INFECTION AND IMMUNITY, June 1996, p. 1913–1917 Vol. 64, No. 6
0019-9567/96/$04.00+0

Substitution of Cysteine 192 in a Highly Conserved *Streptococcus pyogenes* Extracellular Cysteine Protease (Interleukin 1β Convertase) Alters Proteolytic Activity and Ablates Zymogen Processing

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Received 27 December 1995/Returned for modification 2 February 1996/Accepted 7 March 1996

Virtually all strains of the human pathogenic bacterium *Streptococcus pyogenes* express a highly conserved extracellular cysteine protease. The protein is made as an inactive zymogen of 40,000 Da and undergoes autocatalytic truncation to result in a 28,000-Da active protease. Numerous independent lines of investigation suggest that this enzyme participates in one or more phases of host-parasite interaction, such as inflammation and soft tissue invasion. Replacement of the single cysteine residue (C-192) with serine (C192S mutation) resulted in loss of detectable proteolytic activity against bovine casein, human fibronectin, and the low-molecular-weight synthetic substrate 7-amino-4-trifluoromethyl coumarin. The C192S mutant molecule does not undergo autocatalytic processing of zymogen to mature form. Taken together, these data support the hypothesis that C-192 participates in active-site formation and enzyme catalysis.

Microbial extracellular cysteine proteases have emerged in recent years as important participants in host-parasite interactions (5, 16, 17, 34, 35, 41). Several lines of evidence demonstrate that this family of virulence factors is involved in processes such as host colonization, mucosal invasion, avoidance of normal defense systems, spread to deeper tissues, and other pathological processes (16, 17, 28, 35, 37, 41). Many extracellular cysteine proteases are also recognized by the host immune system during infection, and evidence that these enzymes may be critical targets for immunoprophylaxis strategies has been presented (5, 33).

The human pathogenic bacterium *Streptococcus pyogenes* (group A streptococcus) secretes a cysteine protease that is thought to be an important virulence factor. The protein is made as an inactive zymogen of 40,000 Da, which can be converted to a 28,000-Da active form by proteolysis or autocatalytic truncation in reducing conditions (6–9, 31). The zymogen or active protease is also known as streptococcal pyrogenic exotoxin B (SPE B) (9, 14). Virtually all *S. pyogenes* strains have the structural gene (*speB*) (36) and express active enzyme (24). Sequence analysis of the 1,197-bp *speB* gene (14) from 67 isolates has demonstrated that the gene is highly conserved in natural populations of *S. pyogenes* (24). Although the exact role of the zymogen or protease in group A *Streptococcus* pathogenesis is not yet known, the demonstration that the protease cleaves several biologically important molecules has been interpreted as evidence for a role in virulence (1, 23, 24, 46). For example, the protease cleaves fibronectin (FN) and vitronectin, two abundant extracellular matrix proteins involved in maintaining host tissue integrity (24). In addition, the protease cleaves human interleukin-1β precursor to generate mature interleukin-1β with full biologic activity, a result suggesting a critical role in inflammation and shock (23). Consistent with an important virulence role is the observation that infected humans are more likely to die if they have low levels of acute-phase serum antibody against SPE B (15). Moreover, recent data demonstrate that immunization of mice with cysteine protease generates protection against intraperitoneal challenge (22). These findings and other evidence (1, 2, 26, 39, 40, 43) are consistent with the idea that the group A streptococcal cysteine protease participates in host-parasite interactions.

Biochemical and genetic analyses have shown that both zymogen and active enzyme contain only one cysteine residue (C-192), and it has been proposed that this amino acid participates in enzyme active-site formation and catalysis (27, 30–32, 42, 47). With the goal of testing this hypothesis and generating an enzymatically altered form of SPE B for immunoprophylaxis and pathogenesis studies, site-specific mutagenesis was used to replace C-192 with Ser, and the resulting C192S mutant molecule was characterized.

MATERIALS AND METHODS

Construction of the *speB* C192S mutant. The *speB* gene was amplified from *S. pyogenes* MGAS 1719, a strain that contains the *speB7* allele (24), and the PCR fragment was cloned into the pCR-Script vector (Stratagene, La Jolla, Calif.). The oligonucleotides used in PCR amplification corresponded to positions −160 to −138 (primer SPEBX) and 1303 to 1325 (primer SPEB2) of a published *speB* gene sequence (14). The 30-μl PCR mixture contained 1 μg of MGAS 1719 genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 2 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 2 μM each primer, and 2.5 U of *Pfu* DNA polymerase. Amplification was performed in a Perkin-Elmer Cetus model 480 thermocycler, with the following parameters: 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. A 1.5-kb amplification fragment was cloned into pCR-Script, and plasmid DNAs obtained from *Escherichia coli* transformants were characterized by automated DNA sequencing (24) to verify that no mutations had occurred in the inserted *speB* gene.

One clone (pSEB1719) with the same nucleotide sequence from positions −155 to 1325 as the *speB7* allele was chosen for use in mutagenesis. A PCR-based mutagenesis procedure was used to generate a C-192 (TGT)-to-S-192 (AGT) substitution. The sense-strand primer (TGT2) had the sequence CAG GAAGTGTTGCTACTGCAA, and the sequence of the antisense-strand primer (TGT1) was CAACACTTCCTGTAGCTGCATG. Separate PCRs were done with primers TGT2 and SPEB2 in one reaction and primers TGT1 and SPEBX in a second reaction. These reactions were carried out with 1 ng of plasmid

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pSEB1719 and the PCR conditions described above except that primer annealing was done at 40°C for 1 min. As expected, two DNA fragments (740 and 757 bp) were generated by the reactions. Following agarose gel electrophoresis, these fragments were recovered and pooled, and 1 μl of the mixture was used in a second round of PCR amplification with primers SPEBX and SPEB2 and an annealing temperature of 55°C. A resulting 1.5-kb full-length *speB* gene was then cloned into pCR-Script. Screening of plasmids from 183 colonies by agarose gel electrophoresis identified five organisms with appropriately sized inserts. The target region was sequenced in these five clones, and one (pSEBC2S) that had the desired TGT→AGT (C192S) substitution was identified. The 1.5-kb insert was then sequenced in its entirety by an automated strategy (24), and no additional nucleotide substitutions were identified.

Generation and characterization of antiprotease murine monoclonal antibodies. BALB/c mice were immunized by intraperitoneal injection with five doses of 10 μg each of the purified 28-kDa form of the cysteine protease. After the mice had detectable serum antiprotease, as assessed by Western blot (immunoblot) analysis (24), spleens were harvested and fusions with myeloma cell line P3 were performed.

To determine the specificity of three arbitrarily chosen anti-cysteine protease monoclonal antibodies (2A3-B2-C12, 2A3-B2-F12, and 2A3-H5-H10), solid-phase enzyme immunoassay was used to scan 235 overlapping cleaved biotinylated synthetic peptides, each of 10 amino acid residues, with an offset of 2 amino acids (Chiron Mimotopes, Clayton, Victoria, Australia) (11, 12). These peptides represent 13 distinct allelic variants of the full-length secreted protease precursor (24). Reactivity of the antibodies was determined by enzyme-linked immunosorbent assay as instructed by the manufacturer. Preliminary characterization of the antibodies has been reported elsewhere (21). The B-cell epitope mapping analysis identified a linear epitope located in a region of the molecule known to contain two naturally occurring amino acid substitutions (positions 308 and 317) that differentiate three mature SPE B variants that together are expressed by approximately 80% of all group A streptococci (24). Although all three monoclonal antibodies (2A3-B2-C12, 2A3-B2-F12, and 2A3-H5-H10) recognize all known streptococcal cysteine protease variants, interestingly, the specificity of reactivity is considerably altered against peptides representing the three naturally occurring cysteine protease variants. For example, these monoclonal antibodies recognize the sequence 302-VHQINR-307 in cysteine protease variant SPE B1 and the sequence 305-INRG-308 in variant SPE B4. One of these monoclonal antibodies (2A3-B2-C12) was arbitrarily chosen for experimental use.

Immunoblot assay with murine monoclonal antibody 2A3-B2-C12. Test material (100 μl) was spotted onto a nitrocellulose membrane (Hybond-ECL; Amersham), and unabsorbed sites were blocked by incubation with 0.5% blocking agent (Amersham) for 1 h at room temperature. The membrane was rinsed with phosphate-buffered saline (PBS; pH 7.4)-Tween 20 (0.1%) and incubated for 60 min with murine monoclonal antibody 2A3-B2-C12 (1:200 dilution in PBS [pH 7.4]-Tween 20 [0.1%]). The membrane was rinsed with PBS (pH 7.4)-Tween 20 (0.1%) and incubated with a secondary antibody (goat or rabbit anti-mouse-horseradish peroxidase conjugate [Bio-Rad, Inc., Hercules, Calif.] diluted 1:2,000 in PBS [pH 7.4]-Tween 20 [0.1%]). After being washed with PBS (pH 7.4)-Tween 20 (0.1%), the antigen-antibody complex was visualized by enhanced chemiluminescence (ECL developing reagents; Amersham).

Purification of cysteine protease expressed by *S. pyogenes*. The streptococcal cysteine protease was purified from culture supernatants of strain MGAS 1719 by a procedure described previously (23). Briefly, bacteria were grown in a chemically defined medium (JRH Biosciences, Lenexa, Kans.) maintained at pH 5.5 to 6.0, and the protease was purified from the concentrated culture supernatant by column chromatography with Matrex Red A (Amicon, Mass.). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining of the resulting proteolytically active protein showed a single major band with an apparent molecular mass of 28 kDa. SPE B purified by this strategy is approximately 95% pure, as assessed by Coomassie blue staining (23).

Purification of recombinant wild-type and mutant forms of SPE B. All procedures were performed at 0 to 4°C unless specified otherwise. *E. coli* containing appropriate plasmids was grown in stationary culture overnight at 37°C in 4 liters of LB medium. The cells were harvested by centrifugation and resuspended in 40 ml of PBS (pH 7.4), and 0.4 ml of lysozyme (50 mg/ml) was added. After incubation for 30 min, the bacteria were disrupted by three 20-s bursts of sonication with a Sonifier 250 (Branson, Danbury, Conn.) set at 70% power output. After each 20-s sonication, the cell slurry was cooled undisturbed for 5 min.

The cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The supernatant was dialyzed for 6 to 12 h against 40% ammonium sulfate dissolved in water. The precipitate was removed by centrifugation at 20,000 × *g* for 15 min, and the supernatant was dialyzed against 75% ammonium sulfate (pH 8.0). The precipitate was collected by centrifugation and dissolved in 15 ml of water, and the buffer was changed to 0.02 M acetate buffer (pH 5.1) by dialysis. The protein (150 mg) was loaded onto a Macro-S (Bio-Rad) column (2.5 by 13 cm) equilibrated with 0.02 M acetate buffer (pH 5.1). The Macro-S matrix is a strong cation-exchange support. The column was washed with this buffer until the optical density at 280 nm of the flowthrough liquid was less than 0.05. The protein was then eluted from the column with a linear gradient of 500 ml of 0 to 0.5 M NaCl in 0.02 M acetate buffer (pH 5.1), and 5-ml fractions were collected.

SPE B was identified by immunoblot assay using murine monoclonal antibody 2A3-B2-C12, the peak fractions were pooled, and the material was dialyzed for 6 to 12 h against a buffer composed of 20 mM Tris-HCl (pH 7.0) dissolved in 20% ethanol (buffer A). The material was loaded onto a Matrex Red A (Amicon) column (1.5 by 15 cm), and the protein was eluted with 50 ml of a linear gradient of 0 to 2 M NaCl dissolved in buffer A. Fractions (2 ml) containing SPE B were identified by immunoblot assay, and the peak fractions were pooled. The material was desalted, and the buffer was changed to PBS (pH 7.4) by centrifugation three times at 3,000 × *g* for 30 min in a Centriprep 10 spin column (Amicon). After the first two centrifugations, the volume was restored to 15 ml by addition of PBS (pH 7.4), and after the third centrifugation, the volume was adjusted to 2 ml with PBS (pH 7.4). Wild-type and C192S mutant proteins recovered with this protocol are approximately 90% pure, as assessed by Coomassie blue staining following gel electrophoresis.

Protease assays. Three distinct assays were used to characterize enzymatic activity of the mutant proteins. When appropriate, 2-mercaptoethanol (final concentration, 10 mM) was included in the assays to activate the zymogen or protease. The 2-mercaptoethanol was added to the protein and incubated for 30 min before the protease assays were performed. In addition, a streptococcal cysteine protease inhibitor (*N*-benzyloxycarbonyl-leucyl-valyl-glycine diazomethyl ketone [Z-LVG-CHN2; Enzyme Systems Products, Livermore, Calif.]) (2) was also sometimes included in the assays at a final concentration of 10 μg/ml to demonstrate specificity of the observed proteolysis. All protease assays were conducted at least in triplicate.

One assay detects cleavage of bovine casein incorporated into an agarose gel matrix (Bio-Rad). Briefly, 1 μg of purified protein was deposited into a small circular well cut in the bovine casein-agar matrix. The plates were incubated for 2 h at 37°C, and protease activity was visualized by formation of a transparent ring of matrix clearing made as the enzyme diffused outward from the well.

A second assay detects cleavage of the extracellular matrix protein FN by Western immunoblot assay (24). In this assay, 5 μg of human FN (Sigma) suspended in PBS (pH 7.4) was incubated with 0.5 μg of protein at 37°C for 2 h. The reaction was stopped by addition of an equal volume of 2× SDS-PAGE sample buffer followed by boiling for 5 min. The mixture was resolved by 4 to 12% gradient SDS-PAGE, and the FN and degradation fragments were transferred to a nitrocellulose membrane (Hybond-ECL; Amersham). The uncleaved FN and cleavage products were reacted with a rabbit polyclonal antiserum specific for human FN (Gibco BRL, Gaithersburg, Md.). FN and FN fragments were then visualized by use of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad).

A third protease assay used the synthetic substrate 7-amino-4-trifluoromethyl coumarin (Z-Arg-AFC; Enzyme Systems Products), which yields a fluorescent product when cleaved. The assay mixture contained 50 mM acetate buffer (pH 5.5), 10 mM cysteine, 40 μM Z-Arg-AFC, and 1 μg of SPE B. Fluorescence was measured at 15-min intervals for 1 h, and again at 20 h, with a model 112 Turner fluorometer (Sequoia-Turner Corp., Mountain View, Calif.) (excitation at 400 nm; emission at 505 nm).

RESULTS

Assessment of expression of recombinant wild-type and mutant SPE B proteins by *E. coli*. A Western immunoblot assay was used to test if wild-type and mutant SPE B proteins were synthesized by *E. coli*. Bacteria containing the wild-type (pSEB1719) and mutant (pSEBC2S) genes were grown overnight at 37°C in LB broth on an orbital shaker, and the cells were harvested by centrifugation. The cell pellet obtained from 1.0 ml of overnight LB broth cultures of *E. coli* containing pSEB1719 was suspended in 1.0 ml of SDS sample buffer and boiled for 5 min, and a 15-μl aliquot was analyzed by SDS-PAGE. After transfer to a Hybond-ECL nitrocellulose membrane (Amersham), Western blotting with anti-SPE B mouse monoclonal antibody 2A3-B2-C12 identified multiple bands ranging from 40 to 28 kDa. In addition, several bands of lower molecular mass were observed (Fig. 1). In contrast, the cell lysate from bacteria with pSEBC2S yielded only a single band with an apparent molecular mass of 40 kDa. No immunoreactive material was identified in lysates obtained from *E. coli* harboring only the vector.

To determine if immunoreactive SPE B was located extracellularly in abundant quantity, 0.5 ml of 100% ethanol was added to 1.0 ml of the culture supernatant, and the mixture was incubated at 4°C for 1 h. The precipitated proteins were collected by centrifugation, reconstituted in 15 μl of distilled water, and analyzed by Western blotting with anti-SPE B

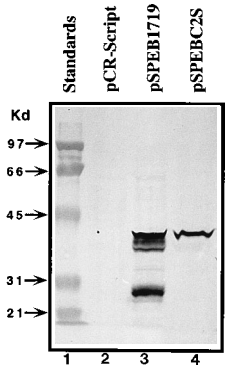

FIG. 1. SDS-PAGE and immunoblot analysis of wild-type and mutant SPE B made by *E. coli*. Lanes: 1, molecular weight standards of the sizes indicated at the left; 2, cell lysate of *E. coli* containing vector plasmid pCR-Script; 3, cell lysate of *E. coli* with recombinant plasmid pSPEB1719 containing the cloned wild-type *speB7* allele; 4, cell lysate of *E. coli* with recombinant plasmid pSPEBC2S containing the codon 192 TGT→AGT (C192S) *speB* derivative generated by site-specific mutagenesis.

mouse monoclonal antibody 2A3-B2-C12. A single faint band with an apparent molecular mass of 40 kDa was identified in the culture supernatant of *E. coli* cells with plasmid pSEBC2S. In contrast, analysis of the cell supernatant from *E. coli* with pSEB1719 containing the wild-type *speB* gene showed multiple faint bands ranging from 40 to 28 kDa (data not shown).

The C192S SPE B mutant lacks virtually all protease activity. To assess if replacement of C-192 with S-192 altered proteolysis capability, the mutant molecule was tested for enzymatic activity in three separate assays. Wild-type and C192S mutant proteins were purified from *E. coli* strains containing appropriate plasmids by procedures described in Materials and Methods. The mutant SPE B had no detectable proteolytic activity against bovine casein (data not shown). In addition, the C192S mutant protein was tested for proteolytic activity against human FN with an assay described previously (24). Virtually no FN cleavage activity was identified in this assay (Fig. 2). To further assess the proteolytic potential of the mutant, we performed a third assay that used a low-molecular-weight substrate (Z-Arg-AFC) that fluoresces when cleaved. The results (Table 1) show that the C192S mutant streptococcal protein lacked detectable proteolytic activity. Recombinant wild-type SPE B made in *E. coli* and purified by the identical protocol used to recover the C192S mutant protein had abundant proteolytic activity against the test substrates.

TABLE 1. Enzymatic activities of purified streptococcal cysteine protease and the C192S mutant, using Z-Arg-AFC as the substrate[a]

Enzyme	Emission value at 505 nm			
	No inhibitor		Plus inhibitor	
	0 h	20 h	0 h	20 h
Wild type	2.4	161	3.4	4.0
C192S mutant	1.8	2.5	1.9	2.3

[a] All assays were conducted with 5 μg of purified protein. The slight increase in the 20-h emission value observed in the wild-type-plus-inhibitor (inhibitor is Z-LVG-CHN2) and the C192S assays is due to nonenzymatic degradation of the Z-Arg-AFC substrate.

Absence of detectable zymogen processing by the C192S mutant. Autocatalytic cleavage of the 40-kDa zymogen is expected to yield a 28-kDa mature form of the cysteine protease. The ability of the C192S mutant to carry out this processing was examined by Western blot analysis using the purified protein and an antiprotease rabbit polyclonal antibody. Addition of the reducing agent 2-mercaptoethanol resulted in efficient cleavage of the wild-type protease precursor to yield the anticipated processed 28-kDa mature form (Fig. 3). In contrast, the C192S mutant had no autocatalytic activity, as shown by the lack of detectable conversion of precursor protein to mature form (Fig. 3).

DISCUSSION

The hypothesis that C-192 participates in streptococcal cysteine protease active-site formation is based on biochemical data that have accrued over many decades (6, 10, 27, 30–32). Liu et al. (31) showed that after conversion from zymogen to enzyme, reduction with thiol compounds (thioethanol, thioglycolic acid, cysteine, or 2,3-dimercaptoethanol) is essential before full catalytic activity is manifested. The reduced, active enzyme is readily inactivated by reagents known to react with sulfhydryl groups, such as iodoacetic acid (6) or *N*-ethylmaleimide (31). These data, the occurrence of a single cysteine

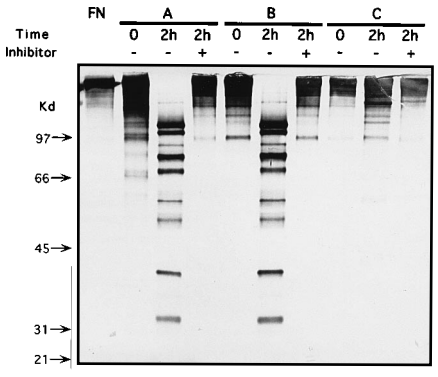

FIG. 2. SDS-PAGE and immunoblot analysis showing virtual lack of FN cleavage by the C192S SPE B mutant. Lanes: FN, purified FN; A, FN incubated with purified SPE B made by *S. pyogenes*; B, FN incubated with purified recombinant SPE B made by *E. coli*; C, FN incubated with purified recombinant mutant SPE B (C192S) made by *E. coli*. Incubation was carried out at 37°C and for the times indicated. Inhibitor refers to the presence (+) or absence (−) of the specific streptococcal cysteine protease inhibitor Z-LVG-CHN2 (2).

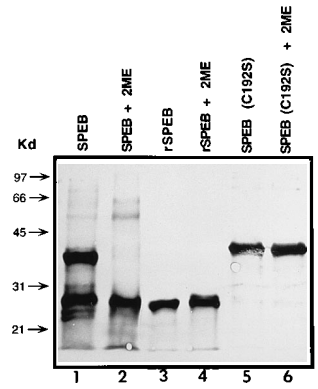

FIG. 3. SDS-PAGE and immunoblot analysis showing lack of zymogen processing by the C192S SPE B mutant. Incubation was carried out for 6 h at 37°C. Lane 1, purified SPE B made by *S. pyogenes*; lane 3, purified recombinant wild-type SPE B; lane 5, purified recombinant mutant (C192S) SPE B. Lanes 2, 4, and 6 are identical to lanes 1, 3, and 5, respectively, except that 2-mercaptoethanol (2-ME) was added.

residue in both the zymogen and the active enzyme (31), and results from additional studies (27) strongly suggested that C-192 is involved in active-site formation. Our results provide direct genetic and biochemical evidence in support of this idea. It is noteworthy that site-directed mutagenesis studies of several prokaryotic, eukaryotic, and viral cysteine proteases have generally shown that replacement of the catalytic cysteine residue with another amino acid results in a molecule with substantially decreased proteolytic activity (3, 5, 13, 18, 44).

Many proteases, including the streptococcal cysteine protease, are synthesized as inactive precursors that must be processed to form a lower-molecular-weight active protease (45). The streptococcal protease is initially made as a 398-amino-acid preproenzyme, and removal of a putative signal peptide results in a 371-residue proenzyme. Under reducing conditions, the zymogen is then converted to a 253-amino-acid mature protease. Although the exact process whereby the zymogen-to-protease transformation occurs has not been elucidated, evidence indicates that the conversion may first involve formation of an intermediate that has 17 amino acids less than the zymogen (30). Biochemical data have shown that a series of truncated intermediate products is formed during the transition from 40-kDa precursor to 28-kDa mature form (9, 30). Our data demonstrate persistence of the 40-kDa high-molecular-mass precursor of the C192S mutant protein under the reducing conditions that convert wild-type zymogen to an active mature protease. Although we cannot exclude the formal possibility that the C192S replacement produced conformational changes that resulted in nonspecific inactivation of zymogen autoprocessing, our findings are consistent with data from studies of other cysteine proteases (44) that replacement of the active-site Cys residue alters precursor processing. In addition, we note that purification of recombinant wild-type SPE B by the identical procedure used to isolate the C192S mutant molecule retained appropriate autoprocessing and proteolytic activity.

Study of microbial cysteine proteases has emerged as an area of considerable research activity, largely because many of these enzymes are believed to mediate one or more phases of infection (5, 16, 17, 34, 35, 41) and because they represent potential targets for chemotherapeutic intervention (34) and immunoprophylaxis (33, 34). Several lines of evidence suggesting that the streptococcal cysteine protease participates in host-parasite interactions have accumulated (1, 2, 2a, 15, 22–24, 35a, 46). Strong data supporting this notion were provided by the observation that among humans with group A streptococcal invasive disease, those with low acute-phase serum antibody against SPE B are more likely to die (15). Moreover, immunization of mice with cysteine protease generated protection against intraperitoneal challenge (22). The availability of precisely defined mutant molecules will assist studies of the role of this streptococcal product in host-parasite interactions and studies of structure-function relationships in the protease and zymogen. Moreover, inasmuch as active immunization of mice with mature streptococcal protease protected against intraperitoneal challenge, and the zymogen contains at least one epitope that the active protease lacks (7), the C192S mutant 40-kDa protein will permit more detailed study of potential immunoprophylaxis applicability of this protein.

By analogy with papain (the canonical cysteine protease) and related proteases (4, 25, 44), several other amino acid residues can be hypothesized to play a role in enzyme active-site formation. For example, crystal structure analysis, other biochemical data, and site-directed mutagenesis studies have demonstrated that the active site of papain is composed of a catalytic triad consisting of Cys-25, His-159, and Asn-175 (20, 44). As judged from sequence alignments and biochemical data (25, 29, 38), it is possible that residues His-340 and Trp-357 in the streptococcal protease also participate in active-site formation. Moreover, cysteine proteases such as papain, actinidin, and cathepsins B, H, and L have a conserved Gln located six residues toward the amino terminus from the catalytic-site Cys (4). The streptococcal cysteine protease also has an identically placed Gln residue (Gln-186). Preliminary studies clearly indicate that Gln-186 and His-340 also participate in active-site formation (38a). Additional site-specific mutagenesis and biochemical characterization studies will be needed to test these hypotheses.

ACKNOWLEDGMENTS

We are indebted to C. White for assistance with the fluorometric assays and S. Highlander for suggestions regarding mutagenesis strategies.

This work was supported by Public Health Service grant AI-33119 and by awards from the Texas Advanced Technology Program and the Moran Foundation. J.M.M. is an Established Investigator of the American Heart Association.

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Editor: V. A. Fischetti