The previously determined crystal structure of the superantigen staphylococcal enterotoxin C2 (SEC2) showed binding of a single zinc ion located between the N- and C-terminal domains (Papageorgiou, A. C., Acharya, K. R., Shapiro, R., Passalacqua, E. F., Brehm, R. D., and Tranter, H. S. (1995) Structure 3, 769-779). Here we present the crystal structure of SEC2 determined to 2.0 Å resolution in the presence of additional zinc. The structure revealed the presence of a secondary zinc-binding site close to the major histocompatibility complex (MHC)-binding site of the toxin and some 28 Å away from the primary zinc-binding site of the toxin found in previous studies. T cell stimulation assays showed that varying the concentration of zinc ions present affected the activity of the toxin and we observed that high zinc concentrations considerably inhibited T cell responses. This indicates that SEC2 may have multiple modes of interaction with the immune system that are dependent on serum zinc levels. The potential role of the secondary zinc-binding site and that of the primary zinc-binding site as Zn I). Here we report a third crystal form (form III) of this toxin grown in the presence of 2.5 mM zinc acetate. The structure revealed the binding of a second zinc atom some 28 Å away from the primary zinc-binding site as Zn I). The crystal structures of SEC2 in complex with a TCR Vβ/H18528/SEC2/MHC complex are considered, and the possibility that zinc may regulate the activity of SEC2 as a toxin facilitating different T cell responses is discussed.

A number of toxins produced by Staphylococcus aureus and Streptococcus pyogenes, known as “superantigens,” are potent inducers of T cell activity. In contrast to conventional antigens, superantigens bind to MHC1 class II molecules on antigen-presenting cells outside the antigen binding cleft and are presented as unprocessed proteins to T cell receptors carrying particular Vβ chains. Very low concentrations of superantigens are able to activate a large amount of resting T cells, thereby inducing massive cytokine release (1, 2), which may result in acute systemic illness and clinical shock (3). Members of the superantigen family include the staphylococcal enterotoxins (SEs) A, B, C, D, E, and H, toxic shock syndrome toxin-1 (TSST-1), and streptococcal pyrogenic exotoxins (Speps) A, B, and C. Amino acid sequence comparison shows that the staphylococcal enterotoxins fall into two main groups with SEA, -D, -E, and -H comprising one group and SEB and the SECs, the second group. The SECs can be classified further into at least three serotypes (C1 to C3) depending on minor epitope differences. TSST-1 shows little sequence homology (less than 28%) with the SEs (4), but it is more similar to SpeC. Crystallographic studies have led to the elucidation of the crystal structures of SEA (5), SEC2 (6), SEB (7), TSST-1 (8), SED (9), and SpeA1 (10) and have revealed a common fold shared among the superantigenes. Moreover, the crystal structures of TSST-1 and SEB in the presence of a MHC class II molecule and SEC2/SEC3 in complex with a TCR Vβ chain (11) have been reported. From these studies, it is now well established that despite sequence and structural similarity, each superantigen appears to have adopted a different mechanism in forming the MHC-superantigen-TCR tri-molecular complex. The presence of one (SEA, SEC2, SpeA1) or two (SED, SpeC) zinc-binding sites plays a central role in this diversity.

The crystal structure of SEC2 has been determined previously in two different space groups: tetragonal P4_3212 (crystal form I (6)) and monoclinic P2_1 (crystal form II (12)) at 2.0 and 2.7 Å, respectively. A striking difference between these two SEC2 structures is the presence of a zinc-binding site found in crystal form I but not in crystal form II (we refer to this primary site as Zn I). Here we report a third crystal form (form III) of this toxin grown in the presence of 2.5 mM zinc acetate. The structure revealed the binding of a second zinc atom some 28 Å away from the primary site (we refer to this secondary site as Zn II). It is shown that the presence of zinc ions in the crystallization medium results in conformational changes and the formation of a secondary zinc-binding site close to the normal MHC-binding site of the toxin. In addition, the recent crystal structures of SpeC and SEH in complex MHC class II molecules via their zinc-binding sites have demonstrated that a single zinc ion can act as a high affinity binding site for MHC class II molecules (13, 14). Thus, the presence of a secondary zinc site.
may have implications for the binding of the MHC class II molecules, suggesting different mechanisms of T cell activation under variable zinc concentrations, which might be linked to the function of SEC2. Moreover, it could guide the efforts to control the effects of enterotoxins on the immune system by controlling the zinc concentrations.

**EXPERIMENTAL PROCEDURES**

**T cell Stimulation**—Peripheral blood mononuclear cells were obtained from healthy donors and isolated by Ficoll density gradient centrifugation. The buoyant layer was removed, and following several washes in phosphate-buffered saline to remove platelets, purified resting T cells were isolated as described previously (15). T cells were stimulated by the addition of HLADR4-C8D8 transfectedants the presence or absence of 100 ng/ml of SEC. Assays were carried out at a range of concentrations of ZnCl₂ or MgCl₂ as indicated. 1

| Table I | Data processing and refinement statistics |
|---------|----------------------------------------|
| **Data set I** | **Data set II** |
| Unit cell a = 42.50, b = 44.54, c = 131.65 (Å) | 40.0–2.0 |
| Space group P2₁2₁2₁ | 0.8835 |
| Resolution range (Å) | 0.9199, 1.1418 |
| Wavelength (Å) | 91.598 |
| No. of measurements | 10,054 |
| No. of unique reflections | 95.8 (91.7) |
| Overall completeness (%) | 7.1 (19.0) |
| Resolution range used in refinement | 40.0–2.0 |
| No. of reflections used in refinement | 16,364 |
| R cryst (%) | 19.82 |
| R free (%) | 22.54 |
| r.m.s. deviation | 0.006 |
| Bond lengths (Å) | 2.06–2.0 |
| Angles (°) | 1.18 |
| B-factors (Å²) | 20.7 |
| Wilson | 26.04 |
| Main chain | 26.78 |
| Side chain | 13.90 |
| Zn I | 20.68 |
| Zn II | 30.9 |

ρ° Outermost shell, 2.49–2.4 Å. ρ° Outermost shell, 2.06–2.0 Å.

1 Refinement was carried out with the 2.4 Å free data set with an initial R cryst and R free (5% of the reflections set aside (20)) of 32.8 and 33.9%, respectively. 2 An unambiguous solution was obtained in 0.1 Å steps using the simulated annealing protocol in X-PLOR (version 3.851 (21)). Reflections flagged for R free were used as a control and did not contribute putative active sites. 3 Refinement was carried out with the 2.4 Å data set with an initial R cryst and R free (5% of the reflections set aside (20)) of 32.8 and 33.9%, respectively.

**RESULTS AND DISCUSSION**

**Quality of the Structure**—The final model, comprising 1851 protein atoms, 77 water molecules, and two zinc ions, has a

**Zinc Binding to Superantigen-SEC2**

**Data processing and refinement statistics**

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**Quality of the Structure**—The final model, comprising 1851 protein atoms, 77 water molecules, and two zinc ions, has a
crystallographic R-factor ($R_{\text{cryst}}$) of 19.82% and an $R_{\text{free}}$ of 22.54%. The r.m.s. deviation in bond lengths and bond angles is 0.006 Å and 1.18°, respectively. Examination of the Ramachandran plot with PROCHECK (24) showed 90.6% of non-glycine and non-proline residues to be in the most favored regions and none in the disallowed regions. Details of data processing and refinement statistics are shown in Table I. The mean coordinate errors derived from a plot of sigmaA versus (sinθ/λ)$^2$ is 0.22 Å. Residues 98–106 from the disulfide loop have been assigned an occupancy of 0 due to high mobility. The unique feature of the SEC2 structure presented here is the presence of two zinc-binding sites. The primary zinc-binding site was reported previously as a strong feature of the difference electron map at 9σ level. The secondary zinc-binding site (reported here) is some 28 Å away from the primary site. Both zinc ions are characterized by low temperature factors (13.9 and 20.68 Å$^2$) with the former one having similar temperature factor as that reported in crystal form I (6).

Description of the Structure—The structure of SEC2 is similar to that of other superantigens of staphylococcal or streptococcal origin (25, 26). Briefly, it consists of two domains, an N-terminal and a C-terminal one (Fig. 1A). The N-terminal domain comprises a β-barrel known as the oligosaccharide/oligonucleotide binding fold (OB fold). At the top of the barrel, a disulfide bridge is formed by residues Cys-93 and Cys-110 between strands β4 and β5. The loop linking the disulfide bridge is highly flexible, a characteristic feature common among all the staphylococcal and streptococcal superantigens with the exception of TSST-1 and SpeC. This loop cannot be defined with a definite single conformation, and it is most likely able to adopt different conformations. The C-terminal domain has an altered β-grasp motif (a β-sheet packed against an α-helix) structure. In superantigens, this helix is a long central helix (α5) packed diagonally across the molecule. Strands β6, β10, and β12 are parallel to each other and antiparallel to β7 and β9. The N-terminal tail consists of two 310 helices (αN1 and αN2) located on top of the C-terminal domain, and thus it is considered part of this domain.

Comparison with Other SEC2 Structures—Superposition of the three crystal forms of SEC2 shows an r.m.s. deviation in Cα atoms of 0.21 and 0.73 for form I–form III and form II–form III, respectively (Table II and Fig. 2A). Residues from the highly flexible disulfide loop were excluded from the calculations because their position is ambiguous. Regions that deviate most include the residues at the N- and C-termini, residues 17–21, 54–58, residues flanking the flexible disulfide loop, and residues 122–130.

Zn I Site—This zinc-binding site, site Zn I, is analogous to that found in crystal form I (6), which is located in a solvent exposed region of the protein at the stretch connecting the N- and C-terminal domains at the bottom of the molecule. In both SEC2 structures the zinc ion is tetrahedrally coordinated to His-118, His-122, Asp-83 from one molecule and Asp-9 (from a symmetry-related molecule in the crystal lattice). The temperature factor for the zinc ion is 13.90 Å$^2$. No zinc site was found in crystal form II, probably because of the removal of the zinc ion after extensive dialysis before crystallization (12). The r.m.s. deviation of main-chain atoms in this region ranges from 0.33 to 0.62. When compared with the zinc free SEC2 structure, the side-chain atoms of crystal form I Asp-9, Asp-83, His-118, and His-122 have an r.m.s. deviation of 0.9–5.6, 0.38–2.1, 0.43–1.0, and 0.56–0.58 Å$^2$, respectively.

Zn II Site—The secondary zinc-binding site in SEC2 (Fig. 1B) was found after co-crystallization of SEC2 with 2.5 mM zinc acetate. This site involves two ligands from one molecule (His-47 and Glu-71) and another two from a symmetry-related molecule (Glu-119, Glu-80). For this site to be created, His-47 had to adopt a different conformation by a swing of about 90° from its original position (Fig. 1C). Moreover, Glu-71 had to
Zinc Binding to Superantigen-SEC2

| Space group | Orthorhombic | Tetragonal | Monoclinic |
|-------------|--------------|------------|------------|
| Cell dimensions (Å) | 42.54 × 44.69 × 131.42 | 43.05 × 43.05 × 290.00 | 43.43 × 69.92 × 42.42 |
| Molecules/asymmetric unit | 1 | 1 | 1 |
| r.m.s.d. | 2.0 | 2.0 | 2.7 |
| Co | 0.21 | 0.73 |
| Main chain | 0.22 | 0.81 |
| All atoms | 0.55 | 1.79 |
| No. of water molecules | 77 | 71 | |
| No. of zinc ions | 2 | 1 | 0 |

* Residues 96–107 were excluded from the calculations.

The zinc ion is 20.68 Å away from its original position to accommodate the zinc ion. Glu-119 and Glu-80 both move toward the zinc ion (about 3.1 and 2.0 Å shift from their original position, respectively) to form a ligand for the zinc ion. Structure-based alignment of SEC2 with SEB shows the presence of a phenylalanine residue, Phe-47 (SEB), in a structurally equivalent to that of His-47 (SEC2). This indicates that SEB would be unlikely to form a similar zinc-binding site. No other major conformational changes in the region are observed. The B-factor for the zinc ion is 20.68 Å², indicating a well ordered metal ion.

**T cell Activation**—T cell activation assays were carried out to establish the effect of zinc on the T cell stimulatory capacity of SEC2. T cell proliferation assays were therefore carried out either in the presence of a range of zinc or magnesium ion concentrations. The results from these experiments (Fig. 3A) demonstrated a complex pattern of T cell response. Firstly, it was clear that SEC2 stimulation of T cells did not absolutely require zinc and that T cells were stimulated by the toxin in its absence. Secondly, at zinc concentrations between 10–100 nm we routinely observed a small but reproducible decrease in responses, however, in the presence of higher concentrations of zinc (up to 100 µM) T cell responses were enhanced ~1.5-fold on average compared with controls. Most strikingly, however, was the abolition of cell responses in the presence of high levels of zinc. It was notable that at all parallel doses of magnesium, no effect on T cell responses was observed (Fig. 3B). These T cell responses were clearly MHC class II-dependent, as control CHO transfectants did not stimulate significant T cell responses (data not shown). Overall, these data demonstrate that the effect of zinc on T cell responses to SEC2 was concentration-dependent, and at high doses, highly significant inhibition was observed.

**Biological Implications**—Multiple zinc-binding sites in superantigens have been identified previously (9, 13, 27). In the case of SpeC, one zinc site is involved in dimerization of the toxin, the other in binding MHC class II molecules. As such, several possible mechanisms for the interaction of this toxin with MHC II and TCR have been proposed (Fig. 2B).

The role of the Zn I site in SEC2 has been discussed previously (6). It was suggested that this site could be used for the binding of a second MHC class II molecule, away from the normal SEB-like MHC-binding site. Binding could take place at both the generic and Zn I sites or exclusively in only one site depending on the conditions. In support of this argument, MHC class II molecules have previously been shown to bind superantigen zinc sites via His-81 from the β-chain of the MHC molecule (13, 14). The presentation of some superantigens to T cell receptors is enhanced by the binding of specific peptides (28). As the SEB-DR1 crystal structure revealed no contacts between the superantigen and the antigen peptide (in contrast to the TSST-1-DR1 complex), a second MHC-binding site could provide the necessary contacts for the peptide. Interestingly, the zinc mediated SpeC-HLA-DR2 (13) and SEH-HLA-DR1 complexes (14) show extensive contact (approximately one-third of the contact surface area) between the toxin and the MHC class II-associated antigenic peptide.

It has also been suggested that this site may have a structural role, i.e. by providing stability in this region. Nevertheless, the crystal structure of SEC2 without zinc (12) revealed no major conformational changes in this region. A comparison with the corresponding region in SEB (which does not bind zinc) shows B-factors similar to the average B-factor for the protein (7) and no major conformational change compared with SEC2. Other possibilities for the role of the Zn I site could also be considered, for example, the cross-linking of two SEC2 molecules. This possibility was not discussed previously because of the lack of the TCR-SEC2/SEC3 structures at that time (6). Based on the arrangement of the two symmetry-related SEC2 molecules, binding of a TCR molecule to each of them shows no clashes between the two TCR molecules (Fig. 2C). Moreover, binding of an MHC-class II molecule could also be accommodated to each of the SEC2 molecule. Thus, the cross-linking of two SEC2 molecules leads to the formation of two TCR/SEC2/MHC complexes. However direct experimental data to support this model have yet to be obtained.

An SEC2-like zinc-binding site has been found in SEA (29) and SED (9). These two superantigens, however, possess a primary zinc-binding site at their respective C-terminal domains. Biochemical evidence suggests that its role is either to participate in an MHC-class II molecule binding (SEA) or to create homodimers (SED). The role of the SEC2-like zinc-binding site in SEA and SED is still unknown, but some suggestions have been put forward (29, 30).

**The Zn II Site**—The Zn II site is formed in the presence of zinc and is close to the normal MHC-binding site expected for SEC2 based on the DR1-SEB structure (31) and the structural similarity of SEC2 with SEB. Examination of the crystallographic packing suggests it is possible that the Zn II site may have a structural role (i.e. by providing stability in this region). Nevertheless, the crystal structure of SEC2 without zinc (12) revealed no major conformational changes in this region. A comparison with the corresponding region in SEB (which does not bind zinc) shows B-factors similar to the average B-factor for the protein (7) and no major conformational change compared with SEC2. Other possibilities for the role of the Zn I site could also be considered, for example, the cross-linking of two SEC2 molecules. This possibility was not discussed previously because of the lack of the TCR-SEC2/SEC3 structures at that time (6). Based on the arrangement of the two symmetry-related SEC2 molecules, binding of a TCR molecule to each of them shows no clashes between the two TCR molecules (Fig. 2C). Moreover, binding of an MHC-class II molecule could also be accommodated to each of the SEC2 molecule. Thus, the cross-linking of two SEC2 molecules leads to the formation of two TCR/SEC2/MHC complexes. However direct experimental data to support this model have yet to be obtained.

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lies in opposite directions, thus no clashes are observed. However, this model should be interpreted with caution. Taking into account the polarity of the cell membrane, the formation of SEC2-TCR complexes as suggested by the crystallographic crystal packing might be difficult. In which case, the arrangement of the TCR molecules would not correspond to their actual position on the cell surface, and hence binding of only one TCR molecule could have a biological significance.

The presence of zinc-binding sites in superantigens indicates that multiple binding modes to MHC class II molecules are feasible. There appears to be two possible mechanisms of superantigen function regarding the role of zinc in these toxins: A) zinc is involved in MHC binding and B) zinc mediates homodimerization of the toxins. The reason behind this variability is still unclear. The two mechanisms suggested here could allow for enhanced T cell activation by promoting clustering of TCR molecules at the interface between the T cell and the APC prolonging the lifetime of TCR-superantigen-MHC

**Fig. 2.** A, Co-superposition of the three SEC2 structures. SEC2 (present work) is in blue, SEC2 (6) in red, and SEC2 (12) in green. B, different modes of zinc-mediated MHC class II recognition in superantigens. C, model of SEC2-TCR binding based on the crystal packing and the existence of zinc-binding site I.
complex and allowing a more apposite response. It is also possible that it may allow utilization of different subsets of residues from the TCR Vβ. The third explanation is that dimerization in this fashion may serve simply to increase the local toxin concentration to bring about a T cell response mimicking that of conventional antigen presentation (33, 34).

Conversely, these multiple zinc sites may be serving to inhibit the binding of the toxin to MHC class II molecules by the formation of SEC2 homodimers. Recently, work performed by Lavoie et al. (35) using SEA has indicated that in order for superantigenic T cell activation to occur less than 0.3% of the MHC class II molecules must be occupied with superantigen. At higher concentrations of bound toxin, the resulting T cell response is aborted by apoptosis after a few cell divisions. The extra regulation afforded to the toxin by the two zinc sites may allow modulation of its binding to MHC class II i.e. dimerization via the Zn II site reduces its ability to bind to MHC class II molecules and therefore alters the subsequent T cell response.

How the Experimental Data Fit With the Crystallography—
The activity of SEC2 in the presence of different zinc concentrations was tested. It was found that zinc ions activate SEC2 but at concentrations higher than 100 μM, the activity drops. In these experiments, SEC2 samples containing no zinc were used. The increase in activity could be explained by the ability of MHC class II molecules to bind to the Zn I site. It is not known, in this case, if two MHC class II molecules can bind to one SEC2 molecule. A bivalent MHC-binding has been reported for SEA with the identification of SEA(DR1)2 complexes (36).

At elevated concentrations of zinc ions, the normal MHC-binding site may be blocked by the binding of an SEC2 molecule through zinc-binding site II. In this case, only zinc-binding site I would be able to bind an MHC class II molecule, possibly accounting for the significant inhibition of T cell stimulation that we observed. The recent work of Chi et al. (37) has shed new light on these possibilities. Gel filtration experiments demonstrated that SEC1 was indeed able to form zinc-dependent dimers. Mutagenesis studies of the Zn I site revealed that the presence of zinc in this site was not essential for T cell proliferation, consistent with our findings here. Furthermore, mutant toxins with reduced zinc-binding ability retained the ability to induce emesis in adult pigtail monkeys and cause lethal
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