IdeS work together, showing cooperativity at substrate binding (*44*). The latter fits well with our cleavage specificity results (Figure 2), which indicated the presence of a Fc binding exosite in addition to the active site. The satisfaction of the exosite in IdeS could induce a conformational change exposing the vacant catalytic site and thereby increasing its affinity for the cleavage site in the substrate. A reaction scheme fitting to this hypothesis would look like eq 1:

$$\text{IdeS} + \text{IgG} \leftrightarrows [\text{IdeS}-\text{IgG}]_{T_1} \leftrightarrows [\text{IdeS}-\text{IgG}]_{T_2} \leftrightarrows \text{IdeS} + \text{Fc} + \text{F(ab)}_2 \quad (1)$$

where IdeS binds first to the Fc part of IgG, forming a transitory complex ($[\text{IdeS}-\text{IgG}]_{T_1}$). Subsequently, IdeS binds to the cleavage site of IgG, forming a second transitory complex ($[\text{IdeS}-\text{IgG}]_{T_2}$) prior to hydrolysis. Hydrolysis results in the two products Fc and $F(ab)_2$. This, however, is not strictly true cooperativity, where binding of the first substrate molecule increases the affinity of the vacant site for the next substrate molecule. Rather, in this hypothesis, only one substrate molecule binds at two sites on IdeS.

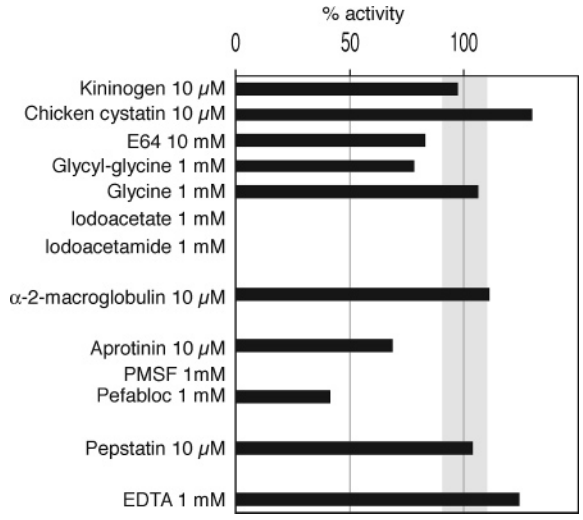

FIGURE 7: Inhibitor scan of IdeS. The possible inhibition of IdeS activity by high- and low-molecular mass protease specific inhibitors was scanned, as described in detail in Experimental Procedures. The results are related to the activity of IdeS without an inhibitor, set to 100%. The uncertainty of the assay is indicated by the gray bar around the l00% activity line.

Nevertheless, the two sites may interact in the same kind of way as identical sites showing true cooperativity. In relation to the sigmoidal velocity curve, formation of $[\text{IdeS}-\text{IgG}]_{T_1}$ would be a rate-limiting step and a high substrate concentration is needed to drive eq 1 in the forward direction.

Indications of cooperativity in monomeric enzymes from Hill plots can easily be confused with product inhibition (*44*), which provides another explanation for the unusual velocity curve for IdeS acting on IgG as a substrate. After reaching a maximum at 175 μM, the enzyme velocity declines at higher IgG concentrations (Figure 6A). This must be due to enzyme inhibition by the substrate or product, of which the latter seems the most likely. If an exosite on IdeS is interacting with the Fc part of IgG and Fc_{papain}, it is not difficult to imagine that the Fc product could also interact well with IdeS. Upon hydrolysis, the Fc product will be present as a competitive inhibitor in continuously increasing amounts in the reaction tube. A competitive product inhibition seen above 175 μM IgG would mean that the Fc concentration has reached a sufficiently high level to result in significant interference with the kinetic factors favoring eq 1, and thus, the enzyme velocity will be reduced. Whether the unusual velocity curve is a result of allostery, i.e., binding site cooperativity, or product inhibition is difficult to say. Nonetheless, both explanations point to the presence of an exosite that is important for the hydrolytic action of IdeS.

We determined V_{max} and the pseudoequilibrium constant (K') for this enzymatic reaction directly from the velocity curve (Figure 6A). V_{max} was 42 mAU/min, and K' was determined as the substrate concentration at $V_{max}/2$, which gave 90 μM. The *x*-intercept of the fitted line in the Hill plot (Figure 6B) also equals K' and gave 91 μM, in good agreement with the estimation from the velocity curve. The value of K' was on the same order of magnitude as the *in vivo* concentration of IgG in blood, 40−105 μM (*45*).

Scanning for Inhibitors of IdeS Activity. Possible inhibition of the enzymatic activity of IdeS was tested with natural and synthetic class specific inhibitors in the routine FPLC assay (Figure 7). No inhibitory effect was observed for E-64,

which is a highly specific inhibitor of cysteine proteases of the papain family (C1). Chicken cystatin, which is a natural inhibitor of cysteine proteases of the C1 family but also legumain-like cysteine proteases in family C13 (*46*), did not show any significant inhibition of IdeS activity. Neither did kininogen, which is a natural inhibitor of C1 family enzymes but also of cysteine proteases of the calpain family (C2) (*47*). Clear inhibition was, however, seen with iodoacetate and iodoacetamide, strongly supporting the idea that IdeS should be classified as a cysteine protease (*10*). The human protease inhibitor α_2M was also tested, but no inhibition was detected. Glycine and glycylglycine were tested for inhibition or competition for the active site, but did not show any effect. We also tested inhibitors of the serine protease class, with some surprising results as PMSF exhibited inhibition of IdeS activity. Pefabloc also inhibited IdeS activity, although to a minor degree. Bovine aprotinin did not have any effect on IdeS activity. Pepstatin, an inhibitor of aspartic acid proteases, and EDTA inhibiting metal ion-dependent proteases were both devoid of an effect on IdeS activity.

Thus, quite strong experimental support for IdeS belonging to the cysteine protease class was obtained by the inhibitor scanning. Inhibition by iodoacetate and iodoacetamide strongly favors this classification, although there can be pitfalls using only these reagents for classification of cysteine proteases (*48*). Upon testing IdeS for inhibition by synthetic serine protease specific reagents, we found that PMSF and to some extent Pefabloc were capable of inhibiting IdeS. On rare occasions, active site cysteines can be reversibly inhibited by PMSF (*49*), and given its similar chemical structure, we assume the same could be valid for Pefabloc; therefore, these results must not be in disagreement with IdeS being a cysteine protease. When the classical cysteine protease inhibitor, E-64, and natural family specific cysteine protease inhibitors directed against C1, C2, and C13 enzymes were tested, none of these showed an effect. A lack of inhibition by E-64 has also been described for other cysteine proteases, i.e., the caspases and legumain (*50*). However, IdeS is inhibited by Z-LVG-CHN_2 (*10*), a tripeptide carrying a diazomethane group which has been shown to bind irreversibly to cysteine proteases in family C1 (*51*), which strongly points toward a cysteine protease classification.

Biochemical Classification of IdeS. To verify that Z-LVG-CHN_2 inhibits IdeS by binding covalently to its single Cys residue, which then can be concluded to be part of the active site, we used an experiment to chemically cleave IdeS with NTCB. NTCB reacts covalently with free and unmodified cysteine residues, and an increase in pH induces a cyclization reaction by NTCB; subsequently, the N-terminal peptide bond is hydrolyzed (*20*, *52*). If Z-LVG-CHN_2 binds covalently to the Cys residue of IdeS, it should obstruct NTCB from reacting with this residue. NTCB treatment of IdeS that had been preincubated with Z-LVG-CHN_2 did not give rise to any chemical cleavage of the enzyme (Figure 8, lane 1). IdeS treated with NTCB, however, gave rise to the expected chemical cleavage at Cys_{94} (Figure 1), hence reducing its molecular mass by approximately 7 kDa as verified by SDS−PAGE analysis (Figure 8, lane 3). These data demonstrate that the single Cys in IdeS constitutes the active site residue and that IdeS must be classified as a cysteine protease. A cysteine protease classification of IdeS has been questioned (*53*), but is in accordance with conclusions based on mutagenesis experiments demonstrating that recombinant Cys94Gly substituted IdeS is enzymatically inactive (*54*).

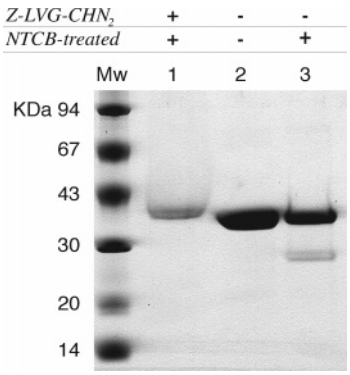

FIGURE 8: NTCB assay for elucidating a cysteine protease classification of IdeS. IdeS samples were incubated with or without the diazomethane cysteine protease inhibitor, Z-LVG-CHN_2, and then analyzed by chemical cleavage with NTCB, as described in detail in Experimental Procedures. The incubation mixtures were analyzed by 4 to 12% SDS−PAGE as described in the legend of Figure 2: lane 1, IdeS preincubated with Z-LVG-CHN_2 and treated with NTCB; lane 2, IdeS; and lane 3, IdeS treated with NTCB.

Concluding Remarks. Taken together, our experimental results demonstrate that IdeS is an unusual protease, most probably representing a novel family of cysteine proteases. The study thus lends experimental support to the sequence-based classification of IdeS in a novel family (C66) of the MEROPS system. The high specificity of the enzyme must be due to the presence of an exosite recognizing the C_H2 domain in the Fc part of IgG. Thus, *S. pyogenes* has evolved a highly specific and tightly regulated enzyme for interfering with human antibody defense, further underlining the molecular complexity of the host−microbe relationship.

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