CONTRACT NO: 87PP7852

TITLE: The 3D Structure of Some Diarrheal Causing Bacterial Toxins

PRINCIPAL INVESTIGATOR: Martin Sax

PI ADDRESS: Veterans Administration Medical Center
University Drive C
Pittsburgh, Pennsylvania 15240

REPORT DATE: July 23, 1992

TYPE OF REPORT: Annual/Final Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21702-5012

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 23, 1992	Annual/Final 7/1/87 - 6/30/92

4. TITLE AND SUBTITLE
The 3D Structure of Some Diarrheal Causing Bacterial Toxins

5. FUNDING NUMBERS
87PP7852
62787A
3M162787A871 AA
DA313380

6. AUTHOR(S)
Martin Sax

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Veterans Administration Medical Center
University Drive C
Pittsburgh, Pennsylvania 15240

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING, MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The major objective of this research project, to determine the 3D crystal structure of the staphylococcal enterotoxin B (SEB), was attained. The structure was solved to a resolution of 2.5 Å and the final result has been submitted for publication to the scientific journal, Nature. The T cell receptor binding site was identified and was demonstrated in a sterically detailed manner with the aid of knowledge obtained independently in a mutational analysis. Regions implicated in the binding of SEB to MHC class II molecules also were elucidated in steric detail. All of the SEs (A to E inclus are predicted to possess the SEB main chain fold.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Staphylococcal enterotoxins; 3D structures; X-ray diffraction; Protein crystallography; Superantigen; RA 4	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTI
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____Where copyrighted material is quoted, permission has been obtained to use such material.

_____Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institute of Health.

YJ Martin Lee July 23, 1992

PI Signature Date

TABLE OF CONTENTS

	PAGE
INTRODUCTION	5
EXPERIMENTAL	7
DISCUSSION	10
CONCLUSIONS.	15
OTHER WORK ON SEs.	16
REFERENCES	17
BIBLIOGRAPHY	18
PERSONNEL	18

INTRODUCTION:

When this research project was initiated five years ago, the mechanism of action of the diarrheal causing staphylococcal entertoxins (SEs) was largely unknown although considerable knowledge had been accumulated about their biochemical, immunological, mitogenic and physiological properties and activities. This situation was described fully in a review paper (1) which indicated that further progress in understanding the mechanism of SE activity would be facilitated markedly by knowledge of their 3D structures. Among the biologically important data covered in the review article were included the similarities and differences in the primary sequences of the SEs, the importance of histidine carboxymethylation in blocking SE intoxication, and the assignment of immunogenic, serologic, and emetic activities to various segments of the primary structure. Comments were comprised also in the review concerning efforts to predict secondary structural features of SEA, presumably in order to visualize the stereochemical nature of the active moieties.

An independent discovery stimulated widespread interest in SEs when they were shown to be superantigens (2). Following this development, a spate of research activity on SEs culminated in the elucidation of many features in the mechanism of action of superantigens. Briefly stated it was learned that the SE molecule first must form a binary complex with an MHC class II molecule, which then adds the beta chain of a T cell to yield a

ternary complex. The formation of the latter triggers T cell proliferation and cytokine production on a scale orders of magnitude greater than o· ·ırs when a normal antigen induces a T cell response. The superantigenic aspect of SEs was reviewed fairly recently (3), and again it was emphasized that crystallographic knowledge was needed before the mechanism of superantigenicity could be understood completely at the molecular level.

The purpose of the present work was to provide missing crystallographic data which were needed for understanding the mechanism of intoxication resulting from food poisoning by S. anreus and for elucidating stereochemical details in the mechanism of superantigencity. The method of approach was to use classical methods of protein crystallography to determine the 3D structures of the SEs. Our strategy consisted of solving the crystal structure of SEB first since we already had grown suitably diffracting specimens. We anticipated that the main chain folding would be similar in all of the SEs since the group (SEA to SEE) shows sequence homology ranging from approximately 45 to 75 percent for the different members (3). The 3D structure of the protein, we reasoned, would show the detailed steric arrangement of the chemical groups which have been identified as sites of biological activity, for example the amino acid residues involved in binding the class II MHC molecule or those that combine with the beta chain of the T cell, or the region of the molecule involved in mitogenesis, etc.

EXPERIMENTAL:

Attempts to crystallize Staphylococcal Enterotoxin B (SEB) were started long before this project period. The protein provided for crystallization looked clean and pure in SDS gel, but crystallization attempts were not successful. At this stage purification by chromatofocusing was tried. The IEF gel clearly showed two or three bands which we were able to separate. Different crystallization conditions were tried and finally the right conditions were found.

Single crystals of SEB were grown by vapor diffusion against PEG 4000 at pH 8.8 (4). The crystals exhibited polymorphism. Three crystal forms were identified. All of them possessed space group $P2_12_12_1$ symmetry but differed slightly in their cell lengths. It was difficult to distinguish the three crystal forms under a microscope, since the morphology was nearly the same for all of them. Initially it was thought that the three forms were due to the heterogeneity of the protein - different forms corresponding to proteins of different pI values. However, it was later found that different crystal forms were present in the same crystallization well. Therefore, native data were collected from all three forms. Their cell dimensions are as follows:

Form I	a=45.3A	b=70.6A	c=78.1A
Form II	a=45.3A	b=68.3A	c=79.4A
Form III	a=45.3A	b=71.2A	c=78.4A

The disagreement in the structure factors of the three forms (R factors on intensities ranging from 26% to 38%) also confirmed that they were quite distinct from one another. Although Form III crystals diffracted to a higher resolution than Forms I and II, the crystal structure analysis was carried out using Form II data since the derivative data were better for Form II crystals. As mentioned before, the main obstacle was distinguishing the three forms. Nominal changes in the cell dimensions of the crystals are normally expected when they are soaked in heavy atom reagents. But in the case of SEB it was difficult to decide whether the change was due to a phase transformation to another form or due to perturbations in the crystal lattice caused by introducing the heavy atom into the unit cell. In view of this, only those derivative data sets, in which the cell lengths agreed with Form II crystals within experimental errors, were considered for use in protein phasing. This made the derivative search a much more complicated and lengthy process than is usually the case. A total of seven isomorphous and two anomalous data sets were collected. The data sets were combined to get MIRAS phases which were further improved by solvent leveling.

The resulting map was in general of good quality but the segments connecting the well defined structural elements, such as beta sheet strands or alpha helices, were not clear. Several models were tried but none of them would refine to a reasonable value. At this stage a phase refinement was done using the solvent leveled phases as protein phases to improve the heavy atom parameters. In

addition this procedure improved the heavy atom phasing by locating new minor heavy atom sites which had been unrecognized previously for some of the derivative data sets. Most importantly, however, the refinement helped in identifying two data sets (one isomorphous and one anomalous, both from the same heavy atom reagent) whose phases were inconsistent with those derived from other data sets. This was judged from the decrease in the phasing power of these derivatives. Accordingly, these two data sets were eliminated from the phase calculations and a new map was computed after the solvent leveling procedure. The final figure of merit was 0.82 for 3.0A resolution. For all these calculations the program package PHASES (5) was used. The electron density map was of very good quality. The C alpha atoms were traced with the help of mini-maps and the bones trace from FRODO (6). The sequence alignment was done by visual inspection of the side chain density and using the slider option in the program 'O' (7). Two models differing in sequence alignment were tried and the one which gave a significantly lower R-factor was chosen as the correct one. After several cycles of alternate model building and X-PLOR (8) refinement the present R-factor is 0.193 for the resolution range 8 to 2.5A. Water molecules have not yet been located and hence not included in the refinement. All residues could be located in the electron density map except for seven residues in the disulphide loop. However, they were built into the model from stereochemical considerations.

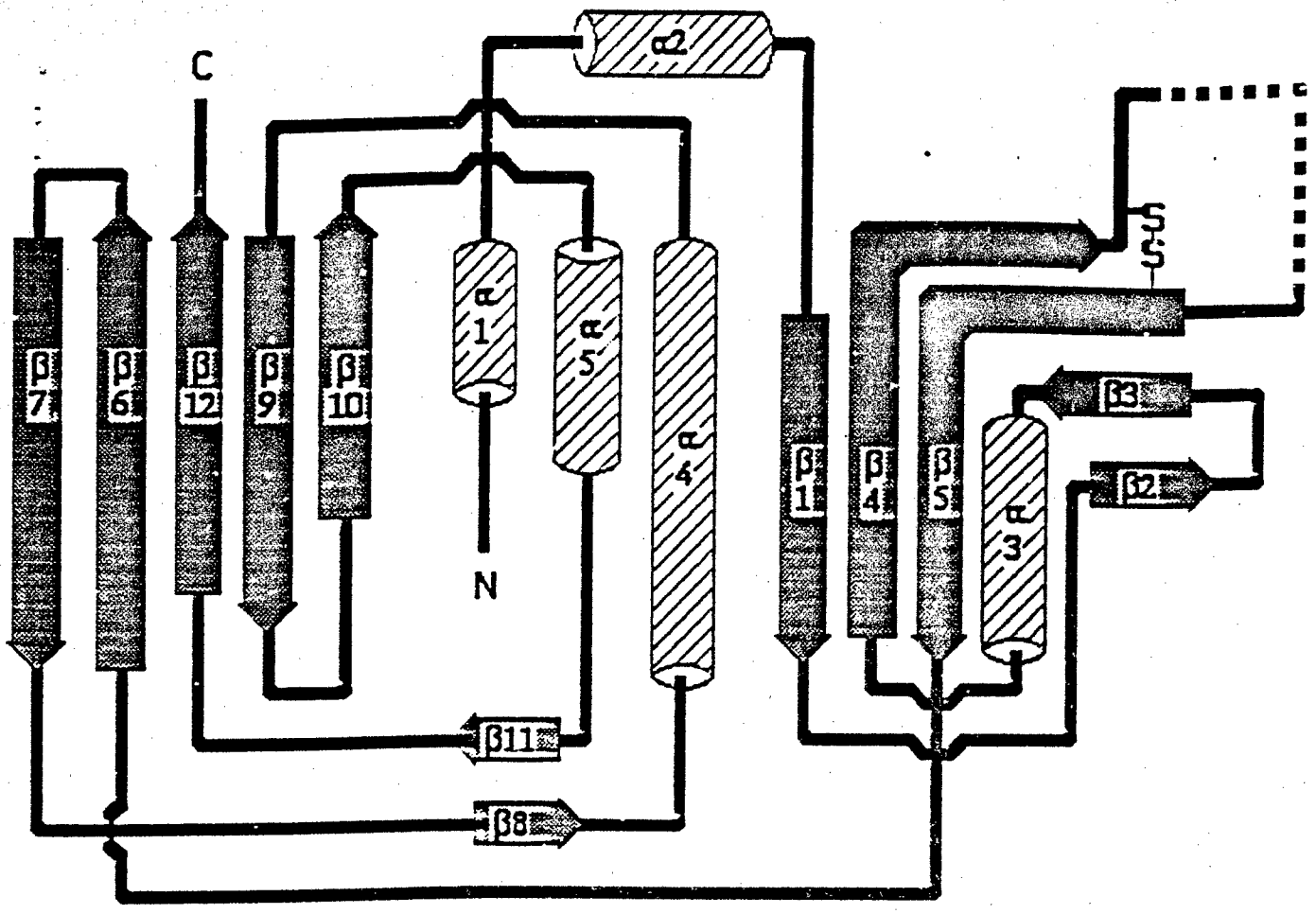
The following facts support the correctness of the model. (a) The

free R-value (9) for the current model is 0.35 and was calculated with 10% of the reflections chosen randomly. (b) The rms deviations for bond lengths and bond angles are 0.020A and 3.5°, respectively. (c) The pep-flip (10) values are less than 2.5A for all except the seven residues in the loop. (d) All non-glycine residues are within the allowed regions in the Ramachandran (11) plot except for 10 residues, seven of which are in the disulphide loop. (e) Omit maps with 2Fo-Fc as coefficients clearly bring back the densities. (f) F_{PH}-F_P maps computed with model phases show the peaks corresponding to the heavy atoms.

DISCUSSION:

A schematic drawing and a ribbon representation of the SEB molecule are shown in figures 1 and 2, where it is evident that the SEB molecule consists of three domains. Two of them are mainly β sheets separated by a third domain which is composed of mainly α helices. The disulphide loop, part of which was not located in the electron density map, is exposed to the solvent. As shown in figures 1 and 2, domain 1 consists of two β sheets, one three stranded and one four stranded and an α-helix, while domain 2 comprises four α-helices and two very short β strands. Domain 3 is made up of five β strands forming a twisted β sheet. The exterior surface of the molecule contains a large proportion of β sheet.

It is known that SEB in functioning as a superantigen must be presented by class II molecules of the major histocompatibility



α1: 13–17	β3: 63–68	β6: 127–138	β9: 182–190
α2: 21–29	α3: 70–78	β7: 141–151	β10: 195–200
β1: 33–39	β4: 81–89	β8: 154–156	α5: 210–217
β2: 48–52	β5: 112–120	α4: 157–172	β11: 222–224
			β12: 229–236

Figure 1. Schematic drawing of the main chain fold in SEB. Residues comprised in alpha helices and beta strands are tabulated.

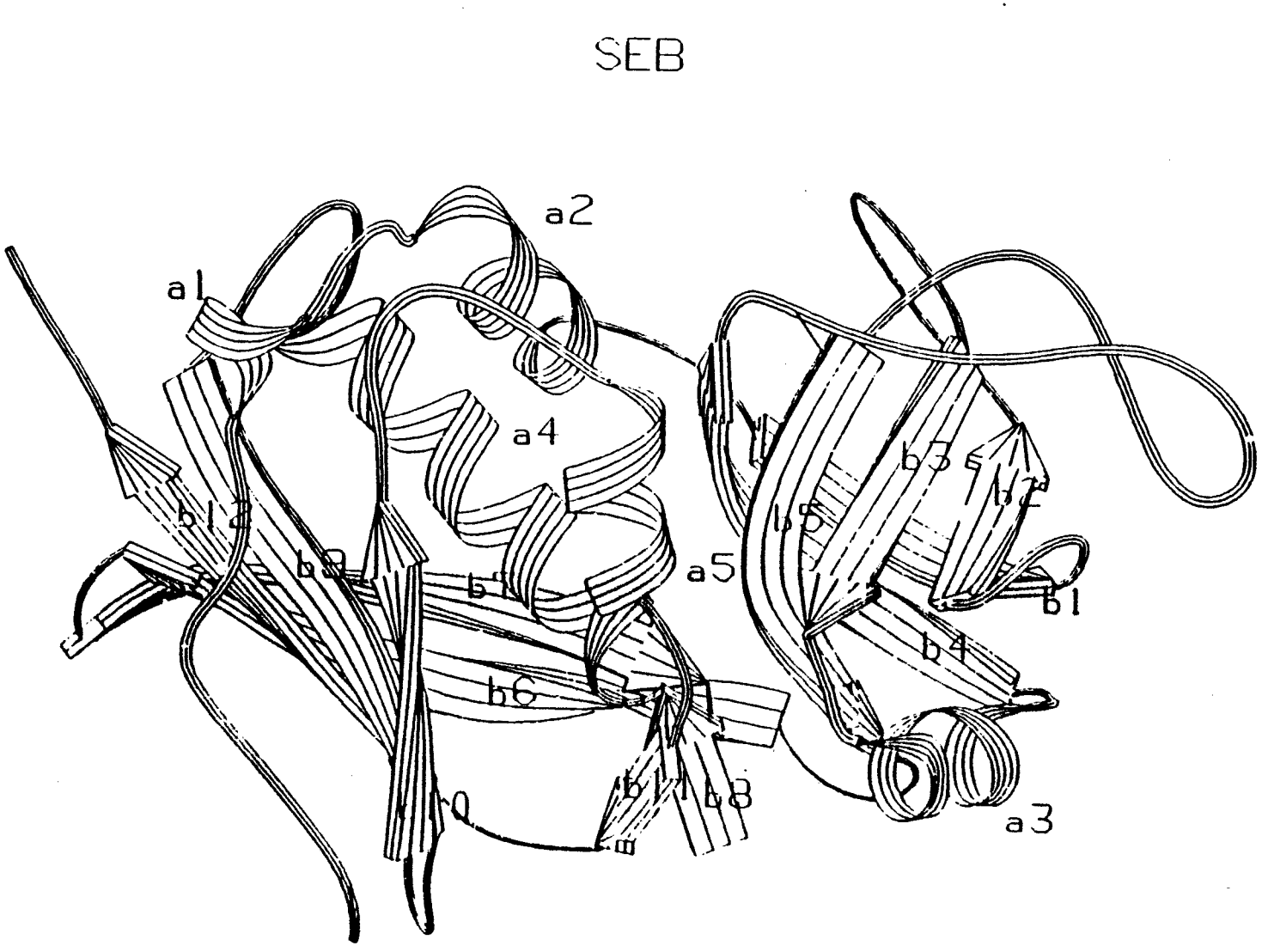


Figure 2. Ribbon trace of the main chain in SEB.

complex (MHCII) to the T-cell receptor (TCR) for T-cell activation. Mutational studies (12) have been done on SEB which identify the MHCII and TCR binding residues. The present crystal structure determination of SEB clearly reveals the steric details of the TCR binding site. A view of it is presented in Figure 3. It consists of a shallow oval-shaped cavity possessing a rim which is slightly elevated above the surrounding protein surface. At the top opening the cavity has diameters of 13 and 14 Å measured from C alpha to C alpha. It comprises residues 22E, 23N, 26V, 27L, 29D, 30D, 31N, 32H, 33V, 55D, 58L, 60N, 61Y, 87A, 88N, 89Y, 90Y, 91Y, 92Q, 112T, 208 F, 209 D, 210Q, and 214L. The mutational study pinpointed two regions, residues 9-23 and 60-61, that are involved in the TCR binding. The presence of residues 23N, 60N and 61Y in this site correlates with those results. However from the architecture of the site as revealed by the crystal structure, it is obvious that there are other resides making up the site that were not identified in the mutational study. From the three dimensional structure it was concluded that residues 208-214 of SEB also are important for TCR activity. This conclusion later was confirmed by an unpublished mutational analysis conducted on SEA (13), which has identified two residues 220 and 221, corresponding to 208 and 209 of SEB, to be important for TCR binding. Residue 23N and 61Y are situated on top of the rim with their side chains oriented outwardly from the protein surface whereas N60 is tilted slightly toward the interior of the cavity. F208 and D209 are on the outer part of the rim (see Figure 3) All these results put together confirm that the TCR binding site has been correctly identified.

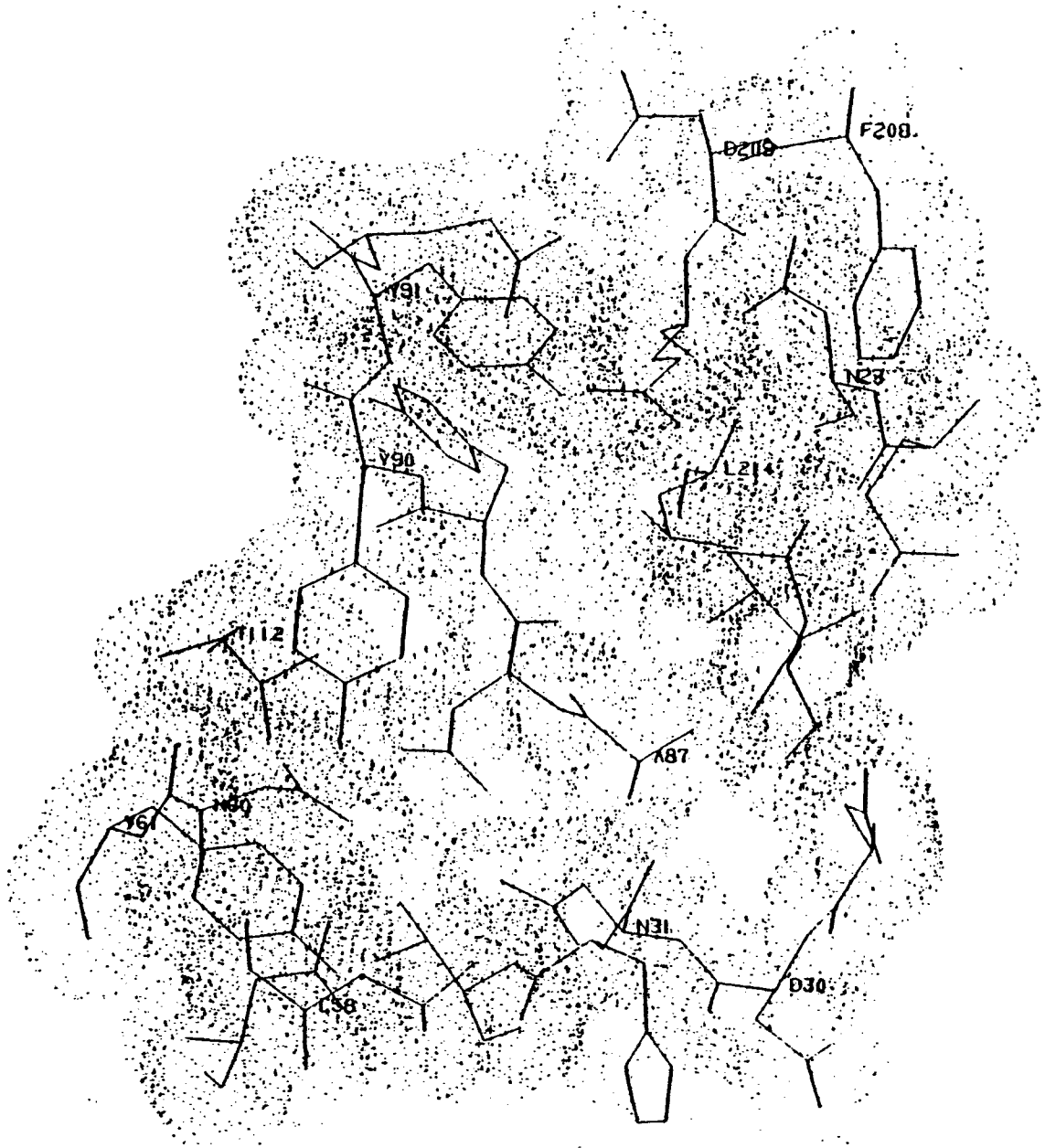


Figure 3. The TCR binding site on SEB. Residues on a slightly elevated rim surround a shallow cavity in the protein surface. Residues 23N, 60N and 61Y are essential for T-cell activation by SEB [illegible]

There are two long grooves adjacent to and on the opposite sides of the TCR binding site. One groove involves the exposed side of α4. This groove also involves residues from region 113-166 which is homologous to the human and mouse invariant chain (3). It has also reported that carboxymethylation of the histidines kills all the activities of the staphylococcal enterotoxins. Three of the five histidines in SEB are situated in groove 1. The other groove involves the exposed side of α5 and is made up mostly of conserved residues (3) in the SEs. Residue 191I is on the outside of this groove. It has been pointed out that (14) residue 186H of SEA, which correspond to 191I of SEB, is on the interface of the MHC binding site and that it forms a zinc bridge with 81H of DRβ. Therefore, groove 1 or groove 2 or both may be involved in MHCII binding. Moreover, according to the suggested model (15) of the MHCII-SEB-TCR ternary complex, MHCII and TCR might make some contact with each other. The two grooves described above are adjacent to the TCR binding site, and if they are indeed MHC binding sites, it is possible that the MHCII molecule and the TCR are in close proximity in the ternary complex. Further analysis is needed to confirm the MHCII binding site.

CONCLUSIONS:

The main chain of SEB assumes a unique folding pattern. The spatial organization of the model neatly accounts for the distribution in the amino acid sequence of those residues which are critical for TCR interaction. Also, it should be mentioned that

the TCR binding site is made up of residues from both the N and C ends of the molecule. Residues identified in the mutational studies of SEB and SEA come together in three dimensions to form the TCR binding site suggesting that the 3D structures of SEB and SEA are similar. This may well be true since there is a high sequence homology among the staphylococcal enterotoxins. All variable residues (3) are either on turns, loops or in regions of the molecule exposed to the solvent. All these facts suggest that the unique folding pattern of SEB is representative of a common folding pattern exhibited by all staphylococcal enterotoxins.

OTHER WORK ON STAPHYLOCOCCAL ENTEROTOXINS:

Single crystals of SEC1 and SEA have been grown in our laboratory, but they are of unsuitable quality for use in collecting x-ray diffraction data. Efforts to improve crystal quality are in progress. The previous work on SEC1 involved the growing of S. aureus 137, a known SEC producer, isolating, and purifying the exotoxin. A new purification protocol was developed for SEC1 (16).

REFERENCES:

1. Spero, L., Johnson-Winogar, A., and Schmidt, J.J. Enterotoxins of Staphylococci, in "Handbook of Natural Toxins" (Hardegree, M.C., and Tir, A.T.,) Vol 4, pp. 131-163, Dekker, New York (1988).

2. White, J., Herman, A., Pullen, A.M., Kubo, R., Kappler, J.W. & Marrack, P. Cell 56, 27-35 (1989).

3. Marrack, P. & Kappler, J. Science 248, 705-711 (1990).

4. Swaminathan, S., Yang, D.S.C., Furey, W., Abrams, L., Pletcher, J. & Sax, M. J. Mol. Biol., 199, 397 (1988).

5. Furey, W. & Swaminathan, S. "Phases-A Program Package for the Processing and Analysis of Diffraction Package for Processing and Analysis of Diffraction Data from Macromolecules," PA33, American Crystallographic Association Meeting, New Orleans, LA, 73 (1990).

6. Jones, T. A. J. Appl. Crystallogr. 11, 268-272 (1978).

7. Jones, T.A. In Computational Crystallography (ed. Sayer, D.) 303-317 (Clarendon Press, Oxford, 1982).

8. Brünger, A. T. Kuriyan, J & Karplus, M. Science 235, 458-460 (1987).

9. Brünger, A. T. Nature 355, 472-475 (1992).

10. Jones, T. A., Zou, J.-Y., Cowan, S.W. & Kjeldgaard, N. Acta Crystallogr. A47, 100-119 (1991).

11. Ramakrishanan, C. & Ramachandran, G. N. Biophys. J. 5, 909-933 (1965).

12. Kappler, J. W., Herman, A., Clements, J. & Marrack, P. J. Exp. Med. 175, 387-396 (1992).

13. Gascoigne, N. J., Private Communication.
14. Fraser, J. D., Private Communication.

15. Janeway, Jr., C.A., Yagi, J., Conrad, P.J., Katz, M. E., Jones, B., Vroegop, S. & Buxser, S. In Immunological Reviews (ed. Göran Müller) 107, 61-88 (Munsgaard, Copenhage, Denmark, 1989).

16. St. Clair, N. L. and Sax, M. Protein Expression and Purification 1, 97-103, (1990).

BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS:

1. Crystallization and Preliminary X-ray Study of Staphylococcal Enterotoxin B., Swaminathan, S., Yang, D.S.C., Furey, W., Abrams, L., Pletcher, J. and Sax, M. J. Mole. Biol., 199, 397 (1988).

2, Progress Report on the Structure Determination of Staphylococcal Enterotoxin B., Swaminathan, S., Furey, W., Abrams, L., St. Clair, N., and Sax, M. ACA Annual Meeting, 1988, Philadelphia.

3. Progress Report on the Structure Determination of Staphylococcal Enterotoxin B., Swaminathan, S., Furey, W., Abrams, L., St. Clair, N., and Sax, M. ACA Annual Meeting, 1989, Seattle.

4. The Crystal Structure of Staphylococcal Enterotoxin B. A Superantigen., Submitted to Nature.

5. Free-Solution Isoelectric Focusing for the Purification of Staphylococcus aureus Enterotoxin C1, St. Clair, N. L. and Sax, M. Protein Expression and Purification 1, 97-103 (1990).

PERSONNEL:

The following personnel received pay from Army Project Order No. 87PP7852: Dr. S. Swaminathan, Ph.D. and Mr. Timothy Umland, graduate student.