Substitution of a Single Amino Acid Switches the Tentoxin-resistant Thermophilic F$_1$-ATPase into a Tentoxin-sensitive Enzyme*

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Tentoxin is a cyclic tetrapeptide derived from phytopathogenic fungi of the *Alternaria* species, causing chlorosis in sensitive plant species. It acts as an inhibitor of the chloroplast F$_0$F$_1$-ATP synthase from these species, but not of the homologous enzymes from other bacteria and animals (1–4). In membrane-bound F$_0$F$_1$-ATP synthase, both ATP synthesis and ATP hydrolysis are inhibited by tentoxin (1), with the soluble F$_1$ subcomplex, which is not capable of ATP synthesis; ATP hydrolysis is inhibited (2). Although binding studies suggested an uncompetitive manner of inhibition by interference with cooperation of nucleotides from the enzyme (2, 5), the precise mechanism of tentoxin is not known. Based on labeling studies, one high affinity inhibitory binding site and additionally one to two low affinity binding sites have been proposed (6, 7). Binding of tentoxin to low affinity sites relieves inhibition caused by binding to the high affinity site (6, 7).

The F$_1$ subcomplex of F$_0$F$_1$-ATP synthase is also referred to as F$_1$-ATPase and consists of the subunits $\alpha_3\beta_3\gamma_3$. Its $\alpha_3\beta_3\gamma$ subunits make up the smallest entity capable of continuous ATP hydrolysis (8, 9). High resolution structures of the $\alpha_3\beta_3\gamma$ complex from bovine heart mitochondria as well as of the $\alpha_3\beta_3$ region from the thermophilic *Bacillus* PS3 and from spinach chloroplast revealed an alternating, hexagonal arrangement of the three $\alpha$ and three $\beta$ subunits (10–12). These subunits consist of three domains: N-terminal $\beta$-barrels, a central nucleotide-binding domain, and a C-terminal bundle of $\alpha$-helices (10).

Recent results obtained by co-crystallization of spinach chloroplast F$_1$-ATPase (CF$_1$) and tentoxin shed more light on the binding of the inhibitor (13) and showed that tentoxin is bound at the $\alpha\beta$-interface in a cleft near the N-terminal $\beta$-barrel domains. The structure of the CF$_1$-tentoxin complex suggests a critical role of residue $\beta$Asp$^{355}$ for tentoxin binding and/or inhibition, which has been concluded from mutagenesis experiments in the past (14, 15), but it displayed at the same time structural differences in the vicinity of $\beta$Asp$^{355}$ between CF$_1$ and tentoxin-resistant F$_1$-ATPases, e.g. from *Escherichia coli* (EF$_1$) or from the thermophilic *Bacillus* PS3 (TF$_1$).

Another critical region for tentoxin inhibition seems to be located in the chloroplast ATPase $\alpha$ subunit. Studies using chimeric $\alpha_3\beta_3\gamma$ complexes that have been assembled from subunits originating from the tentoxin-sensitive CF$_1$ and from the insensitive *Rhodospirillum rubrum* F$_1$-ATPase (16, 17) indicated that the poorly conserved residues $\alpha$120–133 might be crucial.

In this report we superimposed the structures of the CF$_1$-tentoxin complex (13) and the corresponding parts of TF$_1$ (11) to pinpoint crucial amino acid residues involved in tentoxin binding. We predicted that $\beta$Ser$^{66}$ and $\alpha$Asp$^{132}$ of TF$_1$ (corresponding to $\beta$Ala$^{61}$ and $\alpha$Pro$^{133}$ of CF$_1$) play a central role in conferring tentoxin resistance to TF$_1$. To test these predictions based on the static picture provided by the crystal structure, we prepared two mutants of the $\alpha_3\beta_3\gamma$ complex of the tentoxin-sensitive TF$_1$, where the two critical residues $\beta$Ser$^{66}$ and $\alpha$Asp$^{132}$ were replaced by alanine and by proline, respectively, as found in the corresponding position of CF$_1$. The results of this mutagenesis study show how inhibition of ATP hydrolysis responds to subtle changes of the protein structure.

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**Experimental Procedures**

*Chemicals*—Tentoxin was purchased from Sigma, a pyruvate kinase/lactate dehydrogenase mixture was obtained from Roche Molecular Biochemicals, and restriction enzymes were from New England Biolabs. All other chemicals were of analytical grade.

*Bacterial Strains*—Plasmid construction and amplification was done using the strain *E. coli* JM109; for overexpression of TF$_1$, $\alpha_3\beta_3\gamma$ *E. coli* JM103(luncB-D used was used.

*Plasmid Construction*—The pkkHC5 expression plasmid coding for the $\alpha$, $\gamma$, and $\beta$ subunits of the thermophilic *Bacillus* PS3 $F_1$-ATPase, 1 The abbreviations used are: CF$_1$, chloroplast F$_1$-ATPase; TF$_1$, F$_1$-ATPase obtained from thermophilic *Bacillus* PS3; $\alpha_3\beta_3\gamma$ (1526A) and $\alpha_3\beta_3\gamma$ (R132P), thermophilic $\alpha_3\beta_3\gamma$ complexes carrying the mutations $\beta$Ser$^{66}$ → Ala or $\alpha$Asp$^{132}$ → Pro, respectively; MOPS, 4-morpholinopropanesulfonic acid.
Engineered Tentoxin Sensitivity of F$_1$-ATPase

Fig. 1. Structure of the tentoxin binding site. A, location of the tentoxin binding site (circle) near the N-terminal $\beta$-barrel domain of one $\alpha$-$\beta$ pair from spinach chloroplast F$_1$-ATPase (CF$_1$, (12, 13). Subunit $\alpha$ is colored in yellow, and subunit $\beta$ is shown in green. B and C, stereo images of the tentoxin binding pocket of CF$_1$ (13), complexed with one molecule tentoxin (backbone and residue numbers in blue), superimposed with the corresponding part of F$_1$-ATPase from the thermophilic Bacillus PS3 (TF$_1$, backbone and residue numbers in red) (11). The positions of the residues mutated in this study, $\beta$Ser$^{86}$ (B) and $\alpha$Arg$^{132}$ (C), are indicated.

RESULTS AND DISCUSSION

Superimposition of the CF$_1$ and TF$_1$ Structures at the Tentoxin Binding Site—A superimposition of the structure of the CF$_1$-tentoxin complex (13) with the structure of the $\alpha_3\beta_3$ subcomplex of TF$_1$ (11), which is shown in Fig. 1, B and C, revealed that the positions of residues $\alpha$Leu$^{65}$, $\alpha$Val$^{175}$, and $\alpha$Leu$^{238}$, which probably form important hydrophobic contacts with the inhibitor, are essentially conserved as well as the position of the crucial residue Asp$^{83}$ in the $\beta$ subunit (Fig. 1B). Calculation of potential hydrogen bonds showed that the carboxyl side chain of $\beta$Asp$^{83}$ is hydrogen-bonded to the amide hydrogens of leucine 2 and glycine 4 in the tentoxin molecule, which aligns the inhibitor in the binding cleft formed at the $\alpha$-$\beta$ interface (13). In TF$_1$, this critical interaction is probably impaired by a potential hydrogen bond formed between $\beta$Asp$^{83}$ ($\beta$Asp$^{84}$ of CF$_1$) and the hydroxyl group of $\beta$Ser$^{86}$ (corresponding to $\beta$Ser$^{84}$ in CF$_1$), which prevents correct tentoxin binding (13). In addition, the superimposition of the two F$_1$ structures clearly visualizes the potential critical role of residue $\alpha$Pro$^{131}$ in tentoxin binding in CF$_1$. In TF$_1$, this residue is replaced by arginine ($\alpha$Arg$^{131}$), whose bulky side chain seems to block access to the tentoxin binding site (Fig. 1C).

Tentoxin Sensitivity of Mutant $\alpha_3\beta_3\gamma$—ATP hydrolysis activities of the thermophilic $\alpha_3\beta_3\gamma$ complexes carrying the mutations $\beta$Ser$^{86}$ → Ala ($\alpha_3\beta_3\gamma$BS66A) or $\alpha$Arg$^{132}$ → Pro ($\alpha_3\beta_3\gamma$AR132P) were measured with an ATP regenerating system and compared with wild-type TF$_1$ $\alpha_3\beta_3\gamma$ (Fig. 2). The specific activity in the absence of tentoxin was 9–10 units/mg for all three enzymes, comparable with values previously reported for wild-type TF$_1$ $\alpha_3\beta_3\gamma$ (9, 18). These results indicate that the mutations per se do not significantly influence ATP hydrolysis activity. After preincubation with tentoxin, ATP hydrolysis by TF$_1$ $\alpha_3\beta_3\gamma$ (BS66A) declined remarkably (Fig. 2). Significant inhibition was observed using $>1 \mu$M tentoxin; the concentration dependence of inhibition was exceptionally steep when 5–20 $\mu$M tentoxin were used. Half-maximal inhibition was obtained with 5–10 $\mu$M tentoxin, and maximal inhibition of 65–70% was achieved with about 20 $\mu$M of the inhibitor. Increasing the tentoxin concentration up to 100 $\mu$M did not enhance the inhibition, and concentrations $>100$ $\mu$M led to a slight re-activation of the enzyme.

In contrast, only a slight decrease of activity, amounting to about 10% inhibition, was observed when 20–100 $\mu$M tentoxin were added to wild-type TF$_1$ $\alpha_3\beta_3\gamma$ or the mutant TF$_1$ $\alpha_3\beta_3\gamma$ (AR132P) (Fig. 2).
The degree of inhibition, about 70% determined for TF1 $\alpha_2\beta_2\gamma$ (B66A), is comparable with values reported earlier for the Mg-ATPase activity of chloroplast F1-ATPase (16, 17) and for chimeric mutants constructed by reconstitution of mutated $\alpha$ subunits derived from R. rubrum and $\beta$ and $\gamma$ subunits derived from CF1 (16). The $K_i$ value measured here was significantly lower than the $K_i$ value of about 10–8 published for CF1 (6, 7), but comparable with values reported for the above-mentioned chimeric enzymes (16). A re-activation of the enzyme as observed here in the presence of tentoxin concentrations >100 $\mu$M was previously also reported for CF1 and explained by the binding of a second and possibly a third tentoxin molecule to the F1 complex, which by an unknown mechanism may relieve inhibition.

The Role of $\beta$Asp83 for the Binding of Tentoxin—Functional binding of tentoxin seems to depend essentially on correct hydrogen bonding between the amide hydrogens from the tentoxin backbone and residue $\beta$Asp83 (13). In the thermophilic F1 this important hydrogen bonding is obviously affected by a potential hydrogen bond formed between $\beta$Asp83 and the side chain of the adjacent residue $\beta$Ser66 (3.3 Å). A similar competition for intermolecular ($\beta$Asp-TTX) and intramolecular hydrogen bonding (B66–68) is avoided in the tentoxin-sensitive CF1 complex as the chloroplast $\beta$ subunit contains alanine in the equivalent position of the binding site ($\beta$Ala81). For the same reason tentoxin sensitivity can probably be achieved in the F1 complex from Chlamydomonas reinhardtii simply by the replacement of $\beta$Glu83 by aspartate (15), as a proline residue, which has no capability to form hydrogen bonds, is located in the adjacent (n-2) position.

A steric effect on the binding of the inhibitor caused by the side chain located in position 81 seems unlikely as alanine and serine show about the same surface volume of 89 Å2. In addition the even more bulky threonine (surface volume 116 Å2) or proline side chain (surface volume 113 Å2) is found in the equivalent position of the binding site in the $\beta$Ala81 mutant for the same reason tentoxin sensitivity can probably be achieved in the F1 complex from Chlamydomonas reinhardtii simply by the replacement of $\beta$Glu83 by aspartate (15), as a proline residue, which has no capability to form hydrogen bonds, is located in the adjacent (n-2) position.

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Steric Blockage of the Tentoxin Binding Site Caused by $\alpha$Arg122—Although the available structural information (Fig. 1C; see also Ref. 13) strongly suggested that in wild-type TF1 $\alpha_2\beta_2\gamma$ the bulky side chain $\alpha$Arg122 blocks access to the tentoxin binding niche; its replacement by proline did not have a significant effect on tentoxin sensitivity. Furthermore, the double mutant TF1 $\alpha_2\beta_2\gamma$ (R122P/B66A) displayed the same tentoxin sensitivity as the single mutant TF1 $\alpha_2\beta_2\gamma$ (B66A) (data not shown), indicating that steric hindrance by this bulky side chain is not a predominant factor for tentoxin binding. The reason why the mutation failed to show the result expected from the CF1 and TF1 structures might be related to a substitution of $\alpha$Leu255 by proline in the native TF1 complex (surface volume: Leu, 167 Å2; Pro, 112 Å2), which might compensate for the effect caused by the more bulky arginine side chain in position 132. In addition, the wild-type-like activity of the mutant might be explained by dynamic movements of this part of the $\alpha$ subunit during tentoxin binding, which are not visible in a static protein structure, but might be resolved by a set of intermediate structures (alternative conformations) or by dynamic studies.

Requirements for Tentoxin Binding in F1—The point mutation $\beta$Ser66 $\rightarrow$ Ala is sufficient to achieve maximal inhibition, underscoring the importance of the crucial residue $\beta$Asp83 ($\beta$Asp83 in CF1) to form hydrogen bonds with the tentoxin peptide backbone. The results from our mutagenesis studies indicate that prerequisites for inhibition by tentoxin are a tentoxin binding cleft, an aspartate side chain for correct hydrogen binding, and the absence of other residues that might interfere with this crucial hydrogen bond. The comparably high $K_i$ value determined here for tentoxin inhibition indicates that other amino acid residues, which probably are located in the $\alpha$ subunit, also influence the affinity for tentoxin. Experiments to elucidate the role of these residues in fine-tuning the affinity for tentoxin are presently under way in our laboratory.

The results presented in this paper demonstrate in a remarkable way the feasibility of functional predictions based on structural information, e.g. for the design of special characteristics in a target protein. On the other hand, they also stress that, as it comes to functional considerations, structural dynamics should be taken into account.

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