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TITLE: X-Ray Crystallographic Studies on Acetylcholinesterase and Related Enzymes

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X-Ray Crystallographic Studies on Acetylcholinesterase and Related Enzymes

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The objective of the project was to establish a procedure for designing and producing species-specific protein inhibitors, i.e. chimeras, utilizing the scaffold of the 'three-finger toxins'. Theoretical analysis of the 3D structure of the chimera of α-neurotoxin and fasciculin II showed that the loops of fasciculin II grafted onto the core of α-neurotoxin maintain their secondary structure. This supports the feasibility of employing protein engineering, using the 'three-finger toxin' core as a platform, for generating new proteins that mimic 'three-finger toxin' activity but display modified specificity. We have succeeded in producing chimera II biosynthetically, by expression in E. coli, and have been able to reach adequate purity by HPLC purification. Kinetic assays showed that the chimera displays substantial inhibitory activity towards acetylcholinesterase. Due to non-specific proteolysis encountered during cleavage of the fusion protein used for expression of chimera II, it was decided to adopt a synthetic approach to its production. Chemical synthesis provided a more convenient route for obtaining chimera II, in good yield and with high purity.
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Introduction

Acetylcholinesterase

Acetylcholinesterase (AChE) plays a key role in cholinergic neurotransmission. By rapid hydrolysis of the neurotransmitter, acetylcholine (ACh), it terminates transmission, thereby permitting rapid repetitive responses (1). AChE is, accordingly, characterized by a remarkably high turnover number, especially for a serine hydrolase (2), functioning at a rate approaching that of a diffusion-controlled reaction (3, 4). The powerful acute toxicity of organophosphorus (OP) poisons (as well as of carbamates and sulfonyl halides, which function analogously) is attributed primarily to the fact that they are potent inhibitors of AChE (5, 6). Inhibition is achieved by their covalent attachment to a serine residue within the active site (2, 7). AChE inhibitors are utilized in the treatment of various disorders such as myasthenia gravis, glaucoma (6, 8), and in the management of Alzheimer's disease (9). Finally, many carbamates and OPs serve as potent insecticides, exerting their action by selectively inhibiting insect AChE (10).

Elucidation of the three-dimensional (3D) structure of AChE is, therefore, of fundamental interest for understanding its remarkable catalytic efficacy, and of applied importance in developing therapeutic approaches to OP poisoning, as well as in insecticide and drug design. Furthermore, information concerning the ACh-binding site of AChE is also pertinent to an understanding of the molecular basis for the recognition of ACh by other ACh-binding proteins, such as the various ACh receptors (11).

A fundamental question, which arises from knowledge of protein structure, concerns how proteins interact with each other. As many more 3D structures of proteins become available - there are now >20,000 in the Protein Data Bank (see: http://www.rcsb.org) - a key challenge will be the prediction, by use of computational techniques, of which proteins interact with each other.

In the late 60s the notions of phylogenetic characterization and structure-function relationships in protein families was pioneered by Dayhoff (12). One of the first families to be characterized was a family of polypeptides purified from snake venoms, later known as the 'three-finger toxins' (13). These polypeptides are secreted in the venoms of snakes, and contain 60-75 amino acids. They display a great variety of inhibitory functions, such as blocking of the nicotinic acetylcholine receptor (nAChR) by bungarotoxin, inhibition of AChE by fasciculins, blocking of L-type Ca\(^{2+}\) channels by calcisentins, and suppression of cell adhesion processes by dendrotoxins. These proteins represent one of the most abundant families (over 150 sequenced) known. Members of the family share a common 3D organization: a globular hydrophobic core, possessing 4-5 disulfide bonds, and 3 extended loops (fingers), comprised of antiparallel \(\beta\)-strands, emerging from the core. It has been suggested that the primary function of the core is to maintain the overall fold of the family, with structural plasticity being conferred by the loops. This plasticity allows the loop residues to adopt a variety of arrangements, thus producing differential biological recognition for a variety of targets. As was demonstrated for 'erabutoxin a', one of the neurotoxins in the family, recognition of the nAChR is determined by amino acids in specific regions of the loops (14, 15). In another example, it was demonstrated that the fifth
disulfide bond of the long-chain (75-residue) α-neurotoxins is responsible for enhanced affinity for the α7 neuronal nACHR (16). In 1996, Le Du and coworkers reported alternating loop conformations in the crystal structures of the fasciculins (17). These studies give credence to the idea that the disulfide bonds act as joints permitting a certain degree of structural freedom (18).

In order to explore subtle differences in key functional regions, two toxins were chosen of the same length and topology. They were fasciculin II (Fas II), the AChE inhibitor found in the venom of the green mamba, Dendroaspis angusticeps, and α-neurotoxin (α-tox), a potent nACHR inhibitor, from the venom of the black neck spitting cobra Naja nigricolis. The 3D structures of both toxins were already known (19-22).

Superimposition of the two 3D structures suggested that the functional regions responsible for specific binding to their respective targets correspond to 3 structurally distinct homologous zones. In order to study the roles of these zones, a protein chimera was constructed, chimera II (ChII), by transferring these 3 zones from Fas II to α-tox (23, 24). ChII was the subject of this project. As expected, in the chimera the original α-tox (21) activity was lost, and Fas II activity was observed, albeit 15-fold lower than that of native Fas II (Table 1). The chimeric protein was crystallized and its 3D structure was compared to the parent structures.

Table 1: Activity comparison of the various proteins.

<table>
<thead>
<tr>
<th>Inhibition of</th>
<th>Fas II</th>
<th>α-tox</th>
<th>ChII</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAChE</td>
<td>1.1 X 10^{-10} M</td>
<td>no binding</td>
<td>unknown</td>
</tr>
<tr>
<td>mAChE</td>
<td>2.3 X 10^{-12} M</td>
<td>no binding</td>
<td>unknown</td>
</tr>
<tr>
<td>TcAChE</td>
<td>unknown</td>
<td>no binding</td>
<td>unknown</td>
</tr>
<tr>
<td>EeAChE</td>
<td>4.2 X 10^{-11} M</td>
<td>no binding</td>
<td>6.8 X 10^{-10} M</td>
</tr>
<tr>
<td>AChR</td>
<td>no binding</td>
<td>10^{-9} - 10^{-12} M</td>
<td>no binding</td>
</tr>
</tbody>
</table>

Fasciculin II

Fasciculins are 61 amino-acid proteins belonging to the three-fingered toxin family. Fas I and II were isolated and purified in 1983 (25) from the venom of Dendroaspis angusticeps (the green mamba). Both these polypeptides are powerful inhibitors of mammalian AChE. By suppression of AChE activity at the neuromuscular junction they provoke muscle fasciculations, from which they derive their name. The toxins were sequenced in 1984 (26). In 1992 the structure of Fas I was solved by X-ray diffraction (19). However, this did not reveal the nature of the interaction between the toxin and its target. During the following years a number of groups tried unsuccessfully to model the interaction (27).

In 1995, the first structure of a three-fingered toxin complexed with its target was published when two groups elucidated the structure of the AChE/Fas II complex in parallel. One group used mAChE to elucidate the structure of the mAChE/Fas II complex at 3.2 Å resolution (21). The other solved the structure of the complex with TcAChE at 3.0 Å resolution (22). These two studies revealed several common features typical of the interactions in the complex:
The tips and exposed side surfaces of loops 1 and 2 govern specificity. The interaction utilizes a three-point anchorage primarily consisting of main-chain/side-chain hydrophilic interactions. Additional segmental flexibility is accorded to the domains of the loops, and the disulfide bonds between the loop tips and the base may serve as joints to effect finger orientation. Loop 1 fits into a crevice near the tip of the gorge leading to the active site of the enzyme, thus orienting loop 2 into the entrance of the gorge. This orientation is responsible for the unusually large contact area (ca. 2000 Å²) (22) and the consequent very high affinity.

One unique feature observed was a match between the dipole moments of Fas II and AChE, which may contribute to the high affinity observed (22). Although the dipole moments of the toxin and the protein line up, no side-chain side-chain interactions between oppositely charged amino acids are seen. Rather, side-chain main-chain interactions are detected which permit much closer interaction and tighter binding between enzyme and toxin.

α-Neurotoxin

α-Neurotoxins were the first members to be identified of the three-finger toxin family. Their affinities, kinetics, and sites of binding to the various nAChRs have been studied extensively (16, 28, 29).

α-tox, from the black neck spitting cobra (*Naja nigricollis*), was first identified as a nAChR inhibitor and partially sequenced in 1966 (30). It was fully sequenced and characterized a year later (31). Subsequently, it was identified and sequenced in many cobra species (32). It is a short-chain 61-residue toxin containing 4 disulfide bonds. Exhaustive mutational analysis revealed that the short-chain neurotoxins recognize the nAChR via a homologous polar surface of 10 residues located on one face of the toxin (14-16, 28, 29). The 3D structure of the toxin was solved by NMR (20).

Use of a three-finger toxin scaffold in the design of proteins mimicking three-finger toxin activity

Preservation of secondary structure of peptide sequences transferred from one protein to another is crucial in protein engineering. Minor and Kim (33) showed that an 11 amino-acid sequence could adopt different secondary structures depending on the protein context in which it was placed. In a broad survey conducted on the PDB in 2000 by Zhou et al. (34) a wide range of 4-8 amino acid peptides were shown to adapt different secondary structures to best match their environment. These studies showed that non-local interactions could play a role in determining the secondary structure of peptide sequences in proteins as well as intrinsic conformational propensities of amino acids in the sequence. They demonstrated that this scaffold’s rigidity could maintain the secondary structure of peptide loops inserted between two disulfide bonds of the scorpion toxin charybdotoxin.

In the ‘three-finger toxins’, due to the rather rigid nature of the core, it appears that it is easier to transfer peptide loops while maintaining the original structure, than in proteins in general, as showed by Zinn-Justin and coworkers (35).
Research Objectives

Our aim was to investigate the concept of ‘structure conservation among diverse sequences’ using the ‘three-finger toxin’ family as a paradigm. Specifically, to evaluate the contention that the common structural motif (a hydrophobic core containing 3 disulfide bonds) provides a rigid scaffold upon which biological activity is conferred by 6 variable, β-strand-containing, flexible ‘fingers’.

The goals of our research were:

1. To produce the chimeric protein (a ‘three-finger toxin’ designed by combining the core of α-tox and two fingers of Fas II, named chimera II (chII) via expression in E. coli, and to purify it.
2. To produce ChII by chemical synthesis, and to compare the quality and ease of production to that of biological expression.
3. To characterize kinetically the activity of the two different synthetic chimeras towards TcAChE.
4. To use x-ray crystallography to determine the structure of the rChII/TcAChE complex, and to compare it to structures of those of the unbound chimera and the Fas II/AChE complex.

A long-term objective was to establish a procedure for designing and producing species-specific protein inhibitors utilizing the scaffold of the ‘three-finger toxins’. This objective would take advantage of the fact that Fas II has low inhibitory activity towards invertebrate AChEs, due to a reduced number of aromatic amino acid residues in their peripheral anionic sites.
Body

SDS-PAGE:
Polyacrylamide gels were cast in a Mighty Small SE250/SE260 mini-vertical unit (Hoefer). Reagents were purchased from Bio-Rad. Staining of gels was performed with Gelcode Blue (Pierce).

zz-rChII was analyzed by SDS-PAGE under reducing and non-reducing conditions, on 15% polyacrylamide gels. rChII was analyzed by SDS-PAGE on 16.5% polyacrylamide gels (36).

Fusion Protein Expression and Purification
The expression construct for chimera II (zz-rChII) was obtained from Dr. Frédéric Ducancel (Département d’Ingénierie et d’Etudes des Protéines, Commissariat à l’Energie Atomique, CEA Saclay, Gif-sur-Yvette, France). The procedure for building the construct is briefly described below.

Expression of zz-rChII:
Starter culture of freshly transformed bacteria was incubated at 37 °C under aeration in 5 mL Tryptic Soy Broth (TSB) (Difco, Detroit, MI). After ~7.5 h, when the O.D₆₀₀ had reached 1.0, the cells were used to inoculate 500 mL batches (a total of 22 batches altogether) of TSB medium (1:100 dilution) supplemented with glucose (5 g/L), ampicillin (200 mg/L), and chloramphenicol (34 mg/L). Cultures were incubated at 37 °C under aeration for approximately 3.5 h. When the O.D₆₀₀ had reached 0.5-0.6 the cells were induced by addition of 0.1 mM IPTG (final concentration). Prior to induction a 10 mL aliquot was put aside to incubate under non-induced conditions as a control. After 2-3 h, when the O.D₆₀₀ had reached 1.8-2.0, the cells were harvested by centrifugation at 4000 g, 4 °C, for 30 min.

Cell lysis:
Cells were resuspended at a ratio of 1:10 TSE buffer (30 mM Tris-HCl/20% sucrose/5 mM EDTA/0.1 mM PMSF, pH 8.0) to culture volume, while kept on ice, and were disrupted by sonication as follows: three cycles of 3 min each, using a 1 cm diameter probe, at 40% output, and a 30% duty cycle of the sonicator (Sonix XL, Ultrasonic, U.S.A) were applied with a pulse duration of 1 sec, and 1 sec delay. Sonication was performed in a 100 mL rosette tube kept on ice.

Affinity chromatography of zz-rChII:
The cell suspension was centrifuged (15,000 g, 4 °C, 30 min) to pellet cell debris. The supernatant was mixed with IgG-Sepharose gel, pre-equilibrated with TST buffer (150 mM NaCl/0.05% Tween 20/50 mM Tris-HCl, pH 7.6), and the slurry was rotated for 2-3 h at 4 °C. It was then loaded into a column (3 X 10 cm, Bio-Rad), and the buffer was allowed to drain. Two washes were then performed. Wash 1 consisted of 10 column volumes of TST; wash 2 consisted
of 3 column volumes of 5 mM ammonium acetate, pH 5.0. Elution was performed with 0.5 M acetic acid, pH 3.4. The eluent was then lyophilized.

**HPLC purification of zz-rChII:**

The lyophilized protein was dissolved in 20% aqueous acetonitrile containing 0.1% TFA, at a concentration of ~5 mg/mL, and 1 mL aliquots were injected onto a C4 semi-preparative column (Vydac). Elution was performed using a linear gradient of 20-30% aqueous acetonitrile, at 3 mL/min. The eluent was lyophilized and redissolved in milli Q water.

**Cleavage of zz-rChII and purification of chimera II**

**Fusion protein cleavage:**

The reaction mixture contained 5 or 10 units of the catalytic subunit of enterokinase (EnterokinaseMax, Invitrogen) and 1 mg of purified zz-rChII in 0.5 mL of 0.1% Tween 20/1 mM CaCl$_2$/50 mM Tris, pH 8.0. The reaction mixture was incubated for 1-5 h at 37 °C. Digestion was terminated either by flash freezing in liquid N$_2$, or by injection into the HPLC column. The cleavage results were analyzed by SDS-PAGE.

**HPLC purification of cleaved rCHII:**

The enterokinase digestion reaction mixture (0.5 mL containing digestion product derived from ~1mg of zz-rChII) was diluted 1:2 with 5% aqueous acetonitrile containing 0.1% TFA, and injected into a C18 analytical column (Vydac). Elution was performed using the aqueous acetonitrile gradient containing 0.1% TFA specified in Table 2, at 0.8 mL/min.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Acetonitrile percent</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5</td>
<td>5</td>
<td>initial wash</td>
</tr>
<tr>
<td>5-35</td>
<td>5-24</td>
<td>acetonitrile gradient</td>
</tr>
<tr>
<td>35-45</td>
<td>24</td>
<td>acetonitrile gradient</td>
</tr>
<tr>
<td>45-60</td>
<td>24-80</td>
<td>acetonitrile gradient</td>
</tr>
<tr>
<td>60-70</td>
<td>80</td>
<td>wash</td>
</tr>
<tr>
<td>77-75</td>
<td>80-5</td>
<td>column regeneration</td>
</tr>
</tbody>
</table>

**Table 2: aqueous acetonitrile gradient for purification of cleaved rChII.**

Peaks were collected, lyophilized, redissolved in milli Q water and subjected to a second HPLC purification step. This step utilized the same column, but the gradient was modified. Elution was performed using a linear gradient of 20-25% aqueous acetonitrile containing 0.1% TFA, at 0.8 mL/min. The eluent was lyophilized and redissolved in milli Q water.
HPLC purifications utilizing 0.1% NaClO₄/0.1% H₃PO₄:

rChII purified as described above (just after the first HPLC purification step) was injected into a C18 analytical column (Vydac). Elution was performed with a linear gradient of 22-38% aqueous acetonitrile, containing 0.1% NaClO₄ /0.1% H₃PO₄, at 0.8 mL/min. The eluent was lyophilized and redissolved in milli Q water, and a second HPLC purification step was applied to desalt the rChII. This step utilized the same column, but elution was performed with a linear gradient of 20-25% aqueous acetonitrile, containing 0.1% TFA, at 0.8 mL/min. The eluent was lyophilized and redissolved in milli Q water.

N-terminal sequencing of cleavage product:

rChII samples after the first HPLC purification step were separated by SDS-PAGE, and the gel was electrophorosed onto a PVDF membrane. Electroblotting was carried out in a 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) transfer buffer (10 mM CAPS/10% methanol, pH 11.0 (37). N-terminal sequencing using Edman degradation was carried out for bands with molecular weight ca. 7 kDa by the Weizmann Institute Protein Microsequencing Facility.

Purification and Cleavage of reduced zz-rChII:

One milligram of zz-rChII was subjected to reduction in 100 mM DTT/100 mM Tris-HCl, pH 8.5, in a total volume of 0.5 mL, for 3 h at room temperature. The mixture was diluted 1:2, and purified by HPLC using the same procedure as above. The eluent was lyophilized, redissolved in milli Q water, and subjected to enterokinase cleavage using the same conditions described above. The cleavage products were purified also as described above and analyzed by SDS-PAGE.

Refolding of rChII:

rChII was refolded overnight at room temperature in 1 mL total volume of 4 mM GSH/2 mM GSSG/0.1 M phosphate, pH 8.0, at a final concentration of ~1 mg/mL rChII.

HPLC purification of refolded rChII:

The refolded rChII was purified on a C18 analytical column (Vydac) as described above. Peaks were collected, lyophilized, and redissolved in milli Q water.

Chemical synthesis of ChII

Reagents for synthesis were purchased from Novabiochem. The chemical strategy used for synthesis was standard solid-phase peptide synthesis (SPPS) methodology using Fmoc-protected amino acids (38). Deprotection utilized 20% piperidine in dimethylformamide (DMF), followed by a DMF wash. Coupling utilized 100 μM Fmoc amino acid/100 μM benzotriazole-1-yl-oxy-tris pyrrolidino-phosphonium hexafluorophosphate (Bop) /200 μM N-methylmorpholine, followed by a DMF wash. A second coupling step using the same reagents and a final DMF
wash were then performed. Once the synthesis was over, cleavage from the resin support was performed, utilizing 5% triethyl-silane/5% thioanisole/5% H$_2$O in trifluoroacetic acid. The crude mixture obtained was lyophilized, and the product was analyzed both by injection into a C18 reverse phase HPLC column and by SDS-PAGE.

Inhibition tests

Purified TcAChE was used to assess the inhibitory activity of zz-rChII, rChII and Fas II towards AChE. Enzyme activity was measured spectrophotometrically using 5mM acetylthiocholine/0.15 mM 5,5’-dithiobis(2-nitrobenzoic acid)/10 mM phosphate, pH 7, containing 0.01 mg/ml BSA (39). Assay mixtures were preincubated for 5 min at 37 °C before initiations of the reaction by addition of substrate.

Secondary structure prediction and structural alignment

Secondary structures of the various polypeptides were calculated using the program DSSP (40). This program computes the secondary structure by comparing the H-bond pattern of the particular 3D structures studied versus values derived from the H-bond patterns of ideal secondary structure elements. The structures of the toxins were obtained from the PDB. Where subtle differences were encountered between several structures of the same protein, secondary structure was assigned on visual inspection of the phi-psi angles.
Key Research Accomplishments

Cloning of zz-rChII

Briefly, the construct was cloned as follows (23). Complementary synthetic oligonucleotides, ranging from 17 to 48 bases, were annealed, and ligated to generate semi-synthetic cDNA. Restriction sites for KpnI and BamHI were introduced, flanking the designed cDNA, to allow directional cloning of the gene into the expression vector (Figure 1). The gene was cloned into the bacterial expression vector pCP (41). The vector contained two synthetic IgG binding domains, derived from Staphylococcus aureus protein A (42), fused 3’ to ChII to aid in purification (Figure 2). The 5’ restriction site was followed by a sequence encoding the residues Asp-Asp-Asp-Lys, which is specifically recognized by bovine enterokinase (43). This cleavage site was used to separate ChII from the fusion tag. To terminate translation, two tandem stop codons, TAA, were introduced at the end of the gene.

Figure 1: Amino acid and DNA sequences of the construct for the fused protein zz-rChII.

<table>
<thead>
<tr>
<th>Bovine enterokinase cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>zz-KpnI-Asp-Asp-Asp-Asp-Lys-ChimeraII-end-end-BAMHI</td>
</tr>
<tr>
<td>-GAT-GAC-GAT-GAC-AAG-ACG... -TAA-TAA</td>
</tr>
</tbody>
</table>

Figure 2: Schematic map of expression vector pCP used to produce the fused protein.

- MCS - Multiple cloning site.
- Ori - Origin of replication.
- Amp⁰ - β-lactamase coding gene.
- pT7 - T7 phage promoter.
- zz - Two synthetic IgG binding domains derived from Staphylococcus aureus.
Secondary structure alignment of toxin structures

Dr. Ducancel designed the chimeric toxin by combining the sequences of the two toxins as follows. Residues 1-16 (the entire loop I), 25-37 (the entire loop II) from Fas II were transferred into the α-tox core. These are the two regions which were seen to be the most different when the the two toxins structures were superimposed. In addition, the C-terminal asparagine of α tox was substituted by the C-terminal tyrosine of Fas II (Figure 3, Figure 4).

**Figure 3:** A. Sequence alignment of FasII, ChII, and α-tox. Amino acids originating from: Fas II colored in red; α-tox colored in blue; shared amino acids colored in green. The α-tox structure was based on a consensus of 8 NMR structures obtained from the PDB, entry code 1NEA (20). The Fas II structure was based on a consensus of 4 different fasciculin structures obtained from the PDB: FasII alone, PDB entry 1FSC (19), and 3 structures of FasII bound to hAChE, PDB entry 1F8U (22), mAChE PDB, entry 1MAH (44), TcAChE, PDB entry 1FSS (45). B. Percent of ChII derived from Fas II and α-tox.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fas II:</td>
<td>TMCYSHTTTSRAILTNCCGENSCYRKSRRHPKVMGLRGCPCPPGDNLVEVKCCTSPDKCN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rChII:</td>
<td>TMCYSHTTTSRAILTNCPGETNCYKKSRRHPKVMGLRGCPCPTVKPGIKLNCCCTT-DKCNY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tox:</td>
<td>LECHNQSSQPPTKTCPGETNCYKKVWRDHRTTIIERGCPCPTVKPGIKLNCCCTT-DKCNN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4:** Comparison of the 3D structures of α-tox (blue), ChII and Fas II (red). ChII is colored blue for regions originating from α-tox, red from Fas II and green where the sequences were identical in the two parent toxins.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimera II</td>
<td>Fas II</td>
<td>Shared residues</td>
</tr>
<tr>
<td></td>
<td>26/62=42%</td>
<td>21/62=34%</td>
</tr>
</tbody>
</table>

We continued by checking whether the structure of the transferred loops was maintained in ChII by examining their secondary structure. It is important to note that very subtle changes in
these sequences can confer different structures as can be seen in comparing Fas I and Fas II. These toxins, which differ by a single amino acid, Tyr 48 in Fas I being replaced by Asn in Fas II, show subtle differences in their structures, probably due to differences in crystallographic packing. Figures 4 and 5 show the sequence alignment between Fas II and α-tox, together with their secondary structures. Figure 5 B shows that ChII adopts the structure of Fas II (the secondary structure was preserved after transformation into the new scaffold).

Figure 5: Alignment of Fas II vs. α-tox, secondary structure comparison, β-Strand amino acids colored pink.

A.  

<table>
<thead>
<tr>
<th></th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>61</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas II</td>
<td>/---Finger1----/</td>
<td>/---Finger2----/</td>
<td>/---Finger3----/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tox</td>
<td>βββ--Tip1---βββ</td>
<td>ββββββββTip2ββββββββTip3--ββββββββ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C++</td>
<td>++</td>
<td>C-GE</td>
<td>++</td>
<td>++</td>
<td>RGCGCP</td>
<td>+++</td>
</tr>
</tbody>
</table>

Fas II: TMCSHTTTCSRAILTCYKSSRRHKPPKMVLGRGCGCPGDDNLLEVKCCTSPDKCNY

B.  

ChII: TMCSHTTTCSRAILTCYKSSRRHKPPKMVLGRGCGCPGDDNLLEVKCCTSPDKCNY

C. Summary of the sequence comparison between α-tox, FasII, and ChII, on the basis of the different structural components.

<table>
<thead>
<tr>
<th>α-Tox vs. Fas II</th>
<th>Identity</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>61AA, 1 gap</td>
<td>21/62=34%</td>
</tr>
<tr>
<td>Core</td>
<td>16/62=26%</td>
<td>8/16=50%</td>
</tr>
<tr>
<td>Fingers</td>
<td>46/62=74%</td>
<td>12/46=26%</td>
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<tr>
<td>Strands</td>
<td>26/62=42%</td>
<td>10/26=38%</td>
</tr>
<tr>
<td>Tips</td>
<td>16/62=26%</td>
<td>1/16=6%</td>
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zz-rChII Expression and Purification

The fusion protein was expressed at high levels, in the soluble fraction of E. coli (Figure 6), under conditions previously described (23).

Figure 6: 15% SDS-PAGE of bacterial soluble fraction from induced and non-induced samples.

<table>
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<th>MW</th>
<th>M</th>
<th>2</th>
<th>3</th>
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<td>97 kDa</td>
<td>M</td>
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<td>3</td>
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<tr>
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<tr>
<td>30 kDa</td>
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<tr>
<td>20.1 kDa</td>
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<td></td>
</tr>
<tr>
<td>14.4 kDa</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

M. Low molecular weight protein markers (LMW).
2. Non-induced sample.
3. Induced sample.
The apparent molecular weight of the expressed protein was 28-29 kDa, as estimated by SDS-PAGE. The theoretical weight for zz-rChII is 26 kDa. Since the difference between the apparent and calculated weight (<10%) was within the expected error for extrapolation of molecular weight from SDS-PAGE, purification was continued.

The fusion protein was enriched by IgG affinity-chromatography, as described by Drevet et al (41), resulting in a yield of 60 mg of crude zz-rChII/L of initial bacterial growth medium (Figure 7).

**Figure 7:** IgG affinity chromatography of zz-rChII. Absorption at 280 nm shown in blue, and conductivity shown in red.

From Figure 8, it is apparent that further purification was required (Figure 9 lane 7) after affinity chromatography, and this was performed by HPLC. The HPLC purification step utilized a reverse phase C4 semi-preparative column (Vydac), and resulted in the separation of four different zz-rChII fractions (Figure 8).

In Figure 9, peaks 1-4 correspond to four populations of zz-rChII (see insert). Reduction with DTT resulted in elimination of this heterogeneity (data not shown), implying that the four zz-rChII populations differ in their disulfide bond arrangement.
**Figure 8**: 15% SDS-PAGE summarizing expression, affinity chromatography and HPLC purification of zz-rChII (only the main two peaks are shown).

<table>
<thead>
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<th>1 2 3 4 5 6 7 8 9 M</th>
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<tbody>
<tr>
<td>97 kDa</td>
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<tr>
<td>30 kDa</td>
</tr>
<tr>
<td>20.1 kDa</td>
</tr>
<tr>
<td>14.4 kDa</td>
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</tbody>
</table>

1. Not-induced sample.
2. Induced sample.
4. IgG column flow-through.
5. Wash 1.
7. IgG column eluted protein
8. First HPLC peak.
M. LMW

**Figure 9**: HPLC chromatogram of zz-rChII (100μg). 220 nM absorption shown in black and acetonitrile percentage concentration shown in red. Insert (top left) 15% SDS-PAGE. Lanes 1-4 correspond to peaks 1-4, respectively.

Thus significant purification of the fusion protein was achieved utilizing an IgG affinity column followed by reverse-phase HPLC (Figure 9).
Fusion protein cleavage
The next step in obtaining ChII was specific proteolytic cleavage of zz-rChII using enterokinase. In order to analyze the cleavage results, SDS-PAGE in the Tris-tricine buffer system was performed, since proteins and peptides with molecular weight under 10-15 kDa are not separated well using the Laemmli discontinuous gel system (36).

Different incubation periods (1-5 h) were tested to optimize proteolytic cleavage (Figure 10).

**Figure 10:** Time-resolved fusion protein cleavage. Tris-tricine-16.5% SDS-PAGE was used to seek the optimal duration of incubation at 37 °C for cleavage. The experiment utilized 5 EKmax™ units and one mg zz-rChII.

![SDS-PAGE Image]

Figure 10 shows that the fusion protein is completely cleaved after 3-4 h incubation with EKmax™. However, non-specific cleavage, resulting in lower ChII yields and more cleavage products than expected, was observed.

Two enzyme concentrations (5 or 10 units) were tested to optimize proteolytic cleavage. For the two main fractions of zz-rChII, separated by HPLC, 5 units of EKmax™ gave a slightly better yield of rChII.

An attempt was also made to improve the proteolysis yield by reducing zz-rChII prior to cleavage. Since four disulfide bonds maintain the toxin in a compact structure, their reduction might expose the peptidase recognition sequence and thus facilitate hydrolysis. However, neither SDS-PAGE nor HPLC (data not shown) revealed any resultant difference in cleavage yield.

Although a wide range of conditions was explored, we were unable to eliminate the non-specific cleavage of zz-rChII by EKmax™. The optimal conditions (1 h incubation at 37 °C with 5 U of EKmax™ per mg of zz-rChII) resulted in 16% of the desired product, or 40μg ChII per mg of fusion protein.
Cleavage product purification

In order to monitor the progress of purification, protein bands separated by SDS-PAGE were analyzed by densitometry after each purification step.

The cleavage mixture was separated by HPLC using the C18 analytical column. Elution was preformed by a linear gradient of aqueous acetonitrile supplemented with 0.1 % TFA; the different peaks were analyzed by Tris-tricine SDS-PAGE. Two proteolysis products possessed similar molecular weights to the desired product. In order to determine which was the desired chimera they were both subjected to N-terminal sequencing (Figure 11).

**Figure 11:** First purification of cleavage products.

A. HPLC chromatogram of cleavage of 1 mg zz-rChII, purified on the C18 analytical column. 220 nm absorption shown in black and acetonitrile percentage shown in red. The relevant peaks were analyzed by 16.5% Tris-tricine SDS-PAGE shown in B. Lanes 1-6 correspond, respectively, to peaks 1-6. At the bottom, N-terminal sequences are shown for two of the bands suspected to be rChII (AA numbering of the rChII sequence).

\[
\begin{align*}
&N\text{-terminal sequencing identified the most intense band as rChII (Figure 11B, lane 3). However, it was determined to be only 35\% of the total protein collected as peak 3. Hence, further purification steps were required to separate rChII from impurities. Several approaches} \\
&\text{were taken in order to reach the >90\% purity desired for x-ray crystallography.} \\
&\text{The most successful approach replaced TFA by 0.1\% NaClO}_4/0.1\% \text{H}_2\text{PO}_4 \text{as the modifier in the mobile phase (46). By interacting both with the stationary phase and the sample constituents, modifiers alter retention selectively, thus allowing separation between sample constituents. Figure 12 shows that 62\% purity was thus achieved. In the final HPLC purification step, aimed at washing out NaClO}_4 \text{from the sample, the toxin was eluted at 90\% purity (Figure 13, insert, lane 2).}
\end{align*}
\]
Figure 12: Second HPLC purification of rChII using 0.1 % NaClO₄, 0.1 % H₃PO₄ as a modifier. 220 nm absorption shown in black and % acetonitrile shown in red. Insert (top left) 16.5% Tris-tricine SDS-PAGE showing the material prior to purification and the relevant peak.

Figure 13: HPLC chromatogram of second purification of rChII after changing the HPLC mobile phase modifier. 220 nm absorption shown in black and acetonitrile percentage shown in red. Insert (top left) 16.5% Tris-tricine SDS-PAGE showing, in lanes 1-3, peaks 1-3, respectively.
Assay of TcAChE inhibition

zz-rChII and rChII were assayed for their capacity to inhibit TcAChE. Both zz-rChII and ChII showed inhibition in the micromolar range (Figure 14). The fact that zz-rChII inhibits AChE supports our previous conclusion that the disulfide bonds necessary for producing the active fold of the toxin had at least partially formed. The two preparations showed 100-fold less inhibition than wild-type Fas II.

Figure 14: Assessing the inhibitory activity of Fas II, zz-rChII and rChII upon TcAChE

Chemical synthesis

Only one major product was obtained upon chemical synthesis of the chimera (Figure 15). SDS-PAGE revealed that the product still requires purification to remove minor impurities (mostly incomplete polypeptide chains), followed by oxidative folding. The synthetic approach has clearly proved to be much quicker and efficient for production of ChII than the biosynthetic approach.
Figure 15: HPLC chromatogram of chemically synthesized ChII (crude), utilizing a C18 reverse phase column, 0.8 ml/min flow. 220 nm absorption shown in red and acetonitrile concentration shown in black. Insert: 16.5% Tris-tricine SDS-PAGE showing the relevant peak.
**Reportable Outcomes and Conclusions**

Our theoretical analysis of the 3D structures of the uncomplexed chimera versus α-tox and FasII showed that the transferred loops maintain their secondary structure. This supports the feasibility of employing protein engineering, using the ‘three-finger toxin’ core as a platform, for generating new ‘mini’-proteins that mimic ‘three-finger toxin’ activity but display modified specificity.

We have succeeded in producing ChII biosynthetically, by expression in *E. coli*, and have been able to reach adequate purity by HPLC purification, though the procedure is somewhat tedious. In the kinetic assays, the chimera showed substantial inhibition of *TcAChE*, even though it was not yet subjected to formation of disulfide bonds by oxidative refolding. It is clear that the poor yield and heterogeneity of the preparation pose a barrier to using the biosynthetic system to produce ChII in adequate amounts for x-ray crystallography.

Since we were not able to overcome the problem of non-specific cleavage we decided to adopt a synthetic approach to production of the polypeptide. Chemical synthesis proved to be much easier for obtaining the chimeric toxin, in good yield and high purity. The product of this synthesis has yet to be purified and folded.

For the long-term objective of finding a procedure for producing biopesticides, chemical synthesis is inadequate since it is an expensive method. The option of using the first procedure we tried will be available once we find a specific peptidase preparation.
References


Appendix:

Degrees Obtained as Supported by this Award

1) Shaya, D. – M.Sc.
CVs of Principal Investigators

Curriculum Vitae

Prof. Joel L. Sussman
Incumbent of the Morton and Gladys Pickman Chair in Structural Biology

Contact Information

Professor Joel L. Sussman
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http://www.weizmann.ac.il/~joel
Work phone: 972-8-934 4531
Fax: 972-8-934 4159
Home phone: 972-8-947 3040

Education

1972 Ph.D. MIT (Biophysics), Cambridge, MA, with Prof. C. Levinthal
1965 B.A. Cornell University (Math & Physics), Ithaca, NY

Professional Experience

2002 - Incumbent of the Morton and Gladys Pickman Chair in Structural Biology
1994-99 Head, Protein Data Bank, Brookhaven National Laboratory, Upton, NY
1992 - Professor, Dept of Structural Biology, Weizmann Institute
1990: John von Neumann Visiting Professor in Residence - Rutgers Univ
1989-92 Visiting Scientist, NCI, Frederick, MD
1988-89 Head, Kimmelman Center for Biomolecular Structure & Assembly, WIS
1984-85 Head, Dept of Structural Chemistry, Weizmann Institute
1982-85 Visiting Scientist, Fox Chase Cancer Center, Philadelphia
1982-84 Visiting Scientist, Lab of Molecular Biology, NIH, Bethesda
1980-92 Associate Prof., Dept of Structural Chemistry, Weizmann Institute
1979: Visiting Prof, Chemistry Dept, UC Berkeley
1976-80 Senior Scientist, Dept of Structural Chemistry, Weizmann Institute
1972-76 Research Associate., Biochemistry, Duke Univ with Prof. S-H Kim
1972: Postdoc, Biochemistry, Hebrew U, Jerusalem, with Prof. Y. Lapidot

Military Service

1980-82 Israel Defense Forces, compulsory service

Honors & Awards

2000 - Honorary Professor, Chinese Academy of Sciences
1994 - Elected Member of European Molecular Biology Organization (EMBO)
1991 - U.S. Army Science Conference Award for outstanding research
1979 - Bergmann Prize - Outstanding Research in Chemistry in Israel
2002 - Director, Israel Structural Proteomics Center

April 2003
**International Positions**

2002 - Member of The European Synchrotron Radiation Facility SAC Committee
2002 - Chairman, Israel Council of Higher Education Bioinformatics Committee
2001 - Member of the Coordinating Committee Int’l Structural Genomics Initiative
2000 - Member of the NIH Task Force on Structural Genomics
2000 - Member of the NIH Council - Structural Genomics Initiative
1998 - Chairman, Israel National Committee for Crystallography
1998 - Member of the Swiss Institute of Bioinformatics SAC Committee
1998 - Member of the Scientific Committee for Middle East Synchrotron - SESAME
1997 - Member of the Synchrotron Priorities Committee EMBL Hamburg Outstation
1997 - Member Israel’s Super Computer Steering Committee
1994-00 Member of the European Molecular Biology Lab (EMBL) SAC Committee
1993-94 ESF Representative to Brookhaven Protein Data Bank Advisory Board
1991-93 President, Israel Crystallographic Society
1991-92 Special consultant to the ESRF (Grenoble) - Biological Crystallography
1987-92 Coordination Committee: ESF Network of Protein Crystallography

**Teaching Experience**

Weizmann Institute of Science – Feinberg Graduate School courses:
Protein Structure and Function
Protein DNA Interactions
Seminar: “Bioinformatics”
2001  Workshop on Protein Crystallography, Oulu: Bioinformatics
1994  Workshop on Protein Crystallography, Turku: Refinement of Macromolecules
1993  ESF School, Como: Refinement Methods in Macromolecular Crystallography
1993  EMBO School, Heidelberg: Refinement Techniques and Map Interpretation
1990  Int'l Crystallography School, Bischenberg: Refinement of Macromolecules
1990  EMBO School, Heidelberg: Protein Cryogenic Crystallography
1988  EMBO-ESF School, Como: Protein Refinement Methods
1984  Int'l Crystallography School, Kyoto: Protein Refinement Methods
1981  EMBO School, Pavia: Mathematical & Physical Aspects of EM & X-rays

**Service Positions at the Weizmann Institute of Science**

2001 - Member of the Weizmann Institute’s Awards Committee
2000 - Coordinator of the Bioinformatics Program, WIS Feinberg Graduate School
1998 - Member of the Weizmann Human Genome Committee
1991 - Member of the Board of Trustees of the Aharon-Katzir Katchalsky Center
1992-95 Member of the Professional Staff Promotions Committee
1992-94 Member of the Ph.D. Thesis Committee of the Feinberg Graduate School
1986-87 Head of the Computing Steering Committee
1984-87 Member of the Faculty Promotions Committee
1981-87 Member of the Computing Steering Committee
1978-81 Member of the Board of Studies of the Feinberg Graduate School

**Current Research Grants & Contracts:**

2002-07 Israel Ministry of Science, “Israel Structural Proteomics Center”. Collaborator Y. Burstein.
2002-05 EC Vth Framework Integrated Project, “Structural Proteomics in Europe (SPINE)” – within the Quality of Life and Management of Living Resources Collaborators: D. Stuart, Oxford Univ (coordinator) with 10 other partners.


2001-04  EU Vth Framework Grant, “European Structural Biology Forum” - within the EC Infrastructure Cooperation Network. Collaborators: Rolf Hilgenfeld, IMB, Jena (coordinator) with 10 other partners.


---

**Ph.D. Students, Postdocs and Visiting Scientists with JLS**

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<th>Ph.D. Students:</th>
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<tr>
<td>Leemor Joshua-Tor</td>
<td>1987-91</td>
<td>Associate Prof. - Cold Spring Harbor Laboratory</td>
</tr>
<tr>
<td>Ron Unger</td>
<td>1987-90</td>
<td>Senior Lecturer - Bar Ilan University, Ramat Gan</td>
</tr>
<tr>
<td>Oded Livnah</td>
<td>1989-93</td>
<td>Senior Lecturer - Hebrew University, Jerusalem</td>
</tr>
<tr>
<td>Orly Dym</td>
<td>1989-93</td>
<td>Research Scientists – Hebrew University, Jerusalem</td>
</tr>
<tr>
<td>Ziv Reich</td>
<td>1991-94</td>
<td>Senior Lecturer – Weizmann Institute of Science</td>
</tr>
<tr>
<td>Mia Raves</td>
<td>1993-97</td>
<td>Research Scientist – Solvay Corp., Utrecht, Holland</td>
</tr>
<tr>
<td>Simone Botti</td>
<td>1996-00</td>
<td>CEO, BioStrX Ltd., Ramat Gan, Israel</td>
</tr>
<tr>
<td>Pazit Bar-On</td>
<td>1997-02</td>
<td>Postdoc – University of California, San Diego, CA</td>
</tr>
<tr>
<td>*Hay Dvir</td>
<td>2000-</td>
<td>Ph.D. Student - Weizmann Institute of Science</td>
</tr>
<tr>
<td>*Lakshmanane Premkumar</td>
<td>2000-</td>
<td>Ph.D. Student - Weizmann Institute of Science</td>
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<tr>
<td>*Tzviya Zeev-Ben-Mordehai</td>
<td>2002-</td>
<td>Ph.D. Student - Weizmann Institute of Science</td>
</tr>
<tr>
<td>*Orna Man</td>
<td>2002-</td>
<td>Ph.D. Student - Weizmann Institute of Science</td>
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<tr>
<td>Ruth Nussinov</td>
<td>1979-80</td>
<td>Professor - Tel Aviv University</td>
</tr>
<tr>
<td>Mark A. Saper</td>
<td>1985-87</td>
<td>Professor - University of Michigan</td>
</tr>
<tr>
<td>Ulrike Wagner</td>
<td>1989-92</td>
<td>Senior Scientist - University of Gratz, Austria</td>
</tr>
<tr>
<td>Kurt Giles</td>
<td>1996-99</td>
<td>Research Professor – UCSF, San Francisco</td>
</tr>
<tr>
<td>Gitay Kryger</td>
<td>1996-00</td>
<td>Research Scientist - Compugen Corp, Tel Aviv</td>
</tr>
<tr>
<td>Harry Greenblatt</td>
<td>1996-</td>
<td>Research Scientist - Weizmann Institute of Science</td>
</tr>
<tr>
<td>*Dawn Wong</td>
<td>2000-</td>
<td>Postdoc - Weizmann Institute of Science</td>
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<tr>
<td>*Edwin Rydberg</td>
<td>2000-</td>
<td>Postdoc - Weizmann Institute of Science</td>
</tr>
<tr>
<td>*Vijayabaskar Veerappan</td>
<td>2001-03</td>
<td>Postdoc - Weizmann Institute of Science</td>
</tr>
<tr>
<td>*Ofer Yifrach</td>
<td>2002-</td>
<td>Postdoc - Weizmann Institute of Science</td>
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<tr>
<td>Name</td>
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</tr>
<tr>
<td>Judith G. Voet</td>
<td>1992, 97</td>
<td>Professor – Swarthmore College, NJ</td>
</tr>
<tr>
<td>Donald Voet</td>
<td>1992, 97</td>
<td>Professor – Univ of Pennsylvania, PA</td>
</tr>
<tr>
<td>Paul H. Axelsen</td>
<td>1993-94</td>
<td>Professor - Univ of Pennsylvania, PA</td>
</tr>
<tr>
<td>Charles B. Millard</td>
<td>1997-99</td>
<td>Research Scientist - USAMRIID, Frederick, MD</td>
</tr>
<tr>
<td>Gertraud Koellner</td>
<td>1998</td>
<td>Research Scientist – Freie Univ of Berlin</td>
</tr>
<tr>
<td>Thomas Steiner</td>
<td>1998</td>
<td>Research Scientist – Freie Univ of Berlin</td>
</tr>
<tr>
<td>Mitch Guss</td>
<td>1998</td>
<td>Professor – Univ of Sydney, Australia</td>
</tr>
<tr>
<td>Arthur Grollman</td>
<td>1999</td>
<td>Professor – State Univ of Stony Brook, NY</td>
</tr>
<tr>
<td>Hua Liang Jiang</td>
<td>1999</td>
<td>Professor - Shanghai Inst of Materia Medica, CAS</td>
</tr>
<tr>
<td>Terry Lewis</td>
<td>2000</td>
<td>Research Scientist, Zeneca Agrochemicals</td>
</tr>
</tbody>
</table>
ISRAEL SILMAN

December, 2002

CURRICULUM VITAE

Date and Place of Birth: December 31, 1935; Shipley, United Kingdom.
Marital Status: Married, with one child.
In Israel since 1950.

Education
1953-55 Service in Israel Defence Forces.
1955-59 Undergraduate at Hebrew University, Jerusalem.
    Major subjects: Biochemistry and Organic Chemistry.
    Minor subjects: Physical and Inorganic Chemistry and
    Microbiology.
    M.Sc. Thesis under the supervision of Prof. Ephraim Katchalski.
1960 Received M.Sc. degree from Hebrew University, Jerusalem.
1959-64 Graduate student under the supervision of Prof. Ephraim Katchalski in the
    Department of Biophysics, Weizmann Institute, Rehovot.
1964 Awarded Ph.D. degree from Hebrew University, Jerusalem.

Principal Academic Positions and Activities
1964-68 Research Assistant, Dept. of Biophysics, Weizmann Institute.
1965-66 Fulbright-Hayes Fellow.
1965-66 Project Associate, Institute for Enzyme Research, University of Wisconsin,
    Madison, Wisconsin, in the laboratory of Dr. David Green.
1966-68 Research Associate, Dept. of Neurology, College of Physicians & Surgeons of
    Columbia University, New York, in the laboratory of Prof. David
    Nachmansohn.
1968-71 Research Associate, Dept. of Biophysics, Weizmann Institute.
1971-75 Senior Scientist, Dept. of Biophysics, Weizmann Institute.
1976-82 Associate Professor, Dept. of Neurobiology, Weizmann Institute.
1977-79 Sabbatical leave, Dept. of Biochemistry, Imperial College of Science and
    Technology, London, as a Senior EMBO Fellow and as a Senior Investigator
    of the Muscular Dystrophy Association of America.
1982- Professor, Dept. of Neurobiology, Weizmann Institute.
1982 Imperial College–Weizmann Institute Exchange Professor.
1984-85 Sabbatical leave, Division of Biochemistry, Walter Reed Army Institute of
    Research, Washington, D.C., as a Senior Research Associate of the U.S.
    National Research Council.
1984 Visiting Professor, Institute of Biochemistry, University of Ancona.
1985- Bernstein–Mason Professor of Neurochemistry, Weizmann Institute.
1986  Coorganizer (with Eric Barnard and Uri Littauer), Aharon Katzir-Katchalsky Conference on Molecular Neurobiology, Maidstone, U.K.

1987  Chairman of Organizing Committee, Satellite Symposium of 9th International Biophysics Congress on Structural and Functional Aspects of the Cholinergic Synapse, Neve Ilan, Israel.
      Coorganizer (with Clement Bordier and Urs Brodbeck), EMBO Workshop on Post translational Modification of Proteins by Lipids, Les Diablerets, Switzerland.

1988-91  Chairman, Dept. of Neurobiology, Weizmann Institute.

1990  Coorganizer (with Israel Pecht), 1st Joseph Cohn Workshop on Membrane Lipids: Biophysics & Biochemistry, Ein Gedi, Israel.
      Coorganizer (with Jonathan Gershoni and Ferdinand Hucho), International Symposium on the Cholinergic Synapse, Berlin, Germany.

1991-92  Sabbatical leave, Laboratoire de Neurobiologie, Ecole Normale Supérieure, Paris, as a visiting member of the CNRS.

1992  Coorganizer (with Michel Lazdunski and Uriel Littauer), Weizmann/Côte d'Azur Workshop on Molecular and Cellular Neurobiology, Nice, France.

1992-95  Chairman, Life Science Faculties Promotion Committee, Weizmann Institute.

1992-98  Director, Benoziyo Center for Neurosciences, Weizmann Institute.

1993-97  Visiting Professor, Laboratoire de Neurobiologie, Ecole Normale Supérieure.

1994-99  Research Scholar, Dept. of Biology, Brookhaven National Laboratory.

1994  Plenary Lecturer, 9th Annual Meeting, Federation of Brazilian Societies for Experimental Biology, Caxambú, Brazil.
      Member of International Scientific Advisory Committee, 5th International Meeting on Cholinesterases, Madras, India.

1995  Coorganizer (with Michel Lazdunski and Uriel Littauer), Weizmann/Côte d'Azur Workshop on Molecular and Cellular Neurobiology, Rehovot, Israel.

1997-98  President, Israel Society for Neuroscience.

1998  I.B. Wilson Plenary Lecturer, 6th International Meeting on Cholinesterases, San Diego, CA.
      Member of International Scientific Advisory Committee, 6th International Meeting on Cholinesterases, San Diego, CA.
      Member of Scientific Committee, 3rd International Meeting on Esterases Reacting with Organophosphorus Compounds, Dubrovnik, Croatia.
      Member of International Advisory Committee, Xth International Symposium on Cholinergic Mechanisms, Arcachon, France.

1999  Distinguished Neuroscientists Seminar Series Lecturer, Hong Kong University of Science & Technology, Kowloon, Hong Kong.
      Cochairman (with Hermena Soreq), International Program Committee, 5th IBRO World Congress of Neuroscience, Jerusalem, Israel.

1999-2001  Visiting Professor, Université de Grenoble, Grenoble, France.

2000  Professor, Honoris Causa, Institute of Materia Medica, Chinese Academy of Science, Shanghai, China.
2001  Member of International Advisory Board, Molecular Neuroscience Center of Hong Kong University of Science & Technology, Kowloon, Hong Kong.
2002  Coordinator and Member of the Organizing Committee, XIth International Symposium on Cholinergic Mechanisms, St. Moritz, Switzerland.
2002  Member of International Organizing Committee, VIIth International Meeting on Cholinesterases, Pucon, Chile