The Co-crystal Structure of Staphylococcal Enterotoxin Type A With Zn$^{2+}$ at 2.7 Å Resolution

IMPLICATIONS FOR MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II BINDING*

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Superantigens form complexes with major histocompatibility complex (MHC) class II molecules and T-cell receptors resulting in extremely strong immunostimulatory properties. Staphylococcus aureus enterotoxin A (SEA) belongs to a subgroup of the staphylococcal superantigens that utilizes Zn$^{2+}$ in the high affinity interaction with MHC class II molecules. A high affinity metal binding site was described previously in SEA co-crystallized with Cd$^{2+}$ in which the metal ion was octahedrally co-ordinated, involving the N-terminal serine. We have now co-crystallized SEA with its native co-factor Zn$^{2+}$ and determined its crystal structure at 2.7 Å resolution. As expected for a Zn$^{2+}$ ion, the co-ordination was found to be tetrahedral. Three of the ligands are located on the SEA surface on a C-terminal domain $\beta$-sheet, while the fourth varies with the conditions. Further analysis of the zinc binding event was performed using titration microcalorimetry, which showed that SEA binds Zn$^{2+}$ with an affinity of $K_D = 0.3$ µM in an entropy driven process. The differential Zn$^{2+}$ co-ordination observed here has implications for the mechanism of the SEA-MHC class II interaction.

Superantigens bind as nonprocessed proteins to major histocompatibility (MHC)$^1$ class II molecules on antigen presenting cells and subsequently activate T-lymphocytes by interactions with T-cell receptors. Superantigen activated T-cells proliferate vigorously, and subsequently T-cell and monocyte derived cytokines are produced in large amounts. The released cytokines contribute to the development of toxin-induced disease processes (for a review see Ref. 1).

The best characterized superantigens are the staphylococcal enterotoxins. Based on sequence similarity, these may be divided into two subgroups: the first consists of staphylococcal enterotoxins A, D, E, and H (SEA SED, SEE, and SEH) and the second of staphylococcal enterotoxins B and C1-C3 (SEB, SEC1, SEC2, and SEC3) (reviewed in Ref. 2). The sequence identity of SEA to other staphylococcal enterotoxins ranges from 25 (SEC1) to 83% (SEE). In addition, SEA, SED, and SEE are all dependent on Zn$^{2+}$ for high affinity binding to MHC class II molecules in contrast to SEB and SEC1–3 that bind MHC class II molecules independently of metal ions (3).

Recently solved crystal structures of the free forms of SEA (4), SEB (5), SEC2 (6), and toxic shock syndrome toxin 1 (7, 8), as well as of the SEB-MHC class II complex (9), have created an understanding for the structural constraints by which superantigens interact with their target receptors. The structure of SEB, bound to a MHC class II molecule, confirmed that the superantigen binds to the $\alpha$-chain of the MHC class II molecule, outside the peptide antigen-binding groove. The more distantly related superantigen, toxic shock syndrome toxin 1, binds in a fashion similar to that of SEB, although it covers a larger area on the receptor and in addition utilizes a bound peptide antigen in the interactions (10).

Site-directed mutagenesis of SEA confirmed that co-ordination of Zn$^{2+}$ is required for high affinity binding to MHC class II molecules. It was also shown that SEA most likely binds bivalently to both the $\alpha$- and the $\beta$-chain, of two separate MHC class II molecules, utilizing a surface corresponding to the site previously defined in SEB in the first case and the Zn$^{2+}$ binding site in the latter (11). SEB in contrast, binds monovalently to only the $\alpha$-chain.

The recently determined crystal structures of the free forms of SEA co-crystallized with Cd$^{2+}$ (SEA-Cd$^{2+}$), and SEC2 revealed a metal binding site in each protein. An octahedrally co-ordinated Cd$^{2+}$ ion in SEA was located on the surface of the $\beta$-sheet of the C-terminal domain, whereas a tetrahedral co-ordination of a Zn$^{2+}$ ion in SEC2 is observed at the interface between the N- and the C-terminal domains.

In this study, the crystal structure of SEA, co-crystallized with its native “co-factor” Zn$^{2+}$ at 2.7 Å resolution is presented and compared with the previously described SEA-Cd$^{2+}$ structure. Further, the Zn$^{2+}$ binding is analyzed using titration microcalorimetry. The biological implications of the mode of Zn$^{2+}$ co-ordination in SEA are discussed with emphasis on metal ion assisted SEA-MHC class II interactions.

MATERIALS AND METHODS

Chemicals and Equipment—If otherwise not stated, all chemicals were purchased from Sigma or Fluka. All protein purification equipment and material were from Pharmacia Biotech Inc.

Cloning, In Vitro Mutagenesis, Expression, and Purification of SEA—SEA used in the protein crystallographic work as well as in the microcalorimetric titration was expressed and purified as described previously (11).

Calorimetric Titrations—The titration microcalorimetric experiments were performed at 30 °C using a titration microcalorimetric 2-ml stainless steel vessel for the multiple channel microcalorimetric system TAM (Thermometric AB, Sweden) (15, 16). Another 2-ml vessel lacking stirring facilities containing 0.8 ml of water was used as a calorimetric reference in the twin microcalorimetric unit. The noise level was estimated to be $\pm 10$ nW. Electrical calibration was performed in connection.
with each experiment, with regard to both energy and time constants of the instrument. The calorimetric vessel was loaded with 900 μl of 30–60 μM SEA. At each titration 10–15 aliquots of 5.5 μl of 1.2 mM ZnCl₂ were added with a 6-min interval between each injection (17) using a Hamilton syringe attached with a hypodermic needle mounted on an automated motor-driven pump. To correct for dilution enthalpies additional dilution experiments of ZnCl₂ in the buffer solution were performed. The SEA solutions were prepared by exhaustive dialyzing of the protein solutions in 20 mM HEPES, adjusted to pH 6.91 at 22 °C giving pH 6.80 at 30 °C. The ZnCl₂ solutions were dissolved in the same buffer solution that the SEA solutions had been prepared in. The concentrations of the proteins were obtained from amino acid analysis.

**Thermodynamic Analysis—**The contributions to the entropy that can be identified are:

\[ \Delta S = \Delta S_{\text{hydr-prot}} + \Delta S_{\text{hydr-Zn}} + \Delta S_{\text{prot-part}} + \Delta S_{\text{ion}} \]  

(1)

\( \Delta S_{\text{hydr-prot}} \) is the change in hydration of the protein when Zn\(^{2+}\) is bound, which is proportional to the change in solvent accessible surface area and is related to the change in heat capacity by \( \Delta C_p = \Delta S_{\text{hydr-prot}} / T_c \), where \( T_c \) is the reference temperature (385.15 K) (18). \( \Delta S_{\text{hydr-Zn}} \) is dominated by the change in hydrophobic hydration (19) and is at this temperature range positive upon dehydration. \( \Delta S_{\text{prot-part}} \) is the change in entropy for transferring the fully hydrated Zn\(^{2+}\)-ion to the protein binding site with additional positive contribution to the total entropy change. This process is analogous to the transfer of Zn\(^{2+}\) from water to a nonaqueous solvent, where the change in entropy ranges between 3060 J (K mol\(^{-1}\)) at 298 K (20). Thus, the transfer of the Zn\(^{2+}\) will significantly contribute to the total entropy change. \( \Delta S_{\text{prot-part}} \) is the entropy contribution due to the change in the degree of conformational freedom. A reduction in conformational degree of freedom will give a negative contribution to the total entropy change. \( \Delta S_{\text{ion}} \) correlates with the change of the number of particles in the system (for 1:1 binding \( \Delta n = -5.8 \) J (K mol\(^{-1}\)), where \( R \) is the gas constant \( 8.314 \) J (K mol\(^{-1}\)). The last term, \( \Delta S_{\text{ion}} \), arises from entropy changes where there is proton linkage (21) and subsequent proton exchange upon binding a ligand to a protein. The sign of this contribution depends on whether there is a positive or a negative proton linkage in the reaction.

**Crystallization of SEA—**SEA was crystallized using vapor diffusion. Crystals in the space group P₃\(^{21}\) were grown by mixing 3 ml of protein solution (10 mg/ml) containing 100 μM ZnSO₄ with 3 ml of 15% (w/v) of polyethylene glycol 6000 in 0.3 M ammonium sulfate and 0.1 M MES buffer at pH 6.25 in a sealed tissue culture 24-well plate (Falcon). The crystallization droplets were equilibrated at +18 °C with 1 ml of the mother liquor for 1–2 weeks to obtain optimal diffraction quality crystals. Crystals were 0.3 × 0.3 × 0.3 mm in size and diffracted to 2.5 Å with a conventional x-ray source. The crystals obtained were difficult to handle using conventional capillary mounting. However, when cryo-genic conditions were applied, the crystals could easily be mounted in cryo-loops after stabilization in the mother liquor with 30% glycerol (v/v) added and frozen directly in the N₂ beam (Oxford Cryosystems).

**Data Collection and Processing—**Data were collected using a MAR image plate system and processed with Mosflm (22) using the rexf algorithm (23) for indexing and point group determination and then further reduced and scaled using the CCP4 program package (22). For crystallographic data see Table I.

**Structure Determination—**At the time this work was initiated, the only superantigen co-ordinates available were those for the SEB-HILA-DRI complex. A modified search molecule for molecular replacement was created where SEB was converted to a polyalanine model except for those residues that were identical in SEA and SEB. The AMORE (24) molecular replacement solution obtained with this modified SEB model was later verified using the SEA-Cd\(^{2+}\) model when these co-ordinates were available. The highest scoring solution in the resolution interval 4–8 Å was found in space group P₃\(^{21}\) with two SEA molecules in the asymmetric unit. A rigid body refinement in X-plor (25) preceded a cyclic process of model building in the program O (26) making corrections for main and side chain differences and Powell minimizations in X-plor using data between 18–2.7 Å. In addition, NCS restraints as well as simulated annealing (27) refinement steps in X-plor were included in the end of the refinement. At this moment, solvent molecules were manually introduced into persistent F_F, densities above 3.0 σ. After three cycles, 132 solvent molecules had been introduced. A final Powell minimization followed by a dynamics run from 2500 to 300 K in 50 ps steps including data between 10 and 2.7 Å was performed. B-value refinement was added as the final step and solvent molecules with high temperature factors (>40 Å\(^2\)) as well as those with absent 2F_F - electron densities at 1 or above were removed leaving 92 solvent molecules in the final model. The free R-value (28) was used to validate the progress of the entire refinement. The quality of the model was assessed using PROCHECK (29), and structural alignments were performed using the least square fit procedure in the program O (28). The co-ordinates of the SEA-Zn\(^{2+}\) structure will be deposited in the Protein Data Bank (Brookhaven National Laboratory, Chemistry Department, Upton NY 11973).

**RESULTS**

**Structure Determination—**The three-dimensional structure of SEA was determined using data from crystals in the space group P₃\(^{21}\) grown at pH 6.25. The initial structure was solved using a modified structure of SEB (co-ordinates kindly provided by Professor D. Wiley); as a search molecule in a molecular replacement procedure and in the final steps of refinement, the SEA-Cd\(^{2+}\) co-ordinates (4) were used. The asymmetric unit contained two SEA molecules, and their structures were refined at 2.7 Å resolution. At the present stage of refinement the SEA model consists of residues 10–233 for both molecules in the asymmetric unit. Furthermore, two Zn\(^{2+}\) ions and 92 well ordered solvent molecules have been included. The bound Zn\(^{2+}\) ions in the asymmetric unit were easily identified as pronounced F_F, densities in the electron density maps. The refined structure at 2.7 Å resolution show a well defined electron density map (Fig. 1). The crystallographical R-factor is 20.6% (R-free 30.2%) in the resolution range 10–2.7 Å.

**The SEA Monomer—**The SEA molecule consists of two closely packed domains and show a topology similar to that observed in other staphylococcal enterotoxin structures. A
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\textbf{\textit{Co-crystal Structure of Staphylococcal Enterotoxin A with Zn\textsuperscript{2+}}}
\end{quote}

\begin{figure}
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Comparison of main and side chain conformations between the two crystal forms of SEA. Comparison of the Ca positions of the SEA-Cd\textsuperscript{2+} (4) (orange) and the current SEA-Zn\textsuperscript{2+} structure (yellow) is shown. Major differences in the main chain of the two protein structures relate to ordered and disordered regions in the N terminus (residues 1–9, ordered in the SEA-Cd\textsuperscript{2+} structure) as well as the loop 59–63 (ordered in the present study). The figure was drawn using Molscript (30) and Raster3D (31).}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{fig3.png}
\caption{The zinc binding site of SEA co-crystallized with Zn\textsuperscript{2+}. A, tetrahedral zinc co-ordination in molecule one (yellow) in the asymmetric unit. Note that the use of His\textsuperscript{61} from the neighboring molecule (cyan) as zinc ligand leads to the loop 59–63, absent in the SEA-Cd\textsuperscript{2+} structure, here becoming ordered. B, tetrahedral zinc co-ordination in the second molecule of the asymmetric unit. The three high affinity SEA ligands are used and in addition a water molecule (H\textsubscript{2}O) is used as the fourth Zn\textsuperscript{2+} ligand. The figures were drawn using Molscript (30) and Raster3D (31).}
\end{figure}

\begin{quote}
\textbf{A \textit{beta-barrel} is comprised in the N-terminal domain (residues 31–116), and a \textit{beta-grasp} motif constitutes the major part of the C-terminal domain (residues 117–233). Nine residues in the N terminus of each SEA molecule lacks electron density in this crystal form, but in the SEA-Cd\textsuperscript{2+} crystal structure packs against the C-terminal domain where it forms a one turn helix (residues 4–7) and covers a partly hydrophobic area on the C-terminal \(\beta\)-sheet around residues Tyr\textsuperscript{229} and Tyr\textsuperscript{231}.}

A detailed description of the SEA structure has previously been published (4), and therefore we will focus this description on major differences between the two crystal forms (Fig. 2). The refined structure reveals an expected close similarity to other structure determined staphylococcal superantigens, with an overall root mean square deviation of 0.74 Å to SEA-Cd\textsuperscript{2+}, 0.89 Å to SED\textsuperscript{2}, 1.53 Å to SEB, and 1.87 Å to toxic shock syndrome toxin 1, comparing 220, 201, 181, and 147 structurally equivalent Ca positions, respectively.

The major difference between the previous SEA-Cd\textsuperscript{2+} structure and the current structure lies in the N terminus; neither molecule in the asymmetric unit shows electron density corresponding to the first 9 residues, thus it appears to be unordered. Furthermore, we observe a tetrahedral metal ion co-ordination in contrast to the octahedral geometry previously found for Cd\textsuperscript{2+}. As a consequence of a zinc mediated protein-protein interaction between the two SEA molecules in the asymmetric unit, the loop 59–63, unordered in the SEA-Cd\textsuperscript{2+} structure, now clearly is visible in one of the molecules and could be included as a polyalanine loop in the other.

\textit{Differential Zn\textsuperscript{2+} Co-ordination—}The Zn\textsuperscript{2+} ion co-ordination differs between the two molecules in the asymmetric unit, although both bound zinc ions are tetrahedrally co-ordinated. His\textsuperscript{187}, His\textsuperscript{225}, and Asp\textsuperscript{227} are conserved zinc ligands, but the fourth ligand differs. In one SEA molecule, His\textsuperscript{61} from the neighboring molecule in the asymmetric unit is used, whereas a water molecule is used in the other (Fig. 3). Thus, a tetrahedral binding of two Zn\textsuperscript{2+} ions in the asymmetric unit is seen. His\textsuperscript{187}, His\textsuperscript{225}, and Asp\textsuperscript{227} as Zn\textsuperscript{2+} ligands have previously been defined by mutagenesis experiments (11) and in the crystal structure of SEA-Cd\textsuperscript{2+} (4). The Cd\textsuperscript{2+} ion in the first SEA structure was octahedrally co-ordinated involving the N-terminal Ser\textsuperscript{1} amino nitrogen and \(\gamma\)-oxygen and a water molecule, in addition to the three high affinity ligands discussed above.

\textit{Biochemical Characterization of Zn\textsuperscript{2+} Binding—}Because we observe that the two SEA molecules in the asymmetric unit are bridged by a Zn\textsuperscript{2+} ion, it indicated that SEA might form Zn\textsuperscript{2+}-dependent dimers similar to what has been observed for SED\textsuperscript{2}. To assess the significance of this observation, gel permeation chromatography on SEA was performed in the presence and in the absence of Zn\textsuperscript{2+}. The protein eluted at an apparent size corresponding to the monomer, irrespective of the addition of
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**DISCUSSION**

Zinc ions are essential for the activity of many enzymes and a structural component in many protein-DNA and protein-protein interactions. A requirement of Zn\(^{2+}\) to obtain the strong affinity between the SEA and MHC class II molecules was first shown by Fraser and co-workers (3). The amino acid residues involved in the coordination of a Zn\(^{2+}\) ion on the SEA surface were identified by site-directed mutagenesis (11, 12). When the residues Phe\(^{47}\), Asn\(^{128}\), His\(^{187}\), His\(^{225}\), or Asp\(^{227}\) were substituted for alanines the ability to induce MHC class II-dependent T-cell proliferation is markedly reduced (11). Because histidines and aspartates are preferred zinc ligands, it was speculated that the lowered bioactivity was due to impaired zinc binding and metal ion-dependent SEA-MHC class II interactions for His\(^{187}\), His\(^{225}\), and Asp\(^{227}\) (and possibly also for Asn\(^{128}\)). A disruption of a SEB-like interaction with the MHC class II molecule \(\alpha\)-chain was expected for the Phe\(^{47}\) to alanine substitution. As shown in the SEA-Cd\(^{2+}\) structure (4), the effects of the substitutions described above could be explained with disruption of the metal binding site. His\(^{187}\), His\(^{225}\), and Asp\(^{227}\) were shown to be direct high affinity zinc ligands, and Asn\(^{128}\) was shown to stabilize the conformation of Asp\(^{227}\) via a strong hydrogen bond. However, a comparison between the SEA-Cd\(^{2+}\) structure and the SEA-Zn\(^{2+}\) structure presented here reveals that the two refined structures are virtually identical, but with one important exception: the co-ordination of the bound metal ion.

In the present structure the metal ion is co-ordinated without involvement of the N-terminal serine. We clearly observe a tetrahedral Zn\(^{2+}\) co-ordination in both molecules of the asymmetric unit. The two independent molecules in the crystal asymmetric unit have their respective metal binding site in different environments. In both molecules the three high affinity Zn\(^{2+}\) ligands are His\(^{187}\), His\(^{225}\), and Asp\(^{227}\). The fourth ligand, however, is His\(^{130}\) of the neighboring molecule in one case and a water molecule in the second. Thus, none of the molecules of the asymmetric unit utilizes the N terminus in Zn\(^{2+}\) co-ordination as observed for SEA co-crystallized with Cd\(^{2+}\). In fact, the N terminus (residues 1–9) is unordered in each of the molecules.

If a Zn\(^{2+}\) ion could be octahedrally co-ordinated as the Cd\(^{2+}\) ion in the SEA-Cd\(^{2+}\) structure, one would expect this situation also in this SEA-Zn\(^{2+}\) structure, at least in the second molecule where no symmetry molecule interactions are observed, instead a water molecule is used as the fourth ligand in tetrahedral co-ordination. However, one possible explanation to this contradiction could be due to different crystallization conditions. Although both Zn\(^{2+}\) and Cd\(^{2+}\) ions could adopt tetrahedral as well as octahedral co-ordination in nonprotein environments, the norm for protein bound Zn\(^{2+}\) ions is tetrahedral (reviewed in Ref. 13).

The thermodynamic properties for the SEA-Zn\(^{2+}\) 1:1 complex are dominated by the large and positive entropy, \(\Delta S_S = 146 \pm 14 \text{ J} \text{ K}^{-1} \text{ mol}^{-1}\). The possibility to perform a rigorous thermodynamic analysis of the process in terms of dissecting the different contributions to the thermodynamic properties is dependent on the available heat capacity data, which at the moment are not available. However, the most likely dominating contribution to the entropy change is \(\Delta S_{\text{hydr-prot}}\), which is the change in hydration of the protein when Zn\(^{2+}\) is bound, and \(\Delta S_{\text{hydr-zn}}\) which is the change in entropy for transferring the fully hydrated Zn\(^{2+}\) ion to the protein binding site. In addition, either positive or negative contributions from \(\Delta S_{\text{mag}}\) the entropy change upon proton exchange when binding a ligand to a protein, can occur. The sign of this latter contribution depends on whether there is a positive or a negative proton linkage in the reaction.

One possible interpretation of these thermodynamic properties is that a reduction in conformational degrees of freedom occurs upon metal binding, subsequently dehydrating hydrophobic surface residues. An ordering of the N terminus upon Zn\(^{2+}\) binding could possibly explain the thermodynamic properties discussed above, although not observed in any of the two molecules in the asymmetric unit of the present crystal structure. In this context it should be stressed that the form of SEA used here is the product from predicted signal peptide processing, whereas SEA purified from its native host *Staphylococcus aureus* is a mixture of this and of two truncated forms lacking three or five N-terminal residues, with the latter two as the major forms. Thus, none of the shorter forms could co-ordinate.
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the metal ion as observed in the SEA-Cd\(^{2+}\) structure. A thermodynamic analysis of such truncated variants of SEA would be invaluable to the interpretation of the biological significance of the differential metal ion binding modes observed in the SEA-Cd\(^{2+}\) and the present structure.

The crystal structure of SEC2 revealed a bound zinc ion in the domain interface region, a metal binding site distinct from the one observed in SEA. The Zn\(^{2+}\) co-ordinating residues were Asp\(^{83}\), His\(^{113}\), and His\(^{122}\) from one molecule and Asp\(^{9}\) from a neighboring molecule in the crystal lattice (6). In contrast to SEA, SED and SEE, SEB/SEC-MHC class II molecule interactions do not require zinc ions. Thus, the most likely explanation for the function of the zinc ion bound to SEC2 is that it serves as an exposed histidine residue, His\(^{81}\) of the neighboring molecule as the fourth Zn\(^{2+}\) ligand. Thus the loop 59–63, that normally is highly mobile, becomes ordered due to the zinc-mediated protein-protein interaction.

Interestingly, in the formation of the SEA-MHC class II complex, three Zn\(^{2+}\) ligands are postulated to be derived from the superantigen and the fourth from the receptor (11). The postulated co-ordinating residue in the MHC class II \(\beta\)-chain is an exposed histidine residue, His\(^{81}\) (14). Thus, the N terminus as oriented in the SEA-Cd\(^{2+}\) structure would have to disengage from the metal ion in order to allow the ligand function of the MHC class II residue. The ligand function of His\(^{81}\) from the neighboring SEA molecule in the asymmetric unit observed in the current structure may thus mimic the SEA-MHC class II \(\beta\)-chain Zn\(^{2+}\)-dependent interaction. Judged from the previous SEA-Cd\(^{2+}\) and the present SEA-Zn\(^{2+}\) co-crystal structure, a model for this interaction could be regarded in which the N terminus of SEA can be utilized in the co-ordination of zinc but is released upon MHC class II molecule interactions. A second option would be that the N terminus is not at all involved in zinc binding. The latter case will most surely exist in vivo where naturally occurring SEA can have three or five residues removed in the N terminus compared with the material used in this study and by Schad and co-workers (4). However, a full understanding of the interactions in the SEA-MHC class II molecule complex will have to await the crystal structure of such a protein complex.

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