The Spectral and Thermodynamic Properties of Staphylococcal Enterotoxin A, E, and Variants Suggest That Structural Modifications Are Important to Control Their Function*

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The superantigens staphylococcal enterotoxin A and E (SEA and SEE) can activate a large number of T-cells. SEA and SEE have approximately 80% sequence identity but show some differences in their biological function. Here, the two superantigens and analogues were characterized biophysically. SEA was shown to have a substantially higher thermal stability than SEE. Both SEA and SEE were thermally stabilized by 0.1 mM Zn$^{2+}$ compared with Zn$^{2+}$-reduced conditions achieved using 1 mM EDTA or specific replacements that affect Zn$^{2+}$ coordination. The higher stability of SEA was only partly caused by the T-cell receptor (TCR) binding regions, whereas regions in the vicinity of the major histocompatibility complex class II binding sites affected the stability to a greater extent. SEA exhibited a biphasic denaturation between pH 5.0–6.5, influenced by residues in the TCR binding regions. Interestingly, enzyme-linked immunosorbent assay, isoelectric focusing, and circular dichroism analysis indicated that conformational changes had occurred in the SEA/E chimerical constructs relative to SEA and SEE. Thus, it is proposed that the Zn$^{2+}$ binding site is very important for the stability and potency of SEA and SEE, whereas residues in the TCR binding site have a substantial influence on the molecular conformation to control specificity and function.

Superantigens (SAgs) such as the staphylococcal enterotoxins (SE) are very potent T-cell-activating proteins known to cause food poisoning or toxic shock (1). SEs bind as unprocessed peptides to MHC class II molecules and activate T-cells displaying certain V$\beta$ regions of the T-cell receptor (TCR) (2, 3). Because the number of V$\beta$-genes is limited, a much larger fraction of the T-cells is activated by SAgs than by normal antigens (2, 4). The strong cytotoxicity induced by these enterotoxins has been explored for cancer therapy by fusing them to tumor-reactive antibodies (5, 6).

Nine different SEs have been identified and these are designated SEA-SEE and SEG-SEJ. The sequence identity of these SAgs with SEA ranges from 20% for SEG to 82% for SEE (described in greater detail in Refs. 7 and 8). The binding to MHC class II of both SEA and SEE is known to be Zn$^{2+}$-dependent (9). Both SEA and SEE have two MHC class II binding sites, one close to the N terminus with low affinity for MHC class II and one close to the C terminus with moderate affinity. These two sites may cooperate and cross-link MHC class II molecules with a higher affinity (10, 11). In addition the ability to cross-link two neighboring MHC class II molecules may stimulate secretion of inflammatory cytokines, such as interleukin-6, interleukin-8 (12), and interleukin-1β (13). One of these interactions is stabilized by a Zn$^{2+}$ ion, coordinated by His-187, His-225, and Asp-227 in SEA (14) and SEA/E chimerical constructs relative to SEA and SEE. Thus, it is proposed that the Zn$^{2+}$ binding site is very important for the stability and potency of SEA and SEE, whereas residues in the TCR binding site have a substantial influence on the molecular conformation to control specificity and function.

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¶ The abbreviations used are: SAg, superantigen; CD, circular dichroism; IgG, immunoglobulin G; MHC class II, major histocompatibility complex class II; SE, staphylococcal enterotoxin; SEA, staphylococcal enterotoxin A; SEE, staphylococcal enterotoxin E; PBS, phosphate-buffered saline; TCR, T-cell receptor; SPA, scintillation proximity assay.
was from Hy-Clone Laboratories Inc. (Logan, UT). Gentamycin sulfate was from Biologische Industrien (Kibbutz Beit hemeek, Israel), and \([\text{H}]\text{thymidine was from NEN Life Science Products.} \) -[4,5-\text{H}]}\text{Leucine for metabolic labeling of cells was from Moravek Biochemicals Inc. (Brea, CA), and SPA-polyvinyl tolune beads were from Amersham Pharmacia Biotech.}

**Thermal Denaturations**—The thermal denaturations were carried out using a HP 8453 UV diode-array spectrophotometer with a heating cell (Hewlett-Packard, Waldbronn, Germany). The spectrum was monitored between 190 and 1100 nm. Before measurement the cuvette was cleansed with 6M guanidine-HCl and Millipore water. SAg solution was added to the cuvette and diluted with 20 mM phosphate buffer to give a final optical density at 280 nm of 0.1 in a final volume of 1 ml. The temperature was increased stepwise by 0.5 °C, and at every temperature the sample solution was left to equilibrate before the absorbance measurement. The temperature interval was 40−75 °C. The denaturation was visualized by plotting the absorbance difference $A_{\text{280}}-A_{\text{280}}$ (18), and the denaturation temperatures were defined as the $E_{\text{C50}}$ value.

**Expression and Purification of SEA/E Chimeras**—The different superantigens were expressed constitutively in the *Escherichia coli* strain UL 635 (xyl-I, ara-I4, T4h, DompT) using a vector (10, 19) with a *staphylococci protein A* promoter, a signal peptide, and a kanamycin resistance gene. In the Fab-SAg vector (5, 6), expression was controlled by a lacUV5 promoter. The SAg's (Table I) were expressed using either shaker flasks or fermenters (Belach Bioteknik, Sweden), whereas the C215Fab-SAg's (Table II) were produced in fermenters when using shaker flasks or bacteria containing the respective production plasmid (Table I) were cultivated for approximately 18 h in 500 ml of 2×YT medium containing 25 μg/ml kanamycin and 12 μg/ml isopropyl β-D-thiogalactoside at 25 °C. The cells, containing the SAg's, were then harvested by centrifugation at 5000 × g for 30 min.

Production of the Fab-Sags in fermenters was carried out essentially as described in Ref. 6. A similar procedure was used for the SAg expression, although no isopropyl β-D-thiogalactoside was added. The fermentations were discontinued after 45 h. Metabolic activity was interrupted by cooling the cultivation to 15 °C, and 1 mM EDTA was added. SAg's were recovered from the medium after fermentation and from the periplasm after shaker flask cultivations.

The periplasmic extraction was carried out by resuspending the cell pellet in 30 ml of 0.5 M Tris-HCl, 1 mM EDTA, 20% sucrose, pH 7.5, and incubating the suspension for 20 min on ice. After centrifugation for 15 min at 9300 × g the pellet was resuspended in 30 ml of 5 mM MgCl₂. The cell debris was removed by centrifugation for 15 min at 6300 × g; the supernatant containing the SEA/E chimeras was filtered to remove remaining debris, and 3 ml of 10×PBS (0.14 M NaCl, 3 mM KCl, 10 mM phosphate buffer, pH 7.4, and 0.05% Tween 20) were added to the SEA/E solution, which was stored at −20 °C until purification.

The periplasmic supernatant was diluted with an approximate 2 millisieemens/cm, adjusted to pH 5.0, and applied on a HiLoad 26/100 SE-phosphate column (Amersham Pharmacia Biotech). The sample was eluted with a linear gradient from 20 mM to 500 mM ammonium acetate, pH 6.0, with 0.02% Tween 80 over 30 min and a flow rate of 5 ml/min. To wash the column between samples, 30 ml of 1 M NaOH was used. The SAg-containing fraction was diluted to approximately 2 millisieemens/cm, adjusted to pH 5.0, and applied on a 1-ml Resource-S column (Amersham Pharmacia Biotech). The sample was eluted with an ammonium acetate buffer gradient from 20 mM to 500 mM, pH 5.0, with 0.02% Tween 80 over 30 min and a flow rate of 1 ml/min. To wash the column between samples, 2 ml of 1 M NaOH was used. The purified SAg was concentrated to at least 0.5 mg/ml using ultrafiltration (Centriprep 10, Amicon Inc., Beverly, MA).

The Fab-Sags were purified using protein G affinity chromatography and ion-exchange chromatography (6). Typical yields for the SAg chimera were 5 mg/l in shaker flasks and 50 mg/l or more in fermenters. The yield for the Fab-Sags in fermenters was typically 50−400 mg/l.

**Analytical Procedures**—SDS-polyacrylamide gel electrophoresis was carried out using precast 10−15% polyacrylamide gradient gels in the Phast System (Amersham Pharmacia Biotech) and isoelectric focusing with gels ranging from pH 3 to 9. For staining, Coomassie Blue was used. The purity of the samples was visually estimated from the SDS-polyacrylamide gel electrophoresis. The protein concentration of the product solutions was determined with a UV-spectrophotometer (Hewlett-Packard) at 280 nm. Mass spectrometry was carried out on a LASERMAT 2000 laser desorption/ionization mass spectrometer (FINNIGAN MAT Ltd, Hemel Hempstead, United Kingdom) with a matrix consisting of 10 mg/ml sinapinic acid (20) in 30% acetonitrile with 0.1% trifluoroacetic acid.

**CD Measurements**—Both the far UV and near UV CD spectra were recorded in a 20 mm NaH₂PO₄ buffer, pH 6.0, using a Jasco J720 (Japan Spectroscopic Co. Ltd., Hachiogi City, Japan). The path length of the cuvet used for far and near UV measurements were 0.1 and 1 cm, respectively. The concentration of the protein solutions used for near UV CD was approximately 0.8 mg/ml and for far UV CD it was approximately 0.2 mg/ml (21). Corrections were made in the CD spectra for concentration differences between samples.

**Enzyme-linked Immunosorbent Assay**—*In vitro* experiments were carried out using the IgG pool at different concentrations in 3% fat-free dry milk in PBS-Tween, and was added and incubated for 2 h. As a negative control, 100 μl of 0.5 μg/ml C215Fab was used. The final serum was then carried out using either human or rabbit antibodies. Using human antibodies, 10 μl of purified IgG pool at different concentrations in 3% fat-free dry milk in PBS-Tween was added to the wells and incubated for 2 h, and 100 μl of 1.66 μg/ml horseradish peroxidase-labeled goat anti-human IgG (γ-specific) were added and incubated for 1 h. Alternatively horseradish peroxidase-labeled rabbit anti-SEA at different concentrations, diluted in blocking solution, was added and incubated for 2 h. The plates were developed with the Sigma Fast™ OPD peroxidase substrate tablet as recommended by the supplier. Between each step of the assay the wells were washed four times with PBS-Tween.

**T-Cell Proliferation**—The growth medium used was RPMI 1640 with 10% fetal calf serum, 50 μM 2-mercaptoethanol, and 0.1 mg/ml gentamycin sulfate. Spleen cells from C57B1/6 mice were obtained in house as a suspension in growth medium. The cells, 2 × 10⁶/ml well, were determined by counting in Burker chambers using trypan blue viability staining, were incubated in 96-well flat bottomed plates (Nalgene Nunc International, Denmark). The SAg's were used in triplicates in the concentration interval 0.001−100 μM. After incubation for 3−4 days, the cells were pulsed with 10 μl of [2H]thymidine (0.05 μCi/ml), and the DNA of the cells was harvested after 4 h with a Micro Cell Harvester (SKATRON, Dølasletta, Norway). The radioactivity of the sample was measured with a 1205 Betaplate Liquid Scintillation Counter (Wallac Sverige AB, Sollentuna, Sweden).

**Scintillation Proximity-based Binding Assay**—Chinese hamster ovary cells transfected with human CD80 and HLA-DR4 were cultured in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, 0.1 mg/ml gentamycin sulfate, and 1 mM 2-methionine sulfoximine. CHO-CD80-DR4 cells, resuspended to a density of 5 × 10⁶ cells/ml in l-leucine-deficient (Leu−) medium (l-leucine-free RPMI 1640 medium supplemented with 2 mg/ml n-glucose, 0.24 mg/ml l-arginine, 0.035 mg/ml iso-inositol, 0.015 mg/ml l-methionine, 0.596 mg/ml disodium cysteine, 0.01 mg/ml l-leucine, 2% (v/v) fetal bovine serum, 1 mg/ml gentamycin sulfate, and 1 mg/ml 2-methionine sulfoximine), were labeled overnight at 37 °C in a CO₂ incubator with 0.2 μCi of l-[4,5-3H]leucine/10⁶ cells. The next day, [3H]CHO-CD80-DR4 cells were resuspended to a density of 3 × 10⁵ cells/ml in assay buffer (Hank's balanced salt solution without phenol red supplemented with 25 mg/ml HEPES, pH 6.8, and 1% (w/v) bovine serum albumin).

The affinity was estimated by calculating half-maximal binding ($E_{1/2}$) from the saturation curve. The C215Fab moiety was used to attach the C215Fab-SEs to the SPA beads coated with anti-mouse antibodies. Fifty microliters of C215Fab-SE (at different concentrations) and 50 μl of anti-mouse SPA-polyvinyl tolune beads (40 mg/ml in assay buffer without bovine serum albumin) were mixed for 1 min in a microtiter plate (OptiPlate, Packard, Greve, Denmark), covered with a plastic film, and incubated for 30 min to 2 h at 4 °C (depending on the time needed for the preparation of cells). The 3H-labeled beads were added to the microtiter plate with preincubated C215Fab-SE-SPA beads at a density of 1.5 × 10⁶ cells/50 μl/well. The plate was sealed by a plastic film, mixed for 1 min on an orbital shaker platform, and incubated in the dark for 8 h at room temperature. The radioactivity in each well was measured during 5 min/well using a β-top counter (Packard). As a negative control, C215Fab was used.

**RESULTS**

**Denaturations of SEA and SEE**—To investigate the stability of SEA and SEE and understand how it depends on variables such as pH, salt concentration, and various additives, thermal
Denaturations were carried out using a UV spectrophotometer with a heating cell and a diode array detector. The sample consisted of 0.1 mg/ml SAg dissolved in 20 mM phosphate buffer of varying pH. At denaturation the UV absorbance spectra are changed, because buried residues become exposed. The denaturation was monitored by measuring the difference in absorbance at 286 and 264 nm, because drastic changes in the absorbance difference of these two wavelengths occur upon denaturation (18). After denaturation, the SAgs usually precipitated.

The melting points for SEE were generally 5–15 °C higher compared with SEA. Notably, a biphasic denaturation was observed for SEE between pH 5.0 and pH 6.5, but at a higher pH the denaturation was monophasic (Fig. 1). A possible biphasic denaturation was observed for SEA at pH 5.0. At pH 4.0–5.0 for SEA and 4.0 for SEE, the melting was less distinct suggesting unfolding in a more complex way. At pH 4.0, both SEA and SEE remained soluble after denaturation while precipitating at the other pHs.

Denaturations were also carried out with SEA and SEE at pH 4.0–9.0 with the addition of 150 mM NaCl, which substantially reduced the stability of both SEA and SEE at lower pH by approximately 6 °C, while increasing the thermal stability of SEE at higher pH with 2–5 °C (data not shown).

To see whether SEE could obtain its original structure from the partially unfolded state, the temperature gradient was stopped there, and the sample slowly cooled. However, during this procedure most material precipitated.

Denaturations of SEA and SEE in guanidine-HCl monitored by far and near UV CD measurements showed that the tertiary structure unfolds with midpoints at 1.52 M for SEA and 1.96 M for SEE (Fig. 2). The secondary structure for SEA and SEE is lost at 5.10 and 4.14 M, respectively (Fig. 2). Thus, at pH 6.0 the tertiary structure of SEE is more resistant than SEA to chemical denaturants, whereas the opposite is true for the secondary structure.

In conclusion SEE has a substantially more stable structure than SEA, and the melting points differ up to 15 °C. At a lower pH the denaturation of SEA and SEE was less distinct.

**Zn**

**Dependence of the Thermal Stability of SEA and SEE**—To investigate the importance of Zn** binding for thermal stability of SEA and SEE, denaturations were made with buffers containing 1 mM EDTA to bind Zn** ions or buffers containing 0.1 mM ZnCl**. Compared with conditions with no Zn**, accomplished by the addition of 1 mM EDTA, the increase in melting points for SEA with Zn** addition was up to 6 °C (Fig. 1). The stabilizing effect of Zn** for SEA was larger at pH 6.0 compared with pH 7.0, but for SEE (Fig. 1) it was almost independent of pH at pH 5.0, 6.0, and 7.0. The melting points of SEA with the addition of 0, 0.01, and 0.1 mM ZnCl** increased almost linearly from 59.5 to 61.4 °C but no detectable increase in melting point occurred with the addition of 1.0 mM ZnCl** compared with 0.1 mM (data not shown) indicating Zn** saturation of the SAgs at 0.1 mM ZnCl**. In contrast 0.1 mM MgCl** or 0.1 mM CaCl** at pH 6.0 stabilized neither the SEA nor the SEE structure (data not shown).

With the Zn** addition, SEE exhibited a biphasic denaturation at pH 5.0 and 6.0, as did SEA at pH 5.0 (Fig. 1). Interestingly, the denaturation curve of SEA at pH 5.0 was much less distinct than at pH 6.0 and 7.0, and no precipitate was formed. Unlike SEA, the EDTA-treated SEE showed a substantially less distinct denaturation behavior at pH 7.0 (Fig. 1).

Denaturations were also made on the mutants SEA_{D227A}, SEA_{D227G}, and SEA_{D227E}.
The residues Asp-227 and His-187 (10) are essential for coordination of Zn$^{2+}$ in the MHC class II $\beta$-chain binding site. The residue Phe-47 is located in the MHC class II $\alpha$-chain binding site, which is unlikely to be metal ion-dependent. Similarly Ser1 may be involved in the Zn$^{2+}$ coordination (22) according to one crystal structure.

The replacement of the Zn$^{2+}$ binding residues strongly affected the thermal stability of the SAgs. The melting point of SEA$\text{D}_{227}$\text{A} was 1.8 °C lower than SEA at pH 6.0 and 6.3 °C at pH 7.0. Table I. Summary of experimental data for the SAgs.

| SAg        | Thermal melting points (°C) | pH 6.0 | pH 7.0 | pI | dCharge from SEE | Proliferation (EC$_{50}$ rel. to SEA) | Theoretical mol. mass (Da) | Observed mol. mass (Da) |
|------------|----------------------------|--------|--------|----|-----------------|------------------------------------|--------------------------|----------------------|
| SEA        |                            | 62.5   | 61.4   | 6.9| 0               | 1.00                               | 27,076                   | 27,070               |
| SEE        |                            | 72.3   | 71.3   | 7.4| 0               | 0.32                               | 26,753                   | 26,696               |
| SEE/A-a    |                            | 74.5   | 75.3   | 6.7| 1               | 0.26                               | 26,583                   | 26,572               |
| SEE/A-f    |                            | 68.2   | 70.0   | 6.5| 1               | 0.84                               | 26,978                   | 26,948               |
| SEE/A-h    |                            | 80.4   | 69.0   | 8.2| 2               | 0.26                               | 26,684                   | 26,708               |
| SEE/A-ah   |                            | 71.9   | 69.1   | 8.0| 1+              | 0.32                               | 26,514                   | 26,508               |
| SEE/E-bdeg |                            | 68.7   | 68.4   | 6.9| 1               | 0.26                               | 26,791                   | 26,797               |
| SEA$\text{D}_{227}$A |                       | 60.7   | 55.1   | 7.3| 1+              | n/a                                | n/a                      | n/a                  |
| SEA$\text{D}_{227}$A,F$_{47}$A |                      | 61.7   | 55.4   | 7.4| n/a             | n/a                                | n/a                      | n/a                  |
| SEA$\text{H}_{187}$A |                       | 62.0   | 57.5   | 7.3| 0               | n/a                                | n/a                      | n/a                  |
| SEA$\text{A-aD}_{227}$A |                      | 69.0   | 69.3   | 6.9| 0               | n/a                                | n/a                      | n/a                  |
| SEA$\text{D}_{227}$A |                       | 70.1   | 67.4   | n/a| n/a             | n/a                                | n/a                      | n/a                  |

SEA$\text{D}_{227}$A, F$_{47}$A; SEA$\text{A-aD}_{227}$A; SEA$\text{H}_{187}$A; SEA$\text{D}_{227}$A; SEE/A-a$\text{D}_{227}$A; and des(1–5)SEA (Fig. 3, Table I). The residues Asp-227 and His-187 (10) are essential for coordination of Zn$^{2+}$ in the MHC class II $\beta$-chain binding site. The residue Phe-47 is located in the MHC class II $\alpha$-chain binding site, which is unlikely to be metal ion-dependent. Similarly Ser1 may be involved in the Zn$^{2+}$ coordination (22) according to one crystal structure.

The replacement of the Zn$^{2+}$ binding residues strongly affected the thermal stability of the SAgs. The melting point of SEA$\text{D}_{227}$A was 1.8 °C lower than SEA at pH 6.0 and 6.3 °C at pH 7.0.

**Fig. 2.** Guanidine denaturations of SEA and SEE. Denaturation of SEA (●) and SEE (○) using guanidine followed by CD at 280 nm (left) and 220 nm (right). Background signal subtracted.

**Fig. 3.** Thermal denaturations of SAg mutants. Melting points (°C) for wild type SAgs (●) and the mutants SEA$\text{D}_{227}$A (○), SEA$\text{H}_{187}$A (□), SEA$\text{D}_{227}$A, SEA$\text{D}_{227}$A, SEA$\text{D}_{227}$A, SEA$\text{D}_{227}$A, and des(1–5)SEA (□). The melting temperatures for the variants with replacements in the MHC class II binding sites (Table I) were generally lower than those for wild type SAgs, although the difference was larger at pH 7.0 compared with pH 6.0.
Characterization of SEA and SEE

The amino acid sequences for SEA and SEE. The regions A, C, F, and H are close to the proposed T-cell receptor binding site, whereas the regions B, D, E, and G are close to the two MHC class II binding sites.

Fig. 4. The amino acid sequences for SEA and SEE. The regions A, C, F, and H are close to the proposed T-cell receptor binding site, whereas the regions B, D, E, and G are close to the two MHC class II binding sites.

pH 7.0 (Fig. 3). For SEA$_{H187A}$, the melting points at pH 6.0 and 7.0 were lowered 0.5 and 3.9 °C, respectively. The stability decrease for SEE$_{D227A}$ relative to SEE was 2 °C at pH 6.0 and 4 °C at pH 7.0 (Fig. 3). SEE/A-a$_{D227A}$ had melting points 5.5 and 6 °C lower than SEE/A-a at pH 6.0 and 7.0, respectively (Fig. 3). SEE$_{D227A, F47A}$ was slightly more stable than SEE$_{A-a D227A}$ but still less stable than SEA (Fig. 3). Des(1–5)SEA denatured at approximately 56 °C at pH 7.0, a thermal stability decrease relative to SEA of 5.5 °C (Fig. 3). Interestingly the D227A/H187A mutants with substantially lower affinity than SEAwt for Zn$^{2+}$ (10) were also to some extent stabilized by the addition of Zn$^{2+}$ (data not shown).

In conclusion, the addition of Zn$^{2+}$ to SEA and SEE increased the stability compared with Zn$^{2+}$-free conditions. The substitution H187A in SEA and D227A in SEE, SEA, and SEE/A-a had a clear destabilizing effect, indicating that these residues are very important for the stability of the SAgs. The destabilizing effect was more pronounced at pH 7.0 than at pH 6.0. The substitution F47A in SEA stabilized the structure at both pH 6.0 and 7.0. Because of the substantial influence of Zn$^{2+}$ on the stability of the SAgs, all denaturations were henceforth carried out in the presence of 0.1 mM ZnCl$_2$.

Characterization of the SEA/E Chimeras—SEA and SEE have a sequence identity of approximately 80%. The main differences are found in eight different regions, four close to the two MHC class II binding sites and four in the vicinity of the TCR binding site (16) (Fig. 4). The chimeras SEA/A-a, SEA/h, SEA/A-h, and SEA/E-bdeg (Table I) were prepared to study the impact of replacement of these regions. Region f in SEE contains two unique histidine residues, which might influence the biphasic denaturation behavior of SEE (Fig. 1), and regions a and h are in the vicinity of region f. In the chimera SEA/E-bdeg the influence of the whole TCR binding site on the denaturation behavior of SEA and SEE was investigated.

The purification of the SAgs was carried out in two ion-exchange chromatography steps, whereas the Fab-SAgs were recovered using a protein G affinity chromatography step followed by ion-exchange chromatography (6). The purity of the products was determined to be at least 90% (data not shown) using SDS-polyacrylamide gel electrophoresis and isoelectric focusing. The identities were confirmed using matrix-assisted laser desorption/ionization-mass spectroscopy (Table I).

Notably, the isoelectric points differed substantially between the chimeras and the wild type SAgs (Table I). SEE/A-a and SEE/A-h, with a positive charge difference of one or two relative to SEE, had isoelectric points at 8.0 or 8.2 compared with 7.4 for SEE, which suggests some conformational differences between the SAgs. Therefore CD spectra were acquired for SEA, SEE, SEE/E-f, and SEE/A-a. The far-UV CD spectra, 200–250 nm, derived primarily from the secondary structure of the proteins (23), were indistinguishable for all SAgs (Fig. 5). The near UV CD spectra, primarily arising from the tertiary structure in the vicinity of the aromatic amino acid residues Tyr and Trp (23), showed larger differences (Fig. 5) indicating subtle conformational differences in the tertiary structure between the SAgs. Further evidence for structural differences was obtained by enzyme-linked immunosorbent assay analysis of SEA, SEE, and the chimeras, which showed that the binding of antibodies to SEE was substantially less than for SEA (data not shown). Interestingly the chimera SEA/A-f, although being sequentially more SEA-like than SEE, produced a lower response against rabbit anti-SEA than SEE. Similarly, SEE/A-a produced a lower or equal response than SEE against a human IgG pool.

The melting points for the chimeras were all lower than for SEE, with the exception of SEE/A-a at pH 6.0 and 7.0 and SEE/A-h at pH 6.0 but substantially higher compared with SEA (Table I). Notably SEE/A-a and SEE/A-h, at pH 6.0, had higher melting points than SEE (Table I), whereas SEE/A-a had a lower melting point, indicating that a combination of these two stabilizing regions reversed this effect. Interestingly the chimeras where region h had been replaced, SEE/A-h and SEE/A-a, were significantly stabilized by the lower pH contrasting the other chimeras.

The melting points of SEE/A-a and SEE/A-f increased from pH 6.0 to 7.0 with approximately 1 °C for SEE/A-a and 2 °C for SEE/A-f (Table I). For the chimeras SEE/A-h and SEE/A-ah the melting points decreased from pH 6.0 to 7.0 with approximately 11 °C for SEE/A-h and 3 °C for SEE/A-ah. The melting point for SEE/E-bdeg was approximately 68–69 °C at both pH 6.0 and 7.0. The differences in melting points between pH 7.0 and 6.0 could be caused by residues, which are important for the structural stability of the protein in the present conformation, becoming protonated between pH 7.0 and 6.0 and thereby acquiring a charge, which might change the stability of the structure. Biphasic denaturations were observed for SEE/E-bdeg at pH 6.0 and by SEE/A-h at pH 7.0. The denaturation behavior of SEE/A-h at pH 6.0 seems to be more complex with the SAg not precipitating at denaturation (Fig. 6). SEE/A-a, -f, and -ah showed no biphasic denaturation behavior.

Interestingly, SEA/E-bdeg composed of SEA with the two MHC class II binding regions from SEE was substantially more stable than SEA, indicating that several different regions contribute to the greater stability of SEE. Of the chimeras, SEE/A-h and SEA/E-bdeg showed biphagic denaturations.

In conclusion, most likely there are conformational differ-
ences between the SEA/E chimeras indicated by isoelectric focusing and near UV CD spectra as well as the low antibody binding properties of SEE/A-a and SEE/A-f. The thermal stability of all the chimeras was higher than for SEA with the highest melting points obtained for SEE/A-a and SEE/A-h, but a combination of these two chimeras, SEE/A-ah, was substantially less stable.

FIG. 5. Far and near UV CD spectra of the SAgs. Far (A) and near (B) UV CD spectra of SEA (●), SEE (○), SEE/A-a (X), and SEE/A-f (■). The spectra are similar in the far UV region, whereas they differ markedly in the near UV region. This indicates that the SAgs have a similar secondary structure, although the tertiary structure differs.

Binding of SE Fusion Proteins to MHC Class II-expressing Cells—The interaction between C215Fab-SEs and human MHC class II (HLA-DR4) presented on cells was studied using a binding assay based on the SPA technology. To detect the binding of SEs to HLA-DR4, the C215Fab-SEs was attached to SPA beads coated with anti-mouse Ig antibodies via the Fab moiety, whereas the cells expressing HLA-DR4 were labeled by the incorporation of [3H]leucine. The concentration yielding half-maximal binding ($B_{\text{max}}/2$) was used as an estimate of the binding affinity. $B_{\text{max}}/2$ concentrations for the different C215Fab-SEs analyzed (Fig. 7) were found to be approximately in the same range (2 × 10^{-8} M) (data not shown). This indicates a similar affinity for HLA-DR4, for SEA, SEE, and the chimeras. A striking difference, however, in the saturation amplitude was noticed for the C215Fab-SEs (Fig. 7). The highest amplitude was seen for C215Fab-SEE/A-h, which was about twice the height of the cluster obtained for the SEA, SEE, SEE/A-h, and SEE/A-ah C215Fab fusion proteins. C215Fab-SEE/A-f and C215Fab-SEA/bdeg formed an additional cluster of C215Fab fusion proteins, with an amplitude half of that obtained for the C215Fab-SEA cluster. These results indicate that the replacement of region a, alone or together with region h, does not affect the number of binding sites, whereas replacement of region h doubles the number of binding sites compared with that for the SEA cluster. In the same way, a replacement of

FIG. 6. Thermal denaturations of the SEA/E chimeras. Thermal denaturation curves at pH 6.0 (●) and 7.0 (—–) for the chimeras SEE/A-a, -f, -h, -ah, and SEA/E-bdeg. SEA/E-bdeg exhibits a biphasic denaturation at pH 6.0 and SEE/A-h at pH 7.0, but the denaturation process of SEE/A-h at pH 6.0 seems to be more complex.
region f or region bdeg results in a \( \approx 50\% \) decrease in the number of binding sites compared with the SEA cluster. These experiments were performed at pH 6.8.

**Biological Assays**—To investigate if structural or stability differences of SEA and SEE could be correlated to murine T-cell proliferative capacity a T-cell proliferation assay was carried out for SEA, SEE, and the chimeras at concentrations varying from 0.001 to 100 pM using spleen cells from C57B1/6 mice. The analysis of the proliferative activity was carried out after 3 and 4 days by pulsing the cells with \(^{3}H\)thymidine, harvesting the DNA of the cells after 4 h of incubation, and measuring the radioactivity.

SEE/A-a, SEE/A-h, SEE/A-ah, and SEA/E-bdeg were equipotent to SEE, whereas SEE/A-f and SEA showed an approximately three times lower proliferating activity (Table I) as determined by EC\(_{50}\) values. Using human T-cells (16), SEE/A-f was less potent, whereas the proliferating activity for SEA was similar to the other SAgs.

**DISCUSSION**

Several studies have speculated in the importance of conformational changes for superantigen function (24, 25). In this study we have investigated the structural properties of several SEA and SEE variants. The results clearly support the previous hypotheses. Although the amino acid sequences of SEA and SEE are very similar, there are differences in biological function. The V\(b\)-specificities for SEA and SEE differ (2, 17) as do their affinities to different MHC class II alleles (9), and SEA may also have a different affinity for the TCR than SEE (16). Interestingly, in many cases chimerical molecules of SEA and SEE acquire properties that are unique and not the predicted combinations between SEA and SEE (Figs. 5–7). Replacing Phe-47 with Ala reduces the number of V\(b\)s that can be acti-\(\)vated (25). This could be caused by the inability of this, more structurally stable variant (Fig. 3) to undergo a necessary conformational change.

These findings can be explained by the subtle differences in the tertiary structure between the SAgs (Fig. 5). The MHC class II binding regions in SEA are mostly responsible for this stabilization, whereas the TCR binding sites seem more flexible. Notably, the tertiary structure of SEE was more stable than that of SEA, although the opposite was true concerning the secondary structure (Fig. 2). Thus, it is likely that some side chains primarily contribute to tertiary interactions, e.g. by burying hydrophobic surface area in the core of the protein whereas others mainly stabilize secondary structure, and that SEE and SEA differ in both types of side chains. Interestingly, both guanidine-induced and thermal (data not shown) denaturation of SEA and SEE monitored using CD showed that the tertiary structure was unfolded before the secondary structure. Tertiary structure is generally lost before secondary structure when the two levels of structure do not disappear concomitantly, as in e.g. the molten globule, and indicates that the transition observed when performing UV monitored thermal denaturations is mainly an unfolding of tertiary structure.

The regions a, f, and h are important for the conformation of SEA, as suggested by isoelectric focusing, CD, antibody recognition, and MHC class II binding assays (Fig. 5 and 7, Table I). We therefore propose that the TCR binding site in SEs can adopt different conformations that are controlled by subtle differences in this region. This is supported by observed differences in V\(b\)-specificity between SEA and SEE (2, 17) and the SEA/E chimeras (16). Thus, several of the residues in the TCR binding site have important functions in controlling the structure, such as the ability for structural modification upon receptor binding and affinity and specificity. Interestingly, these regions may also influence the stoichiometry in binding to MHC class II (Fig. 7), perhaps by having structures that facilitate or hinder binding via the low affinity binding sites.

At certain pH values a biphasic denaturation occurs indicating a partial unfolding in the SAgs. SEE/A-f and SEE/A-ah showed no biphasic denaturation, whereas SEE/A-a, SEE/A-h, and SEA/E-bdeg did. Because the biphasic melting is only observed at pH 5.0 and 6.0 but not at pH 7.0, it was hypothesized that it involved His residues getting ionized. Notably SEE/A-f, with two His residues replaced, lacks a biphasic melting suggesting a role for His-161 or His-164 in this local destabilization. Thus, at least one of these two His residues may form parts of the flexible elements that control the structure of the TCR binding site. This hypothesis is further supported by the similar T-cell-proliferating properties of SEE/A-f and SEA. However, there is a biphasic denaturation with a larger absorbance difference in SEA/E-bdeg, which also lacks the two His-161 and His-164, indicating more complete unfolding or that another region is denatured. Therefore, biphasic melting occurs with certain combinations of residues that destabilize the structure.

Zn\(^{2+}\) is important for the MHC class II affinity of both SEA and SEE (9), as well as the reduction in monokine release triggered by SEA and SEE, but Zn\(^{2+}\) does not influence the V\(b\)-specific T-cell stimulation (26). Loss of Zn\(^{2+}\) coordination significantly lowers the potency of SEA (27). From the data presented here, it is clear that Zn\(^{2+}\) also stabilizes the structures of SEA and SEE, especially at physiological pH. Coordination of metal ions often leads to stabilized structures and can increase melting points with approximately 10 °C including both functional Mg\(^{2+}\) (28) and engineered Zn\(^{2+}\) binding sites (29, 30). The importance of the Zn\(^{2+}\) binding region for the thermal stability of SEA and SEE was further shown by the dramatic decrease in melting point at pH 7.0 for the SEA Zn\(^{2+}\) binding site mutants, such as SEA\(_{322TA}\). Although having a reduced Zn\(^{2+}\) binding, the variants were all stabilized by the addition of a high concentration of Zn\(^{2+}\) indicating that they
bind Zn$^{2+}$ through the known site but with a significantly reduced affinity. Alternatively, an unknown Zn$^{2+}$ binding site could exist (24). Because SEA$_{D227A}$ was less stable than EDTA-treated SEA, this replacement destabilizes or affects the structure of this MHC class II binding site. This suggests that the very low affinity of this variant for MHC class II is caused by two independent mechanisms, removal of the coordinated Zn$^{2+}$ ion and structural disturbance. In contrast SEA$_{H187A}$ is more stable than SEA D227A and has a much higher MHC class II affinity and activity (10, 11), indicating that here the structure is less affected. In the SEA crystal structure Asp-227 is less exposed compared with His-187 (22).

In conclusion residues in the Zn$^{2+}$ binding site are very important for the stability and potency of SEA and SEE, whereas residues in the TCR binding site have a substantial influence on the molecular conformation, which may control specificity and function. Our findings will further guide us to understand how bacterial superantigens have evolved and how their potent T-cell stimulatory capacity is maintained on the molecular level. However, these findings will also help us to design superantigens that could have clinical benefits, such as cancer therapy (5, 31).

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