A case study in applying docking predictions: Modelling the tentoxin binding sites of chloroplast F1-ATPase

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The study presents an ab initio approach for locating a ligand-binding site and demonstrates that relevant conclusions can be deduced from multiple, predicted ligand positions. Tentoxin is a specific inhibitor of plastid CF1-ATPase, its interaction with the α and β-subunits of the enzyme resulting in complex interference with ATPase activity. The uniquely different conformations of the β-subunits in the quasi-symmetrical structure of the F1-ATPase from bovine mitochondria offer an opportunity to model tentoxin binding at multiple sites in plastid CF1-ATPase. Using software for molecular docking, we located and analysed three putative binding sites with approximately equally high complementarity to tentoxin. Complementarity at these sites is sensitive to the nucleotide occupancy state of the β-subunit. The main interactions stabilizing the putative complexes were determined, and homology models for the tentoxin-binding sites of Chlamydomonas plastid CF1-ATPase were created. The predicted binding pocket residues for Site I are at the αβ/βγ interface and include residue Glu-67βγ (codon 83β in plastid CF1-ATPase), previously identified as a molecular-genetic determinant for the high affinity, inhibitory response to tentoxin in Chlamydomonas. This site overlaps with that suggested by others, but ~50% of residues differ. Sites II and III, possibly related to low-affinity binding of tentoxin, are located in functionally active regions: one entirely within the αβ-subunit and sharing a residue with the αβ nucleotide-binding site; the other at the αγ/βγ/γ interface and sharing a residue with the conserved DELSEED sequence. Non-catalytic residues in these putative pockets represent potential targets for mutational analysis.

ANY molecular docking approach trying to predict the structure of a protein–ligand complex faces three major problems: locating the approximate position of the binding site, generating a computationally sufficient number of ligand positions within the binding site, and defining the most probable position for the ligand. Molecular biologists are mainly interested in answering the last point in order to analyse interactions that stabilize the complex. This analysis permits them to predict desired modifications of the ligand (ligand design) and/or protein (protein engineering) in order to regulate the binding. In many, but not all cases, researchers can rely on external experimental information for locating the ligand-binding site. In addition, established docking procedures exist for generating and scoring ligand positions within a presumptive binding site, but in most cases several positions appear to have the same probability. In this study, we present an ab initio approach for locating a binding site and demonstrate that relevant conclusions can be deduced from multiple, predicted ligand positions.

F0F1-ATPase (ATP synthase) is a central enzyme in energy conversion in chloroplasts, mitochondria and bacteria. The main reaction catalysed by this enzyme is ATP formation from ADP and Pi, using energy derived from a transmembrane electrochemical potential gradient. In chloroplasts, a transmembrane electrochemical proton potential difference is built up by photosynthetic electron transport. The soluble part of the proton ATPase (F1 in mitochondria and CF1 in chloroplasts) in the isolated form catalyses the reverse reaction, ATP hydrolysis. The activity of F1 or CF1, both in the forward and backward direction, may be perturbed by specific inhibitors or activators. Among well-studied examples are complexes of bovine mitochondrial F1-ATPase with aurovertin, efrapeptin, nitrozobenzofurazan, dicyclohexylcarbodiimide and non-hydrolysable ATP analogues. Clarification of the interaction of highly specific inhibitors with CF1 or F1 offers a powerful means to unravel the complex structural dynamics associated with ATP formation.

Tentoxin (cyclo-[L-leucyl-N-methyl-(Z)-dehydrophe- nylalanyl-glycyl-N-methyl-alanyl]), a naturally-occurring tetrapeptide, affects chloroplast CF1-ATPase activity of sensitive plant species in a biphasic manner. Non-competitively, it inhibits ATP hydrolysis and phosphorylation at low concentrations (10⁻⁵ to 10⁻⁷ M), while stimulating ATP hydrolysis at higher concentrations (10⁻³ to 10⁻² M). The stimulatory effect has been observed in isolated CF1, in thylakoid membranes, and in
reconstituted proteoliposomes. Tentoxin has not been found to inhibit mitochondrial or bacterial ATPase and does not inhibit plastid ATPase of tentoxin-resistant photosynthetic species. Therefore, tentoxin may be utilized to analyse subtle structural differences in catalytic domains of the enzyme.

The mechanism of tentoxin inhibition and reactivation is not well understood and the number of binding sites involved is controversial. Equilibrium dialysis with synthetic tentoxin analogues and radiolabelled tentoxin revealed two binding sites with high and low affinities, that were correlated with the inhibitory and stimulatory effects respectively. The existence of a third, very-low-affinity binding site that could account for the stimulatory effect has also been suggested. In early studies, the inhibitory, high-affinity site of tentoxin was localized to the α and β-subunits of CF1. More recently, Avni et al. identified codon 83 of the β-subunit as a specific site which confers differential sensitivity to tentoxin in the genus Nicotiana. This conclusion was substantiated by mutagenesis of the corresponding site in the tentoxin-insensitive, transformable green alga Chlamydomonas and in the Rhodospirillum rubrum chromatophore reconstitution system. Thus, the 83β site offers a natural starting point in the search for high-affinity binding pocket. The location(s) of the low affinity site(s), however, is more obscure. On the basis of kinetic and equilibrium experiments, an interaction of the low affinity site with the nucleotide-binding sites has recently been proposed, involving a complex relationship between the catalytic state and tentoxin-induced F1-ATPase stimulation. An alternate proposal, based on heterologous chromatophore reconstitution experiments, implicates codon 83β in the low, as well as high, affinity site.

A perusal of the protein database (PDB) using LPC software readily reveals that a ligand of the size of tentoxin invariably contacts multiple residues. Thus, a structural analysis of the tentoxin-binding sites could add considerably to the existing molecular–genetic and biochemical studies and deepen our understanding of the mechanism by which the toxin inhibits or stimulates chloroplast CF1-ATPase. The structures of mitochondrial and bacterial ATPases have been reported. The structure of the αβγ complex of F1-ATPase from bovine heart mitochondria shows that the three catalytic β-subunits differ in conformation and occupancy of the nucleotide-binding site. The structures of the nucleotide-free αβγ complex from chloroplast CF1-ATPase of spinach and αβ complex of F1-ATPase from the thermophilic Bacillus PS4 were determined by molecular replacement based on the structure of the bovine mitochondrial enzyme. The αβγ complex of F1-ATPase from rat liver mitochondria has all catalytic and non-catalytic sites occupied with nucleotide.

In all these studies, the overall structure and sequence of the catalytic α and β-subunits are highly similar. Indeed, the primary sequences of mitochondrial and plastid α and β-subunits are respectively, ~55 and 70% identical. In particular, a BLAST-P scan of non-redundant F1-ATPase β-subunit sequences from plastids, mitochondria and bacteria reveals >100 files, all of which contain a conserved negative charge at the 83β site.

We have chosen to model the binding site(s) of tentoxin to CF1-ATPase based on the structure of the F1-ATPase from bovine heart mitochondria. The uniquely different conformations of the β-subunits in this quasi-symmetrical structure offer an opportunity to model tentoxin-binding at multiple sites in CF1-ATPase. In this study, we search for theoretical binding sites of the toxin and model the consequences of complex formation. We dock the toxin in the resolved structures, predict the main stabilizing interactions of the resulting putative complexes, and create homology models for the tentoxin-binding site(s) of plastid CF1-ATPase.

Recently, Groth has resolved the structure of spinach CF1-ATPase complexed with tentoxin to a resolution of 3.4 Å. The crystal structure confirms the general location of the high-affinity binding site in the codon 83β pocket, but the weak and averaged electron density did not allow an accurate pinpointing of the inhibitor. The docking data presented by our predictive methodology seem more relevant as regards the geometry of the ligand, the absence of ligand–protein bumping, and the fitting to the shape of the cavities. Interestingly, the fitting found by us at the codon 83β site is significantly shifted towards the α-subunit, in agreement with other biochemical data. Importantly, our predictions, based on the quasi-symmetrical bovine mitochondrial structure, contain original assumptions on the location of low-affinity binding sites in catalytic zones of the complex. These sites were not resolved in the crystal structure of the nucleotide-free CF1-tentoxin complex.

Results

Probe cavities and ligand docking

The entire structure (minus water molecules) of the bovine mitochondrial crystal of the PDB file 1cow was pre-scanned with a probe molecule consisting of the ten toxin ring without methyl-dehydrophenylalanyl and methyl-alanyl side chains. This procedure enabled us to avoid considering ligand side-chain orientations and to retain tight pockets. Since the volume and shape of the ring depended slightly on its conformation, we used only the prevalent A-conformer. In essence, the scan searched for cavities into which the backbone ring of tentoxin could fit sterically. Scanning was performed...
using LIGIN\textsuperscript{10} and assuming all ligand atoms as neutral. The various approximations reduced the time required for docking the probe molecule vs tentoxin itself by $\sim$ 250-fold. The three-dimensional space of the 1cow $\alpha_\beta\gamma$ crystal was divided into $\sim$ 1500 cubes, and the probe was docked in each. Only probe positions separated by 5 Å or more were tallied. The resulting list of cavities, in decreasing order of complementarity, contained 837 members, including those completely burying the probe molecule as well as some with only a few probe atoms in contact with the protein.

We docked all 22 conformations (see the section on ‘Materials and methods’) of tentoxin into the 230 highest-scoring probe cavities. For each cavity, the docked conformation yielding the highest complementarity value was scored. A small number (3\%) of scored structures were discarded due to ligand–protein bumping. Those remaining, ranged in complementarity values from 454 to 226 Å\textsuperscript{2}. All docked conformations in the top 10 percentile were analysed. Three unique sites were found: Site I (probe-cavity 5; complementarity to tentoxin, 445 Å\textsuperscript{2}), formed by $\alpha_\beta$- and $\beta_\gamma$-subunits; Site II (probe-cavity 22; complementarity, 437 Å\textsuperscript{2}), formed by $\alpha_\beta$-subunit alone; Site III (probe-cavity 59; complementarity, 454 Å\textsuperscript{2}), formed by $\alpha_\beta$, $\beta_\gamma$ and $\gamma$. All other structures in the top ten percentile range were lower value variants of Site III. Putative sites in the next ten percentile range were of lower complementarity and are not presented here. (Their location and LPC analysis can be found at http://sgedg.weizmann.ac.il/tentoxin/).

A set of residues forming a binding site is composed of those residues that are in contact with tentoxin in at least one of the 22 conformations docked at that site. The set of residues forming Site I is listed in the leftmost column of Figure 1, while the set of residues forming Site II is: $\alpha_\beta$ (A152, L156, E353, E355, L356, K359, I361, P363, I365, N366, V367, G368, L369, L394, Y397, R398, A401, A402, F403, V422, T425, L428); and that forming Site III is: $\alpha_\beta$ (F406, S408), $\beta_\gamma$ (S383, D386, I387, I390, L391, E395) and $\gamma$ (K24, M25, A28, Y31, A32, E35, M229, T230, D233, N234, K237). Sites I and II take the form of pockets in which the $\sim$ 600 Å\textsuperscript{2} surface of the tentoxin molecule is $\sim$ 90\% complexed with the protein. Site III forms a niche in which the toxin molecule fits with high complementarity, but remains $\sim$ 40\% surface-exposed.

The predicted positions having the highest complementarity for each ring form of tentoxin at Site I are displayed in Figure 1. Site I, which includes E67$\beta_\gamma$ (CFI codon 83$\beta_\gamma$), is spacious, the ligand contacting somewhat different subsets of residues in each case (Figure 1 a–d). Those residues common to all the four ring structures (Figure 1 e) are the most probable to be in contact with tentoxin at this site. The schematic locations of Sites I, II and III within the $\alpha_\beta\gamma$ crystal structure are shown in Figure 2.
Docking of tentoxin at each of the three Site I interfaces 
Tentoxin binding in quasi-symmetrical cavities likely that during functional rotation 
tional counterparts in the crystal structure. However, it is partially with the unique 
binding sites than Sites I and II. Site III, which interfaces 
point cavities are considerably less fit as potential toxin-
(Figure 2). The highest complementarity to tentoxin 
and similar folding topologies arise from partial homology 
ther level of symmetry, arising from partial homology 
for Site I, at the empty nucleotide-binding site. The hypothetical tentoxin-binding sites are shown. Site I, formed by the αTP/βTP interface has ‘sister’ cavities at the αTP/αTP and αTP/βTP interfaces. Site II, in the αTP-subunit, has ‘sister’ cavities in subunits αTP and αTP. There are, altogether, an additional six counterpoint cavities (drawn but not marked) which emanate from similarities in sequence and overall structure between the α and β-subunits. Site III is located at the αTP/βTP/γTP interface.

Symmetries in F1-ATPase
Due to the quasi 3-fold symmetry of the bovine αβγ structure and similar folding topologies of α and β-subunits, adds a set of counterpoint cavities to Sites I and II (cf., Figure 2). The highest complementarity to tentoxin among these is for cavities at the αTP/αTP interface (382 Å²) and in the βTP-subunit (349 Å²). The counterpoint cavities are considerably less fit as potential toxin-binding sites thanSites I and II. Site III, which interfaces partially with the unique γ-subunit, does not have additional counterparts in the crystal structure. However, it is likely that during functional rotation complementary cavities do form at the various αTP/βTP/γTP interfaces.

Tentoxin binding in quasi-symmetrical cavities
Docking of tentoxin at each of the three Site I interfaces (αTP/βTP, αTP/βTP and αTP/βTP) revealed different comple-
mentarities (Table 1). Consequently, the three Site I pockets are not identical. To measure their differences, we superimposed all backbone and side chain atoms of the residues forming the sites. The results showed that the interface cavity of αTP/βTP differs from that of αTP/βTP by a relatively small degree (RMSD = 0.81 Å for backbone atoms and 0.91 Å for backbone plus side chain atoms), while the same αTP/βTP cavity differs from that of αTP/βTP by a larger amount (RMSD = 1.78 Å for backbone atoms and 1.96 Å for backbone plus side chain atoms). Thus, the inherent flexibility of the αTPβTPγ molecule is manifested also by measurement of the Site I region. A second major conclusion from Table 1 is that for all four tentoxin ring structures analysed, Site I at the αTP/βTP interface has the highest complementarity and, consequently, the highest probability to be occupied. In contrast, Site I at the αTP/βTP cavity has the lowest complementarity and, therefore, the lowest probability to be occupied.

Docking tentoxin into the symmetrical Site II cavities of αTP, αTP- and αTP-subunits also produced different complementarities (Table 1). The main conclusion drawn is that the cavity formed in the αTP-subunit has the lowest probability to be occupied by tentoxin, while the remaining two Site II cavities are occupied with approximately the same probability. Site II in the αTP-subunit is favoured since it shows the highest complementarity (437 Å² for ring conformation B; Table 1).

As already noted, Site III is formed in part by residues of the unique γ-subunit and, therefore, lacks quasi-symmetry at the five other interfaces of the αTPβTPγ lattice. The highest complementarity (454 Å²) is for the B-form of the tentoxin ring (Table 1).

Applying the modelling results to CF1-ATPase
The conclusions drawn from modelling binding sites for tentoxin in F1-ATPase were applied to modelling the analogous sites in CF1-ATPase. The amino acid sequences of the α, β and γ-subunits of F1- and CF1-ATPase from bovine mitochondria and the chloroplast of Chlamydomonas were aligned (Figure 3) and compared with regard to their respective, putative tentoxin-binding site residues. Site I residues (located in αTP- and βTP-subunits) are highlighted in yellow. Site II residues (located solely in the αTP-subunit) are highlighted in cyan. Site III residues (located in αTP-, βTP- and γ-subunits) are highlighted in green. The degree of matched F1 and CF1 residues at the three binding sites matched the general level of homology for the relevant subunits of the two ATPases.

The main interactions stabilizing complex formation with tentoxin were analysed. Table 2 lists the relevant residues in the mitochondrial and chloroplast subunits for Site I. Putative hydrogen bonds, or hydrophobic-
Table 1. Complementarity ($\AA^2$) of tentoxin at docked binding sites

<table>
<thead>
<tr>
<th>Tentoxin ring conformation</th>
<th>Site I</th>
<th>Site II</th>
<th>Site III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1/\beta_T$</td>
<td>$\alpha_1/\beta_T$</td>
<td>$\alpha_1/\beta_T$</td>
</tr>
<tr>
<td>A</td>
<td>445</td>
<td>345</td>
<td>241</td>
</tr>
<tr>
<td>B</td>
<td>414</td>
<td>296</td>
<td>195</td>
</tr>
<tr>
<td>C</td>
<td>408</td>
<td>286</td>
<td>146</td>
</tr>
<tr>
<td>D</td>
<td>443</td>
<td>301</td>
<td>255</td>
</tr>
</tbody>
</table>

$\alpha_1/\beta_T$ $\alpha_1/\beta_T$ $\alpha_1/\beta_T$ $\alpha_1/\beta_T$

$|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|$ $|
out of the four ring conformations (Table 4). However, all of the ten putative, hydrophobic–hydrophobic contacts score positive for either four out of four or three out of four ring conformations (Table 4).

**Discussion**

**Biological relevance of Sites I, II and III**

In this study, we analysed the putative binding site(s) for tentoxin in plastid CF1-ATPase based on the structure of bovine mitochondrial F1-ATPase. Docking was to the whole volume of the crystallographic asymmetric unit of the unit cell ($\alpha_6\beta_6\gamma$), without any bias for a particular location. Currently, such docking procedures can readily result in several cavities with a similar level of complementarity and, indeed, this was the case in our study. Independent experimental data are, therefore, important for evaluating and deciding among the various possibilities.

Site I includes residue Glu-67$_{\beta_T}$, which corresponds to Glu-83$_{\beta_T}$ in plastid CF1-ATPase (cf., Figure 3b). Avni et al.\textsuperscript{17} proved, and others have since confirmed\textsuperscript{18,19}, the molecular–genetic importance of this residue in the inhibitory, high-affinity response of CF1 to tentoxin. Moreover, tentoxin is a non-competitive inhibitor of photophosphorylation\textsuperscript{9}; thus, the high-affinity site should be located out of the catalytic nucleotide-binding site. Indeed, Glu-67$_{\beta_T}$ is situated in the crown region of the $\beta$-subunit, spatially distinct from the catalytic site\textsuperscript{23}. Aside from 67$_{\beta_T}$ in Site I, whose phylogenetic amino acid composition was previously used to distinguish between tentoxin resistance and sensitivity\textsuperscript{17}, we do not find another residue at any of the three binding sites described here, that readily distinguishes between toxin sensitivity and resistance.

Not surprisingly, several studies have used codon 83$_{\beta}$ as a starting point in an attempt to derive the composition of the high-affinity tentoxin site. Based on the structure of bovine mitochondrial F1-ATPase, Santolini et al.\textsuperscript{14}...
**b β-Subunits**

**CHL**

MPWGILPLTMSDSEITKMNMGRIQVIIGVLIVDFAKQGQVIPNYNALTIRA2KNSAGTEMA 60
AAQAPSPKANKAGTRLVIIAGAVDVQFDEGLPPILNALEVQGR.....ETR 44

**MIT**

VTCEVQLLGDNCVRAVSMNPAGESTLGLMRGMEVDTGKPLSPVGVKVT2NGRIVNLGEVDPDN 120
LVLEVAQHLGESTVTIAMDCGTEILVRGQKVLDVSGAPIRIPVGPETLGRIMNGEPIDE 104

**CHL**

MGNVKVEETLPIHTRAPAFVDLDDLRTIFSETGIKVVDLLAPYRRGKGLFGGAAGVGTKV 180
RGPPIKTKQFAAIHAEAEFVMVEQEEIIIVRTKIVKVDLLAPYAKGGKIGLGGAGGAGVGTKV 164

**MIT**

LIMELINNAKAHGQVVFAGVGETREGNDLYTEMKESGIVKEKSLSDKSVALVYQGMN 240
LIMELININAVAHGGYVSFAGVGETREGNDLYHMEMIESGIVNLKDATSKVALVYQGMN 223

**CHL**

EPPGARMRVALTALIMAELYFDRNKQDVFIFINDFVRFVQAGAESVSALELGMPAVGYQP 300
EPPGARAVALTGTIVAYFDRQGQVFIVLFIINDFVTQAGSEVSALELGMPAVGYQP 283

**MIT**

TLATEMGLQERITSTKDGSITSIQAVYVPAADDLDPADAPATFFAHDLATTVLSRNLAAG 360
TLATDMGMQERITTTKKGSITSIQAVYVPAADDLDPADAPATFFAHDLATTVLSRAIAE 343

**CHL**

IYPADVPLESTMLQPWIL3GKHEDSAQSVKKTQLRKEQDIIAILKELYELSTDL 420
MYPADVPLDLSTIRMPGIVCHDVARQKILQDLYKSLDIAILKELYELSTDL 403

**MIT**

VARARKIERFLSQQPFVAVFSTGSPKGYVSLAETIEFGRIFAGELEDPLPQFAFVGFLNVGI 480

**CHL**

VARARKIERFLSQQPFVAVFSTGSPKGYVSLAETIEFGRIFAGELEDPLPQFAFVGFLN 463

**MIT**

VARARKIERFLSQQPFVAVFSTGSPKGYVSLAETIEFGRIFAGELEDPLPQFAFVGFLN 463

**CHL**

TEAISKAASLK 491
MIT EEAVALAKDLA 474

**c γ-Subunits**

**CHL**

MAAMLASKQGAFMGRSSFAPAPKGVASREGLSLQVVAQLKEVRDRIASVNTQKIDAMKLV 60
ATLKDITRRLSLQNIKQITKSMRV 26

**MIT**

AAKVRACAVVNGRPFSENLVKYLGVNQRQRQEDVDSPLCAVRPVKSVLIVLVTGDR 120
AAKVRADFELKPAVPVYVGSGSDYEDK.............PEDKHKILIGIVSRSML 104

**CHL**

GLCAGIIIHSVEAKQXMEANAAABGGKEKIIIVGDKNRTSESDQFLTVFKEKVRPP 186

**MIT**

GLCAIIHSVEAKQXMEANAAABGGKEKIIIVGDKNRTSESDQFLTVFKEKVRPP 186

**CHL**

PSTKEAQGIADEIFASFASESQEDKVLEFTKISLINSNPTQIITLPMTPGSELDVGDG 238

**MIT**

PSTKEAQGIADEIFASFASESQEDKVLEFTKISLINSNPTQIITLPMTPGSELDVGDG 238

**CHL**

CVDADDEIFKLTGKEFAVEREKTITTEATEALPDLSLIFEQEPAAQLDALLPLYMSSCLL 298

**MIT**

CVDADDEIFKLTGKEFAVEREKTITTEATEALPDLSLIFEQEPAAQLDALLPLYMSSCLL 298

**CHL**

RSLQFALASEAIALAARVAINDNAKELKGGKLTQYMKQRAKIKTIQLIEAIVGGAATG 358

**MIT**

RSLQFALASEAIALAARVAINDNAKELKGGKLTQYMKQRAKIKTIQLIEAIVGGAATG 358

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**Figure 3 b, c.** Alignment of CF1- and F1-ATPase sequences from *Chlamydomonas reinhardtii* chloroplasts (CHL) and bovine heart mitochondria (MIT). Numbering for CHL sequences as in SwissProt (accession numbers P26526, P06541, P12113). Numbering for MIT sequences as in the ATOM section of PDB file 1bmf. Site I residues are highlighted in yellow and Site III residues in green. The ANP (adenyllyl-imidodiphosphate)-binding site residues at the αβ/β′ interface are underscored in red. The DELSEED motif in the β-subunit is underlined in purple.
suggested the involvement of $\alpha_{TP}$- and $\beta_{TP}$-subunits and residue Tyr-300$\alpha_{TP}$ in the inhibitory binding site. Groth and Pohl\textsuperscript{26} and Tucker \textit{et al}.\textsuperscript{19,20} assumed codon 83$\beta$ as the centre of the binding pocket. Residues at a radius of 10 Å from 83$\beta$ were taken as putative residues forming the inhibitory, high-affinity tentoxin-binding site. However, residue 83$\beta$ is located at a wall, not the centre, of the cavity. We calculated the distance between the average geometric centre of docked tentoxin in Site I (considering rings A–D) and the C$\alpha$ atom of residue Glu-67$\beta$.

Table 3. Site II residues at the $\alpha_{TP}$-subunit forming potential hydrogen bonds or hydrophobic contacts with tentoxin

<table>
<thead>
<tr>
<th>Tentoxin ring conformation$^a$</th>
<th>Bovine mitochondria</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Chlamydomonas chloroplast$^b$</th>
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<td>Potential hydrogen bonds</td>
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<td>Potential hydrophobic contacts</td>
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$^a$See comment in Table 2.
$^b$As aligned in Figure 3a.

Table 4. Site III residues at the $\alpha_{TP}$/\$\beta$/\$\gamma$/interface forming potential hydrogen bonds or hydrophobic contacts with tentoxin

<table>
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<tr>
<th>Tentoxin ring conformation$^a$</th>
<th>Bovine mitochondria</th>
<th>Subunit</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Chlamydomonas chloroplast$^b$</th>
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$^a$See comment in Table 2.
$^b$As aligned in Figure 3a–c.
(codon 83β in CF1), and found it to be > 8 Å. Thus, Site I in our study and the binding site suggested by Groth and Pohl26 and Tucker et al.19,20 are displaced by almost an entire cavity radius. Although both sites include approximately the same number of residues (32 in our study and 31 in Groth and Pohl26), only 16 residues overlap (cf., Figure 1 e). Correspondingly, a number of Site I residues are more than 10 Å from codon 83β while several residues listed by Groth and Pohl26 are located in the protein core and have solvent-accessible surfaces equal to zero.

The full set of 32 residues forming Site I (cf., Figure 1) was obtained by summarizing all residues in contact with tentoxin at least once in the 22 structures docked at this site. However, for any given conformation, tentoxin is in contact with only a subset of these. A number of residues are common to all four ring structures (Figure 1 e) and these are prime candidates for molecular-genetic manipulation. On the other hand, manipulation of residues not in direct contact with the ligand in a given conformation (those coloured green in Figure 1 a–d) may enable subtle changes in cavity structure. Mutation of such residues is unlikely to dramatically affect the protein structure, as they are situated at the cavity surface and not in the protein core.

Based on early experimental data discussed in Pick et al.11, the low-affinity binding site of tentoxin was postulated to be close to the nucleotide-binding sites. Indeed, recent analysis of the kinetics of tentoxin-binding furnishes strong evidence for an interaction between the low-affinity site and the nucleotide-binding ones14. The location of putative Site II in this study (cf., Figure 2) is compatible with this description. Analysis of the 1bmf αβγ structure shows that Site II has a residue (Pro-363δ) in common with the nucleotide-binding pocket of the αγδ-subunit (Figure 3 a).

Biological input exists as well for putative Site III. This site overlaps with residues forming an energy-coupling region between γ and β-subunits in F1-ATPase. Site III residue Glu-395β (corresponding to Glu-412β in CF1) is in the conserved sequence motif DELSEED23 (Figure 3 b). Also, Site III residue Lys-24γ (corresponding to Lys-58γ in CF1) is the nearest neighbour to Met-23γ a residue that plays a key role in energy coupling37, although not rotation38,39 of F1-ATPase. It has been established that high concentrations of tentoxin lead to over-energization of the thylakoid membrane, possibly through interaction with a tentoxin low-affinity binding site40. Thus, Site III also potentially satisfies the requirement of a low-affinity site. As opposed to Sites I and II, which are embedded pockets, Site III is partially surface-exposed and has positional potential for interaction with other macromolecular components, particularly the δ and ε-subunits (and unresolved sections of γ of FI itself). However, our visual analysis of the detailed structure of the central stalk in bovine F1-ATPase indicates that Site III is indeed a surface-exposed niche in which tentoxin can potentially be situated.

**Sites I–III and rotation of the γ-subunit**

There is no direct structural evidence as to how tentoxin binding influences ATPase function. One may speculate9,12 that flexibility of the Site I region is important for function and that by binding, tentoxin freezes this region, possibly by restricting conformational mobility in a manner similar to that suggested for efrapeptin in inhibiting F1-ATPase3,41. Considerable experimental data support the rotation of the γ-subunit during ATPase function33,36,42. The question arises: does tentoxin affect function by interfering with γrotation? It has been shown that tentoxin does not inhibit αβ functions in the absence of the γ-subunit41. Our study shows that Site I residues are not in direct contact with the γ-subunit. However, we can identify several residues dually contacting both the Site I pocket and γ-subunit. Thus, blockage at Site I is structurally feasible. We have shown that Site II peripherally overlaps with the αγδ-subunit nucleotide-binding site, and Site III with the γ-subunit (at residues distinct from those interacting with the βDELSEED sequence; compare Hara et al.38 and Figure 3 c). Thus, blockage at these sites is potentially feasible as well. Theoretical arguments notwithstanding, the actual relationship, if any, of tentoxin binding to γ-subunit rotation needs to be clarified by mutational studies. As some of the hypothetical sites are in the vicinity of regions that play crucial roles in catalysis, only mutations not affecting these activities can be tested.

**Materials and methods**

**F1-ATPase structures analysed**

PDB file 1cow (ref. 2) was used for cavity searching and 1bmf (ref. 23) for docking. Following Abrahams et al.23 the seven peptide chains in these crystals are referred to as αγδ, αβγδ, βγδ, δγδ, βγδ and γ.

**Numbering of F1 and CF1 residues**

Unless otherwise indicated, all numbering of F1 residues is as in the ATOM section of PDB file 1bmf. Numbering of CF1 residues in the α, β and γ-subunits is as in SwissProt, accession numbers P26526, P06541 and P12113 respectively.

**Docking procedure**

LIGIN software for molecular docking30 was used for predicting the position of tentoxin within a putative binding site. The program generates a number of randomly distributed ligand positions within the putative binding site and maximizes complementarity as a function of the
six degrees of freedom of the ligand\textsuperscript{45}. Density of starting points was kept at > 4 points per Å\textsuperscript{3}. Multiple ligand conformations were docked; however, during the optimization procedure, ligand and protein were treated as rigid bodies.

**Ligand flexibility**

A schematic representation of tentoxin is shown in Figure 4. The cyclic tetrapeptide ring has a cis–trans–cis–trans conformation, both in chloroform\textsuperscript{44} and water\textsuperscript{45}. In chloroform, one conformer (B-form) of the ring predominates; however, in aqueous solutions there are four forms – A, B, C and D, in relative proportions 51:37:8:4 respectively\textsuperscript{45}. We incorporated ligand flexibility in our docking procedure to allow for any of several conformers of tentoxin. The rationale was that from many ligand conformers in solution, the apoprotein most likely selects one with high complementarity to the bond site. We considered all four forms of the ring. To determine the structure of forms A and B, the backbone torsion angles given in Pinet \textit{et al.}\textsuperscript{45} were used. We created the C and D structures from the B-form; by adding 180° to $\Phi_{\text{Phe}}$ and $\Psi_{\text{Phe}}$ to obtain C, and by flipping the two non-methylated peptide bond planes to obtain D. The structures were then refined by energy minimization\textsuperscript{46} using AMBER force field\textsuperscript{47}.

We also considered the different side-chain orientations for $\Delta$Phe (dehydrophenylalanyl) and Leu. In tentoxin, $\Delta$Phe almost exclusively assumes a Z-configuration\textsuperscript{48}. However, at equilibrium ~3% of molecules can exist in the E-configuration as isotentoxin. Moreover, the C-conformation of a synthetic analogue of tentoxin, MeSer$^-$tentoxin, takes the E-configuration\textsuperscript{49}. Although it is not yet clear whether the E-conformation is relevant \textit{in vivo}\textsuperscript{50}, we decided not to exclude it a priori, and considered both configurations of $\Delta$Phe in our preliminary calculations. As for Leu, its side chain has two rotatable bonds with three rotamers per bond. We considered all Leu rotamers that did not produce atomic bumping. \textit{In toto}, 44 tentoxin conformations were initially applied in the docking procedure. Since the results for the E- and Z-conformations were similar, we present only data corresponding to binding complementarity of the 22 conformations of the latter. This number of conformations is readily managed by our docking procedure. Data for the 22 tentoxin conformations are presented at the website http://sgedg.weizmann.ac.il/tentoxin/

**Analytical tools**

Complementarity, a geometric and chemical measure of ligand–protein fitness\textsuperscript{40}, was used to gauge complex stability. LPC and CSU software\textsuperscript{22} were used to determine the interactions stabilizing the structures obtained. Tentoxin has four ring conformations: A, B, C and D (ref. 45). For each ring conformation, LPC analysis was performed for the complex structure with the highest complementarity.

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