Zinc-mediated Dimerization and Its Effect on Activity and Conformation of Staphylococcal Enterotoxin Type C*

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Staphylococcal enterotoxins are superantigenic exotoxins that mediate food poisoning and toxic shock syndrome in humans. Despite their structural and functional similarities, superantigens display subtle differences in biological properties and modes of receptor binding as a result of zinc atoms bound differently in their crystal structures. For example, the crystal structures of the staphylococcal enterotoxins in the type C serogroup (SECs) contain a zinc atom coordinated by one aspartate and two histidine residues from one molecule and another aspartate residue from the next molecule, thus forming a dimer. This type of zinc ligation and zinc-mediated dimerization occurs in several SECs, but not in most other staphylococcal enterotoxin serogroups. This prompted us to investigate the potential importance of zinc in SEC-mediated pathogenesis. Site-directed mutagenesis was used to remove the zinc binding ligands with alanine. SEC mutants unable to bind zinc did not have major conformational alterations although they failed to form dimers. Zinc binding was not essential for T cell stimulation, emesis, or lethality although in general the mutants were less pyrogenic. Thus the zinc atom in SECs might represent a non-functional heavy atom in an exotoxin group that has diverged from related bacterial toxins containing crucial zinc atoms.

The atomic coordinates and structure factors (code 1ck1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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¶¶ The abbreviations used are: SE, staphylococcal enterotoxin; TSS, toxic shock syndrome; SAg, superantigen; TCR, T cell receptor, MHC, major histocompatibility complex; ICP MS, inductively coupled plasma mass spectrometry; SEC, staphylococcal enterotoxin in the type C serogroup.

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The hallmark symptom, emesis, can be accompanied by other effects including diarrhea and abdominal cramping. Evidence suggests that SEs interact with receptors on mast cells, and further that neuropeptides generated contribute to the emetic response (10–12).

Although the number of recognized SEs continues to grow, these classical SEs (SEA through SEH) can be divided into general subgroups based on the degree of sequence homology. SEA, SED, SEE, and SEH share higher sequence homology and form one subgroup, whereas SEB and SECs forms another subgroup. The SECs are a highly related heterogeneous group of serologically cross-reactive but distinct molecular variants produced by some staphylococcal species, particularly S. aureus and S. intermedius isolates from several animal hosts (13). The amino acid sequences of the SEC molecular variants share more than 90% identity.

One conserved property of the SECs is their ability to bind zinc (14, 15). Although several SAgS bind zinc, their mechanisms for zinc ligation vary and are usually different from that of SECs (Table I). The SEC zinc binding mechanism is similar to that of metalloproteases such as thermolysin. Three residues in the SECs (His-118, Glu-119, and His-122) form a motif (HEXH), which provides two of the zinc binding ligands. Interestingly, zinc was not found in the structure of SEB (16, 17), which shares 86% sequence homology with SECs. The zinc ligation in the crystal structures of SEA (18, 19), SED (20), SEE at least two human illnesses, staphylococcal food poisoning (SFP) and toxic shock syndrome (TSS) (1, 2). Although the pathogenesis has not been completely elucidated for either disease, TSS results from the superantigen (SAg) activity of the SEs, toxic shock syndrome toxin-1 (TSST-1), or other pyrogenic toxins. SAgS are potent immune cell stimulators and induce the proliferation of T cells in a manner that depends on the Vβ sequences of the T cell receptor (TCR) (3, 4). This type of immunostimulation results from specific interaction of SAgS with the MHC class II molecules (MHCII) on antigen-presenting cells (APCs) in addition to TCR. Activation of large numbers of T cells and APCs causes massive release of cytokines, which contribute to the effects on the cardiovascular and other organ systems leading to TSS. Some studies have suggested that direct cytotoxicity to other cells contributes to the pathogenesis of TSS (5, 6).

Although TSS and staphylococcal food poisoning have overlapping symptoms, the pathogenesis of each is unique, and the biological activities responsible for both diseases are determined by separate regions of the SE molecules (7–9). The route of exposure also determines which disease will result. TSS requires systemic exposure to any pyrogenic toxin, whereas staphylococcal food poisoning results from SEs in the gastrointestinal tract following ingestion in food (2). The hallmark symptom, emesis, can be accompanied by other effects including diarrhea and abdominal cramping. Evidence suggests that SEs interact with receptors on mast cells, and further that neuropeptides generated contribute to the emetic response (10–12).

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Effect of Zinc on SEC Biology and Conformation

Zinc binding sites and homodimerization observed in the superantigen crystal structures

The abbreviations used are SEs (staphylococcal enterotoxins), TSST (toxic shock syndrome toxin), SPEs (streptococcal pyrogenic exotoxins), SSA (streptococcal superantigen A), and SMEZ (streptococcal mitogenic exotoxin Z).

| Toxin     | Zinc atom location | Zinc ligands | Zinc-mediated dimer | PDB accession code |
|-----------|--------------------|--------------|---------------------|-------------------|
| SEA†      | 2                  | H187, H225, D227, H61 | Yes            | 1SXT, 1ESF        |
| SEB       | None               | None         | None               | None             |
| SEC2      | 1                  | D83, H118, H122, D9 | Yes            | 1SEE             |
| SEC3      | 1                  | D83, H118, H122, D9 | Yes            | 1CK1d            |
| SED†      | 2                  | D182, H220, D222, H218 | Yes            | N/A              |
| SEE       | 1                  | B184, H222, D224 | No               | 1SSE             |
| SEH       | 2                  | D187, H206, D208 | No               | 1EWG, 1HXY       |
| TSST-1    | None               | None         | None               | None             |
| SPEA1‡    | None               | None         | None               | None             |
| SPEC      | 2                  | H187, H201, D203 | Yes             | 1BIZ, 1FNU       |
| SPEH      | 2                  | H35, H35, E54, H35, E54 | No            | 1AN8, 1HQR       |
| SSA       | None               | None         | No                 | 1BTX             |
| SMEZ2     | 2                  | H162, H202, D204 | No               | 1EU3             |

† The zinc sites are located at the bottom of the cleft between two domains (no.1), on the wall of the β-grasp C-terminal domain (no.2), and on the edge of the β-barrel small domain surface (no.3).
‡ The residues underlined are from the neighboring molecule that mediates the dimerization.
§ The SEC3 atomic coordinates have been deposited and will be released upon publication.

Table II

Data processing and refinement statistics

| Data statistics | Resolution range 2.6 Å | Reflections 8,889 | Redundancy 4.6 (2.8) | Data coverage (%) 91.5 (76.7) | R1 10.1 (5.8) | Rsym (%) 5.5 (11.2) |
|-----------------|-------------------------|--------------------|---------------------|-----------------------------|--------------|------------------|
| Refinement Statistics | Resolution range 2.6 Å | Number of non-H Protein 1,944 | Number of solvent molecules 83 | R factor for 42922 reflections 16.2 % | Rrup value for 2268 reflections 23.3 % | RMS deviation from ideal geometry Bond lengths 0.008 Å | Bond angles 1.241° | Dihedral angles 25.927° | Improper angles 1.100° | Average temperature factors Main chain atoms only 37.8 Å² | All protein atoms 39.0 Å² | Waters 40.4 Å² |

EXPRESSMENTAL PROCEDURES

Crystalization and Data Collection of the Native Protein—Native SEC3 was purified directly from the Staphylococcus sp. FRI-909 strain (23). Homogenous SEC3 protein preparations were crystalized into two crystal forms, both of which diffracted to atomic resolution (24). The structure presented in this present report was determined from the tetragonal crystal form (P4121) with unit cell dimensions of a = b = 43.7 Å and c = 280.5 Å, with one molecule in the asymmetric unit. The native data set to 2.6 Å was collected at room temperature with a Xentronics/Siemens multiwire area director coupled to an Elliot-20 rotating anode generator and processed using the XDS package (25).

Construction and Purification of Zinc Binding Site Mutants—A commercial kit (Altered Sites II in vitro mutagenesis systems, Promega) was used to create site-specific mutations in secMNDDON, allowing the expression of mutant toxins containing alanine substitutions at one or more of the three positions in SEC1 occupied by zinc binding residues (Asp-83, His-118, His-122). Five different mutant genes were constructed; three encoding proteins with single alanine substitutions and two with double alanine substitutions. The mutant toxins expressed by these genes were designated SEC1(D83A), SEC1(H118A), SEC1(H122A), SEC1(D83A/H118A), and SEC1(D83A/H112A) based on the amino acid positions involved. For expression, each mutant gene or native secMNDDON was subcloned into the chimeric shuttle vector pMIN164 and then transformed via protoplast transformation into the non-toxigenic background of S. aureus RN4220 as described previously (26, 27). Recombinant toxins were purified to apparent homogeneity from culture supernatants by preparative isoelectric focusing as described previously (26). Size and purity of each toxin and mutant were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

Electrophoresis—SEC1 and various mutants (typically 1–10 µg) were resolved by denaturing, discontinuous, SDS-PAGE in 12.5% gels using established techniques (28), with the minor modification of adding 10% glycerol to the resolving gel. Proteins were visualized in the gels by staining with Coomassie Blue. Prestained size markers were used as standards. For native gel experiments, ~5 µg of each toxin, SEC1 and SEC1(D83A/H112A), was loaded onto a continuous native, 12.5% polyacrylamide gel. The gel system used was equivalent to the denaturing gel system above except that SDS was omitted from both the gel and tank buffer, and the sample buffer contained no β-mercaptoethanol.

Gel Filtration—Purified SEC1 or SEC1(D83A/H112A) (125 µg each) were loaded onto a Sephadex G-75 column equilibrated with 10 mM phosphate, 150 mM NaCl, and 10 mM ZnCl2. Monomer and dimer forms of the toxins were eluted and detected as a change in absorbance at 280 nm. Molecular weight markers were used to determine the size of eluted proteins.

Zinc-mediated dimer

PDF accession code

SEC3, 1SEE, 1ESF

SPE1, 1SCHA, 1SB1

SEC1(D83A), 1AN8, 1HQR

1BXT, 1EU4

1EU3
Zinc Binding Assessment—To assess the effect of each mutation on the ability of SEC1 to bind zinc, native SEC1 and each of the five mutant proteins were compared under identical conditions. After exhaustive washing of each protein with buffer (2 mM HEPES, pH 7.0) using a Centrioc filter (Amicon, Beverly, MA), the retentates were analyzed for zinc content by ICP MS (Inductively Coupled Plasma Mass Spectroscopy) using an ISA/Jobin Yvon model JY24 with a high efficiency nebulizer (29).

**T Cell Proliferation Assays**—To determine the effect of zinc or zinc binding residues on the activity of SEC1 as a SaG, SEC1 or mutants were compared for their abilities to induce proliferation of human T cells. Incorporation of [3H]thymidine into cellular DNA during stimulation in culture was used as an index for lymphocyte proliferation (30). Purified mutant toxins or native SEC1 were added to T-lymphocyte-enriched cultures in complete RPMI 1640 medium and incubated at 37 °C in 7% CO2 for 72 h. Cultures were pulsed (18–24 h) with 1.0 μCi of [3H]thymidine. Cellular DNA was harvested on glass filters, and the level of radioactivity incorporated was quantitated by liquid scintillation counting.

**Rabbit Model Experiments**—The rabbit model described by Kim and Watson (31) was used to compare lethality and fever induced in vivo by SEC1 and several selected mutants as an indication of their potential to induce fever and TSS. Each animal (adult New Zealand White rabbit) was given an initial intravenous injection containing SEC1 or a mutant (0.01–10 μg/kg) dissolved in 0.9% NaCl. After monitoring rectal temperature for 4 h, an intravenous injection of endotoxin (Difco) from *Salmonella typhimurium* was administered at a dose equal to one-fiftieth of its LD50 (10 μg/kg). Mortality was monitored for 48 h. Fisher exact probability test was used in the StarView statistical package (Version 4.57, SAS Institute Inc., Cary, NC) to determine statistical significance between treatment groups.

**Emesis Assays**—A modification (9) of the standard monkey feeding assay for SEs (32) was used for comparing the emetic capability of SEC1 and several representative zinc binding mutants. Toxins (10 μg/ml) were administered in sterile saline to manually restrained young adult (5–10 kg) pigtail monkeys (*Macaca nemestrina*) through a nasogastric tube (infant feeding tube; BD PharMingen). Each animal received 1.0 ml of toxin solution per kg of body weight so that the dose received was 10 μg/kg. We had determined previously that this dose of native SEC1 is sufficient to induce emesis in 100% of animals tested using this technique (9). Animals were returned to their cages following removal of the nasogastric tube and observed for 24 h.

**Proteolysis Susceptibility Assays**—Purified preparations of SEC1 or mutants were incubated in the presence of trypsin or pepsin to assess the contribution of zinc binding residues toward overall conformation and potential stability of SEC1 in the gastrointestinal tract. The susceptibility to trypsin was assessed as follows. Each protein (100 μg/ml) was incubated at 37 °C with trypsin type XI (100 ng/ml) (Sigma Chemical Company) using standard reaction conditions (25 mM Tris, pH 8.0, 20 mM CaCl2) as described previously (9). After incubation for desired periods of time, aliquots were removed, and the digestions were terminated by boiling (5 min) in SDS-PAGE sample buffer. The extent of proteolysis was assessed by SDS-PAGE as described above. To assess the relative liability to pepsin, each toxin or mutant (100 μg/ml) was incubated (37 °C) with pepsin (50 μg/ml) in a final volume of 100 μl (100 mM sodium acetate buffer, pH 4.5). At selected time points, aliquots (10 μl) of the digestion mixture were removed, and the proteins were resolved by SDS-PAGE. Control lanes contained toxin or mutant proteins removed from reaction mixtures exposed to identical conditions in the absence of protease.

**RESULTS**

**Structure Determination and Model Refinement**—The structure was solved by the molecular replacement method using the AMORE program (33). Our previously determined SEC3 structure2 from another (P1) crystal form was used as a search model. The refinement of the model was then continued with the group B factor refinement followed by the simulated-annealing protocols implemented in X-PLOR (34). The rebuilding of the model including addition of solvent molecules and a zinc atom was done on the graphic program O (35). A substantial drop in the value of Rfree validated inclusion of individual atomic temperature factor refinement during the final stages. The program PROCHECK (36) was used to check the quality of the structure. At the end of the refinement, the crystallographic R-factor was 16.2% and Rfree was 23.3%. The detailed refinement statistics are shown in Table II. The atomic coordinates have been deposited in the Protein Data Bank with the accession code 1ck1.

**Overall Structure and a Common Architecture**—The structural features of SEC3 are very similar to those found in other bacterial SaGs, characterized by two unequal sized domains. The small domain on the right (Fig. 1A) has the fold of a Greek-key beta barrel capped by an alpha-helix at one end. This domain has the same topology and fold as the oligonucleotide/oligosaccharide binding (OB) fold (37) and contains the disulfide loop. The larger domain on the left (Fig. 1A) is an alpha/beta sandwich or beta-grasp motif made up of a five-strand mixed beta-sheet with over which a group of alpha-helices are laid.

**Zinc Coordination and Dimerization**—A zinc ligation complex was found in the structure of SEC3. This complex is located at the bottom of the cleft between the two domains of SEC3 (Figs. 1 and 2). The zinc atom is coordinated by Asp-83 from a beta-strand of the small domain and His-118/His-122, both from the loop connecting the two domains (Figs. 1 and 2). Unlike the SEC3 structure in the P1 space group where a water molecule acts as the fourth ligand (results not shown), the fourth ligand in P4_22 space group is provided by Asp-9 from the neighboring molecule, thus forming a dimer (Fig. 2). The zinc sits in the center of these residues ~0.80 Å above the plane formed by any three liganding atoms. Standard metal-ligand

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2 Y.-I. Chi, G. A. Bohach, and C. V. Stauffacher, unpublished observations.
distances taken from the structures refined at high resolution are 2.0–2.3 Å (38). The distance between the zinc atom in SEC3 and OD1(Asp-83), ND1(His118), NE2(His122), and OD1(Asp9) is 2.17, 2.22, 2.20, and 2.22 Å, respectively, well within these standard distances. The two histidines also adopt different tautomeric conformations in binding the zinc in the toxin structure. His-118 in SEC3 binds to zinc through ND1 (\textit{syn}) and His-122 through NE2 (\textit{anti}). The formation of the dimer (Fig. 2) buries 590 Å² of solvent-accessible surface per monomer, and the noticeable makeup of the dimer interface is the presence of two salt bridges between Lys-37, Lys-56 from one molecule and Asp-10, Glu-16 from a neighboring molecule, respectively. Because no divalent metal ions were added to the crystallization medium, this zinc atom is believed to be intrinsic, and the homodimerization does not appear to be an artifact of the crystal environment. The same zinc-mediated dimerization was observed in the SEC2 crystal structure (14) that was grown under different conditions. A similar zinc-induced homodimerization has been also observed in human growth hormone (39).

Alteration of Any of the Three Residues Abrogates Zinc Binding—Native SEC1 and each of the five mutant proteins were assessed for ability to bind zinc using ICP MS as described above. Similar to results reported for SEC2 (14) the molar ratio of zinc/SEC1 was less than 1.0. Specifically, a mean molar ratio of 0.42 was obtained from multiple replicate experiments, indicating that less than 50% of the SEC1 molecules contained a bound zinc atom (Fig. 3). Analysis of the mutants showed that zinc binding required the presence of all three native residues at positions 83, 118, and 122. The amount of zinc in purified single or double site mutants was dramatically reduced and similar to background levels detected by ICP MS.

Zinc Binding Is Not Required for in Vivo Toxicity—SEs are able to cause either food poisoning or toxic shock syndrome, depending on the route of exposure to the toxins (1). Mutants SEC1(D83A), SEC1(H118A), SEC1(H122A), SEC1(D83A/H118A), and SEC1(D83A/H122A) were tested for their ability to induce emesis in monkeys and to assess the potential requirement for zinc in inducing food poisoning. SEC1 or mutants were suspended in 0.9% NaCl and administered into the stomach of young adult pigtail monkeys using standard techniques (see above). Each mutant induced emesis in two animals when administered at a standard screening dose consisting of 10 µg/kg body weight.

Demonstration of fever and lethality in a rabbit model is considered an indication of SE potential for inducing TSS (40). Several doses (0.01–10 µg/kg) of SEC1 toxin or mutant toxins

![Fig. 3. Zinc binding by SEC1 and mutants.](http://www.jbc.org/)

![Fig. 4. Lethality and pyrogenicity of zinc binding mutants in a rabbit TSS model.](http://www.jbc.org/)
were administered to New Zealand White rabbits via intravenous injection. After 4 h, during which their rectal temperatures were monitored, a sublethal dose (10 μg/kg) of *S. typhimurium* endotoxin was administered via the intravenous route, and the animals were monitored for 48 h. The results summarized in Fig. 4 clearly indicate that zinc binding by the toxin is not required for induction of lethal shock by SEC1 in this assay. SEC1 and most of the mutants were lethal at doses as low as 0.1 μg/kg. One mutant SEC1(D83A) had potency identical to that of native SEC1 in this assay and induced lethality in 100% of the animals at a low dose of 0.1 μg/kg. SEC1(H122A) was slightly less potent than SEC1 in this assay. However, it is unlikely that this reduced toxicity was attributed directly to loss of zinc binding because the SEC1(D83A) mutant had no significant levels of bound zinc but had a toxicity similar to that of the native toxin. None of the mutants were lethal at the lowest dose tested (0.1 μg/kg) and were not statistically different from native SEC1 in this regard (p > 0.9999).

Interestingly, alteration of the zinc binding residues dissociated lethality and fever induction. Every mutation generated a protein with a significantly reduced pyrogenic response compared with SEC1, despite having minimal or no effect on lethality. Even SEC1(D83A), which had a lethal potency identical to that of SEC1, induced a dramatically reduced fever response compared with SEC1 at all the doses tested.

**Zinc Binding Is Not Necessary for T Cell Proliferation by SEC1—Cation binding by SEA and SEE was initially observed because of their requirement for zinc in induction of T cell proliferation (41). Therefore, it was important to determine whether zinc plays a similar role in SEC-induced T cell proliferation, and the ability of the mutants generated in this study to induce proliferation of human lymphocytes was evaluated.

The differences between the dose-response curves for SEC1 and each of the substitution mutants were only minimal (Fig. 5). In all cases, maximum proliferation was with a toxin dose of 1 ng. One substitution H118A caused a minor, albeit reproducibly stronger, proliferative response at lower doses compared with SEC1. SEC1(D83A) consistently produced a reduced mitogenicity dose response curve. Interestingly, the reduced mitogenicity induced by the D83A substitution could be restored by introducing a second Ala substitution for His at either position 118 or 122. Both double mutations containing D83A induced levels of T cell proliferation equal to or above that for SEC1 in lymphocyte proliferation assays.

**Conformational Alterations Induced by the Mutations**—Initially, each mutant was determined to be indistinguishable from the native toxin in immunological analyses and produced a line of identity with SEC1 when analyzed by immunodiffusion assays (26) (results not shown). Furthermore, the retention of biological activity by all of the mutants generated in this study also suggested that the native SEC1 structure is not grossly affected by either the lack of a bound zinc atom or the different properties of the Ala residue substituted for any three zinc binding residues. Strong evidence for this is that induction of emesis in the primate model is expected to require unique native S.E. structural features that allow these toxins to resist degradation by the harsh conditions in the gastrointestinal tract.

To extend these results, two protease treatment methods were used to assess the extent of minor conformational alterations induced by the mutations in SEC1 generated in this study. First, there was no major alteration in tryptic susceptibility of any of the five mutants tested (compared with native SEC1) when exposed to proteolysis by trypsin. The well characterized major trypsin sensitive sites (42) in native SEC1 were unaltered in regard to rate of hydrolysis, and the profiles of expected tryptic products generated were identical to those generated from SEC1 (Fig. 6). The general conclusion from these experiments was that the zinc atom does not provide a significant degree of conformational stability to SEC1.

However, further analysis revealed a reduced resistance to pepsin for some of the mutants. Two mutants with single Ala substitutions for either His-118 or His-122 demonstrated remarkable resistance to degradation by pepsin, a characteristic typical of all SEs. Like native SEC1, these two mutant toxins were stable for at least 24 h in the presence of pepsin under the conditions employed in this study (Fig. 7). In contrast, the three mutants having alterations at position 83 degraded at a rate that was significantly more rapid than native SEC1. Because all five mutants generated in this study were equally deficient in zinc, the instability resulting from the D83A mutation is not likely to be the result of absence of the cation. Instead, it is probably attributed to the inability of Ala to functionally substitute for Asp at this position and completely preserve the native SEC1 structure.

**Zinc Mutants Lack the Ability to Form Toxin Dimers**—In the presence of 10 mM ZnCl₂, elution of SEC1 in gel filtration experiments occurred in two distinct peaks, corresponding in size to monomeric and dimeric forms of the toxin (Fig. 8A). Consistent with earlier experiments showing that less than half of the SEC1 molecules bound zinc, approximately one-third of the native toxin eluted from the gel in an elution volume consistent with the size of a dimer. The remainder...
eluted at a point in the profile consistent with a protein of ~27,500 daltons, the size of monomeric SEC1. In contrast, the gel elution profile of the double mutant SEC1(D83A/H122A) contained only one peak of protein eluting in a volume consistent with the size of a monomer.

Results from electrophoretic analyses supported gel filtration findings. Both SEC1 and the SEC1(D83A/H122A) mutant resolved as single bands on denaturing SDS-PAGE (Fig. 8B).

However, SEC1 applied to native PAGE (Fig. 8B) formed two bands having intensities consistent with the protein peaks observed in gel filtration profiles. One protein band, which comprised approximately one-third of the sample in the gels, represented the dimer while the remaining two-thirds represented the monomer. In contrast, SEC1(D83A/H122A) appeared as a single monomeric band in native PAGE.

**DISCUSSION**

The crystal structure of SEC3 possessed a structural architecture common to the SEs. In particular, it shares a high degree of structural identity to SEC2 (root mean-squared deviation value of 0.34 Å for backbone atoms when superimposed). It also contained a dimerization-mediating zinc binding site as seen in the SEC2 structure that is different from other members of bacterial SAgs.

The low content of zinc in SEC solutions measured by ICP MS and the two peaks in the gel filtration elution profile indicated that the monomeric and zinc-mediated dimeric SECs are in equilibrium. This is consistent with the fact that SEC3 was crystallized into two different space groups where one space group (P1) exhibited the crystal packing made of a monomer and the other space group (P4_2_2_2) showed the dimeric packing (present structure). Furthermore, abrogation of zinc atom binding by site-directed mutagenesis resulted in minimal disruption of the protein conformation, protein stability, and biological activity.

In living systems, zinc is important in catalysis, gene expression, and immune function (38). In addition, zinc can stabilize the structure of some proteins and nucleic acids, preserve the integrity of subcellular organelles, and participate in transport processes (43). Zinc atoms associated with protein molecules serve two general roles. Structural zinc atoms such as those in zinc finger motifs are typically coordinated by cysteines or histidines, while those having a functional role within a catalytic site are usually coordinated by aspartates, glutamates, or histidines (43). Because the one found in SECs is held by two histidines and one aspartate and resembles the configuration of the catalytic site zinc in thermolysin, it was natural for us to first speculate the zinc had a functional catalytic role. In fact, zinc-mediated catalytic activity is inherent to several bacterial toxins. For example, botulinum and tetanus neurotoxins (44, 45) are proteases that exert their catalytic activity through a zinc atom. Similar to the SECs, the zinc atom in these toxins is coordinated by canonical glutamate and two histidine residues. The anthrax lethal factor also binds zinc through a thermolysin-like motif (46).

However, no absolute function could be attributed to the zinc in the SECs by these present studies. To date, the only functional role for zinc in SEs has been found in the subgroup containing SEA, SED, SEE, and SEH. The zinc atoms in these SEs and related toxins have been suggested to mediate their complex formation with MHCII for the SAg activity. In addition, this function has been proven by the crystal structures of the SEH-MHCII complex (47) and the SpeC-MHCII complex.

**FIG. 6.** Trypsin sensitivity of SEC1 and zinc binding mutants. Each protein (100 μg/ml) was incubated (37 °C) with trypsin type XI (100 ng/ml) as described in the text. At selected time points, aliquots (10 μl) were removed, boiled in SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

**FIG. 7.** Relative liability of native SEC1 and mutants to proteolysis by pepsin. Each toxin or mutant (100 μg/ml) was incubated (37 °C) with pepsin (50 μg/ml) in a final volume of 100:1 (100 mM sodium acetate buffer pH 4.5). At selected time points, aliquots (10 μl) of the digestion mixture were removed and resolved by SDS-PAGE. Control lanes contain toxin or mutant removed from reaction mixtures exposed to identical conditions in the absence of protease.
with MHCI is not known at the moment, a similar binding mode is expected from the sequence homology and similar TCR Vβ specificity with SEB (50). Thus, these findings establish SECs along with SEB as another subgroup of SEs in which the MHCI binding does not require zinc.

There has been evidence suggesting that multimeric interaction of some superantigens with MHCI and TCR enhances their toxicity (51). Although the precise implication of SEC homodimerization will require additional future investigations, our data suggest that the zinc-mediated homodimerization has a minimal effect on its function. In addition a catalytic activity also does not appear to be associated with SEC activity because formation of its homodimer should likely block its putative substrate binding site. These findings are consistent with the fact that the related SEB lacks this zinc binding site but still retains SAgs and emetic activities. Therefore, it appears that the zinc atom in SECs represents a non-functional heavy atom in an exotoxin group that has diverged from related bacterial toxins containing crucial zinc atoms. Its presence may reflect evolutionary divergence from a protein in which zinc had a catalytic or structural role may be purely circumstantial.

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