Complete inhibition of the tentoxin resistant F$_1$-ATPase from
*Escherichia coli* by the phytopathogenic inhibitor tentoxin after
substitution of critical residues in the $\alpha$- and $\beta$-subunit

Claudia Schnick, Nicole Körtgen and Georg Groth*¶

¶Heinrich-Heine-Universitaet, Biochemie der Pflanzen, Universitaetsstr. 1,
D-40225 Duesseldorf, Germany

* To whom correspondence should be addressed.

Phone : +49-211-8112822
Fax:      +49-211-8113706
Email:   georg.groth@uni-duesseldorf.de
SUMMARY

Substitution of critical residues in the α- and β-subunit can turn the typically resistant ATP synthase from the bacterium *Escherichia coli* into an enzyme showing high sensitivity to the phytopathogenic inhibitor tentoxin which usually affects only certain sensitive plant species. In contrast to recent results obtained with the thermophilic F$_1$ (Groth *et al.* (2002), *J. Biol. Chem.* 277, 20117-20119) substitution of a critical serine in the β-subunit (βSer$^{59}$) which is supposed to provide an important intermolecular hydrogen bond in the binding site was not sufficient on its own for conferring tentoxin sensitivity to the EF$_1$-complex. Superimposition of the CF$_1$-tentoxin inhibitor complex on a homology model of the *E. coli* F$_1$-complex provided detailed information on the critical residues in the α-subunit of the binding cleft and allowed us to model the binding site according to the steric requirements of the inhibitor. Substitution of the highly conserved residue αLeu$^{64}$ seems to be most important to allow access of the inhibitor to the binding site. Combination of this substitution either with additional replacements in the α-subunit (Q49A, L95A, E96Q, I273M) or with the replacement of Ser$^{59}$ in the β-subunit enhanced the sensitivity to the inhibitor and resulted in a complete inhibition of the *E. coli* F$_1$-ATPase by the plant specific inhibitor tentoxin.
INTRODUCTION

Tentoxin, a cyclic tetrapeptide produced by phytopathogenic fungi from the *Alternaria* species, acts as a specific inhibitor of photophosphorylation in certain sensitive species of higher plants [1-4]. In these plants the phytotoxin binds specifically to the F₁-complex of the chloroplast ATP synthase causing the inhibition of ATP hydrolysis and ATP synthesis [2, 3]. High affinity binding (10⁻⁸ M) of a single tentoxin molecule seems sufficient for the inhibition, whereas low affinity binding (10⁻⁵ M) of a second and third molecule results in a reactivation of the enzyme [5, 6]. Recently, the structure and location of the inhibitory tentoxin binding site was determined by X-ray analysis of crystals containing the spinach chloroplast α₃β₃γε-core complexed with tentoxin [7]. The inhibitor binds to a cleft localised at the αβ-interface of the F₁-complex. Hydrogen bonding provided by residue Asp₈₃ in the catalytic β-subunit seems exceptionally crucial for the binding of the inhibitor. Most of the binding site, however, is located on the non catalytic α-subunit which forms important hydrophobic contacts with the inhibitor.

The importance of βAsp₈₃ for tentoxin binding and sensitivity was already suggested in mutagenesis studies. Substitution by glutamate, alanine or leucine caused tentoxin resistance, while substitution of glutamate by aspartate in the tentoxin resistant F₁-ATPase from *Chlamydomonas reinhardtii* resulted in a sensitive F₁-complex [8, 9]. Surprisingly, bacterial F₁-ATPases from *E. coli* and *Bacillus PS3* are resistant to tentoxin even though they contain aspartate in the corresponding position of their β-subunit. Thus additional structural elements in the F₁-complex must be required for tentoxin binding and sensitivity. The crystal structure of the CF₁-tentoxin inhibitor complex [7] shed more light on these additional critical residues and identified important hydrophobic contacts between residues L-Leu² and MePhe³ in the tentoxin molecule and Ile⁶³, Leu⁶⁵, Val⁷⁵, Tyr²³⁷, Leu²³⁸ and Met²⁷⁴ in the α-subunit. Moreover, the structure supports the critical role of Ala⁹₆ for tentoxin binding which was
already suggested on the basis of a structural alignment of MF₁, TF₁ and CF₁ [10]. Substitution of alanine by larger side chains like methionine (TF₁), valine (MF₁) or leucine (EF₁) which are found in resistant species will certainly result in steric conflicts with L-Leu² in the tentoxin molecule and will in turn avoid the binding of the inhibitor to the F₁-complex. In order to identify the minimal structural requirement for tentoxin binding in the α- and β-subunit we have substituted several critical residues in the E. coli F₁F₀ ATP synthase which in spite of containing the essential aspartate in a position equivalent to Asp⁸³ in CF₁ is resistant against tentoxin [11]. Failure of βAsp⁶¹ in EF₁ to bind tentoxin might be related to βSer⁵⁹. As already reported for the thermophilic F₁-complex an intramolecular hydrogen bond formed between the βaspartate and the hydroxyl side chain of the adjacent serine residue might impair the critical interaction of the aspartate carboxyl and the amide hydrogens of L-Leu² and Gly⁴ in the peptide backbone of the inhibitor [12]. Hence, we have substituted residues 58-60 in the β-subunit of the E. coli F₁-complex according to the native residues found in CF₁. Moreover, residues Gln⁴⁹, Leu⁶⁴, Leu⁹⁵, Glu⁹⁶ and Ile²⁷³ in the α-subunit were changed for those found in the tentoxin sensitive CF₁-complex or for residues having smaller side chain volumes. Here we report the results of these substitutions on the inhibition of ATP hydrolysis, ATP synthesis and ATP-dependent proton translocation by tentoxin and demonstrate that by subtle changes in the α-subunit the E. coli ATP synthase can be turned into a tentoxin-sensitive enzyme.
EXPERIMENTAL PROCEDURES

Chemicals – DNA restriction and modification enzymes were purchased from New England Biolabs GmbH (Frankfurt, Germany). Oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Tentoxin was obtained from Dr. B. Liebermann (Friedrich-Schiller University, Jena, Germany) and ATP was from Roche (Mannheim, Germany). All chemicals and reagents were of analytical grade.

Bacterial strains – Strain *E. coli* XL1-blue [13] was used for plasmid construction and amplification. Expression of wild type or mutant F1F0 was done using the strain *E. coli* DK8 [14] which is lacking the *unc*-operon coding for the ATP synthase.

Plasmid construction – The vector *pBWU13* [15], a derivative of plasmid *pBR322*, containing the complete *unc*-operon was used as template for the substitution of selected amino acids in the α- and β-subunit by sequential PCR amplification as described in Groth et al. [16]. Single amino acids were substituted by applying the following mutagenesis primer:

1. 5’- ATTTCACCGGCATACAATC-3’ (reverse) for the mutation α Q49A
2. 5’- TCGAGGTTCTCGCTGCGATAG-3’ (reverse) for α L64A
3. 5’- ACCGGAAACTTGCAGCGATCAGG-3’ (reverse) for α L95A/E96Q
4. 5’- TACCGTCAGATGTCCCTGCT-3’ (forward) for α I273M
5. 5’- TCGCAATGAGTGCCACCAGCGTGT-3’ (forward) for β G58S/S59A/S60T

The PCR fragments carrying the α mutations were digested with the restriction enzymes Sph I and Pml I and ligated into the corresponding sites of vector *pBWU13* appropriately predigested with the same restriction enzymes. The PCR fragment containing the β mutations was digested with Rsr II and Sac I and introduced into *pBWU13* cut with these enzymes.

Combinations of different amino acids substitutions in the α-subunit were generated by PCR using single site mutant *pBWU13* as template and the appropriate mutagenesis primer.
mentioned before. Substitutions in the α- and the β-subunit, were obtained by sub-cloning of the PCR generated fragment carrying the mutations in the β-subunit into the Rsr II/Sac I restriction sites of mutant pBWU13 containing the appropriate substitutions in the α-subunit. All substitutions were verified by DNA sequencing (SeqLab, Göttingen, Germany).

Plasmid pBR322 was used as control for cells lacking the ATPase activity.

*Isolation of membrane vesicles –* E. coli cultures (500 ml) were grown on 2YT to an OD\textsubscript{600} of 1.4 – 1.6. Cells were harvested by centrifugation at 5,000 g for 10 minutes at 4°C and resuspended in ice-cold TMG buffer containing 50 mM Tricine pH 8.0, 10 mM MgCl\textsubscript{2} and 10% glycerol (v/v) at a density of 0.3 g cells/ml buffer. Subsequently resuspended cells were disrupted by sonification. After centrifugation at 30,000 g for 10 minutes the cellular debris was removed. The resulting supernatant was applied to an ultracentrifugation step at 278,000 g for 60 minutes at 4°C. Finally pelleted membrane vesicles were resuspended in 0.4 ml TMG buffer.

Protein concentration of the membrane vesicle suspension was determined by the bicinchoninic assay (Pierce Chemicals, Rockford, U.S.A.) using bovine serum albumin as a standard.

*Measurement of ATP synthase activity –* ATP hydrolysis was determined in a reaction medium containing 20 mM Tricine pH 8.0, 150 mM KCl and 5 mM MgCl\textsubscript{2} at 25°C. The reaction was started by the addition of 2 mM ATP to the reaction medium containing 0.3 mg/ml membrane protein. ATPase activity was quenched in intervals of 15 sec by the addition of 1 M trichloroacetic acid. Phosphate release was estimated spectro-photometrically according to [17]. The effect of the inhibitor tentoxin on the ATPase activity of the *E. coli* F\textsubscript{1}F\textsubscript{0}-complex was tested by incubating membrane vesicles for 30 min. on ice at different
concentrations of tentoxin. ATP hydrolysis was measured as described before but in a
reaction medium containing the same concentration of tentoxin as used in the incubation step.
ATP synthesis was measured at 25°C in the same reaction medium, but in the presence of
40 μM P₁,P₅-di(adenosin-5')pentaphosphate, 5 mM KH₂PO₄ and 0.25 mM ADP. The
reaction was started by adding 0.3 mM NADH to the reaction medium. Aliquots were
withdrawn from the reaction mixture in intervals of 15 sec. ATP synthesis was quenched with
50 mM KH₂PO₄, 79 mM NaCl and 2.5 % (v/v) HClO₄. The medium was adjusted to neutral
pH and ATP was determined by the luciferin/luciferase assay using the ATP Bioluminescence
Kit CLS II (Roche Diagnostics, Mannheim, Germany).

ATP-dependent proton translocation was detected by fluorescence quenching of 9-amino-3-
chloro-7-methoxyacridine (0.25 μM) in the reaction medium described for ATP hydrolysis
and ATP synthesis. Quenching was initiated by addition of 1 mM ATP or 0.3 mM NADH. To
correct for non-specific quenching caused by the interaction of the acridine dye and ATP 1
μM nigericin was added at the end of the reaction. Tentoxin inhibition of ATP synthesis and
ATP-dependent proton translocation was tested with membrane vesicles that have been pre-
incubated with 50 μM tentoxin on ice for 30 min. in a reaction medium containing 50 μM of
the inhibitor.
RESULTS AND DISCUSSION

The role of β\textsubscript{Asp}\textsuperscript{83} for tentoxin binding – The F\textsubscript{1}-tentoxin structure of the spinach chloroplast ATPase (PDB accession code 1KMH) suggests that functional tentoxin binding depends critically on hydrogen bonding between amide hydrogens in the peptide backbone of the inhibitor and residue Asp\textsuperscript{83} in the chloroplast β subunit [7]. Based on a super-imposition of the CF\textsubscript{1}- and TF\textsubscript{1}-structure [12] tentoxin-resistance of the thermophilic F\textsubscript{1}-complex was attributed to an intramolecular hydrogen bond formed between the critical aspartate in the β-subunit (β\textsubscript{Asp}\textsuperscript{68}) and the side chain of the adjacent (n-2)-residue (β\textsubscript{Ser}\textsuperscript{66}). In fact, substitution of β\textsubscript{Ser}\textsuperscript{66} in the thermophilic ATPase by alanine which is found in the equivalent position of the chloroplast β-subunit, resulted in a significant inhibition of ATP hydrolysis by tentoxin [12]. Sequence alignment of the chloroplast and the \textit{E. coli} β-subunit (Fig. 1A) suggests a similar intramolecular hydrogen bond between serine (Ser\textsuperscript{59}) and aspartate (Asp\textsuperscript{61}) in the \textit{E. coli} enzyme that affects the correct binding of tentoxin to the F\textsubscript{1}-complex. However, when residues 58-60 in the \textit{E. coli} β-subunit were changed according to the chloroplast enzyme, only a slight decrease in the ATPase activity of the resulting mutant was detected after pre-incubation with tentoxin amounting to about 10 % inhibition (Fig. 2A).

Steric blockage of tentoxin binding in the α-subunit – The minor effect on the inhibition of ATP hydrolysis by tentoxin in the βG58S/S59A/S60T mutant suggested that additional residues probably located in the α-subunit of the \textit{E. coli} ATPase impaired the binding of the inhibitor to the critical aspartate in the β-subunit. Potential targets for modification in the α-subunit seem to be those residues which form important hydrophobic contacts in the crystal structure of the CF\textsubscript{1}-tentoxin inhibitor complex. A sequence alignment (Fig. 1B) of the α-subunits from CF\textsubscript{1} and EF\textsubscript{1} showed that three of the residues forming hydrophobic interactions with the inhibitor in the chloroplast ATPase, Ile\textsuperscript{63}, Leu\textsuperscript{65} and Val\textsuperscript{75} are strictly

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conserved. On the other hand, the small residues Ala$^{50}$ and Ala$^{96}$ (surface volume 88.6 Å$^3$) in CF$_1$ are substituted by the bulky residues glutamine (143.8 Å$^3$) and leucine (166.7 Å$^3$), respectively, in the α-subunit of the E. coli ATP synthase. Furthermore, residue Met$^{274}$ is replaced by the more hydrophobic isoleucine (Ile$^{273}$ in EF$_1$). In order to confer tentoxin sensitivity to the EF$_1$-complex residues Gln$^{49}$, Leu$^{95}$, Glu$^{96}$ and Ile$^{273}$ were changed independently according to the residues of the CF$_1$ α-subunit and mutants αQ49A, αL95A/E96Q and αI273M were tested for tentoxin inhibition of their ATPase activity. However, again no significant inhibition of ATP hydrolysis was observed (data not shown).

**Super-imposition of the CF$_1$ crystal structure and a homology model of EF$_1$ at the tentoxin binding site** – In contrast to the homologous mitochondrial and thermophilic enzymes current structural information of the E. coli ATP synthase is limited to a low resolution structure [18] and a homology model [19] based on the high resolution structure of bovine MF$_1$ [20]. Furthermore exploiting the homology model for structure-based engineering of the αβ-interface in order to confer tentoxin sensitivity to the EF$_1$-complex is complicated by the fact that a sequence of 5 residues located in the N-terminal part of the β-subunit (β56-60 in CF$_1$) which is located adjacent to the tentoxin binding site is missing in the E. coli enzyme. In spite of these critical restrictions we have superimposed the Cα atoms of the EF$_1$ homology model on the tentoxin binding site of the CF$_1$-structure (Fig. 3) intending to identify crucial residues in the α- or β-subunit that might interfere with the binding of the inhibitor. Most evident the structural alignment in Fig. 3 indicates a critical steric clutch formed between αLeu$^{64}$, a residue which is strictly conserved in all species, and the aromatic ring system of MePhe$^3$ in the tentoxin molecule. To remove this steric stress in the binding site Leu$^{64}$ was replaced by alanine. The resulting αL64A mutant showed a clear inhibition of its ATPase activity by tentoxin as shown in Fig. 2B. The maximal inhibition of about 30 % was observed at tentoxin concentrations of 40-50 µM, half maximal inhibition was detected at 23 µM
(Table I). The relative high \(K_i\) of the mutant is probably related to the still imperfect binding site compared to the high affinity binding cleft found in the chloroplast enzyme. However, the affinity of the binding site for the inhibitor was remarkably improved when the critical residues for binding that have been suggested on the basis of the sequence alignment of CF\(_1\) and EF\(_1\) were substituted in addition to Leu\(^{64}\). In mutant \(\alpha\)Q49A/\(\alpha\)L64A/\(\alpha\)L95A/E96Q/I273M having all critical residues replaced by the native residues of the chloroplast F\(_1\)-ATPase ATP hydrolysis declined to about 20% of the initial rate after preincubation with 50 \(\mu\)M tentoxin (Fig. 2 B). The extent of about 80 % inhibition in this combination mutant is comparable to data reported for the inhibition of the Mg\(^{2+}\)-ATPase activity of the chloroplast enzyme by tentoxin [21, 22]. The \(K_i\) of 8.5 \(\mu\)M, however, is still somewhat higher than for the chloroplast enzyme.

Inhibition of ATP-dependent proton translocation by tentoxin was determined by fluorescence quenching of 9-amino-3-chloro-7-methoxyacridine (Fig. 4). No significant decrease in the transmembrane proton gradient generated at the expense of NADH was detected in the presence of tentoxin indicating that all mutations had no effect on the respiratory-driven proton gradient and the proton conductance of the membrane. However, quenching induced by ATP hydrolysis was decreased by 15 % in mutant \(\alpha\)L64A (Fig. 4A) and by 35 % in mutant \(\alpha\)Q49A/\(\alpha\)L64A/\(\alpha\)L95A/E96Q/I273M (Fig. 4C) in the presence of 50 \(\mu\)M tentoxin (see also Table II). In contrast to this less pronounced, partial inhibition of proton translocation ATP synthesis is inhibited to about the same extent as ATP hydrolysis by tentoxin in the mutant EF\(_1\)F\(_0\)-complexes showing 30 % inhibition for substitution \(\alpha\)L64A (Fig. 5A) and 55 % for the combination mutant \(\alpha\)Q49A/\(\alpha\)L64A/\(\alpha\)L95A/E96Q/I273M (Fig. 5C and Table II).

In total a substitution of 5 residues in the \(\alpha\)-subunit seems sufficient to turn the tentoxin-resistant EF\(_1\) into a tentoxin sensitive enzyme of the same extent as the chloroplast F\(_1\)-ATPase. Remarkably the most important substitution is the replacement of a strictly
conserved residue (Leu\textsuperscript{64}) which probably would not have been identified by a sequence alignment.

*Replacement of βSer\textsuperscript{59} in the α-mutants inhibited by tentoxin* – Further improvement of the tentoxin sensitivity of the α-mutant EF\textsubscript{1}-complex was achieved by changing βSer\textsuperscript{59} for alanine as described for turning the tentoxin-resistant TF\textsubscript{1}-complex into a tentoxin sensitive enzyme [12]. After pre-incubation with tentoxin ATP hydrolysis declined to about 40\% in mutant αL64A-βG58S/S59A/S60T (Fig. 2 C). Significant inhibition was observed at concentrations of 20 µM. Half maximum inhibition was obtained with about 12 µM tentoxin (Table I). On the other hand complete inhibition of the ATPase activity was found when βGly\textsuperscript{58}Ser\textsuperscript{59}Ser\textsuperscript{60} were changed for serine, alanine and threonine in the αQ49A/L64A/L95A/E96Q/I273M mutant. The affinity of the binding site was further improved and concentrations of 5 µM showed half maximum inhibition already (Table I).

As for the mutations in the α-subunit of the *E. coli* ATP synthase inhibition of proton translocation by tentoxin is less significant. Fluorescence quenching is reduced by 22\% in mutant αL64A-βG58S/S59A/S60T (Fig. 4B) and 35\% in mutant αQ49A/L64A/L95A/E96Q/I273 carrying the βG58S/S59A/S60T substitution (Fig. 4D). However, similar to the situation found with the substitutions in the α-subunit significant inhibition of ATP synthesis was detected for the αβ-combination mutants which was comparable to the effect of tentoxin on ATP hydrolysis of the mutant *E. coli* ATP synthase (Fig. 5B, D and Table II).

The results obtained with the αβ-combination mutants clearly support the conclusions drawn from the CF\textsubscript{1}-tentoxin structure and mutation experiments with the thermophilic enzyme that intramolecular hydrogen bonding in the β-subunit impairs the binding of the inhibitor to the critical aspartate in the tentoxin binding site. In EF\textsubscript{1}, however, the major problem for the binding of the inhibitor seems to be the imperfect structure of the binding cleft in the
α-subunit which has to be adjusted before correct alignment of the tentoxin molecule by the intermolecular hydrogen bond formed between inhibitor and βAsp61 can take place.

Effect of the substitutions in the α- and β-subunit on kinetic constants $K_m$ and $v_{max}$ – Previous kinetic studies [25] and structural data from a CF$_1$-tentoxin co-complex [7] have established that tentoxin acts as an allosteric inhibitor on the F$_1$-part of the chloroplast ATP synthase. Nevertheless, steady state kinetics and binding studies have suggested different types of inhibition for the phytopathogenic inhibitor tentoxin. While equilibrium ultra filtration experiments and kinetic parameters from steady state studies support an uncompetitive inhibition of the ATP synthase [2], binding studies with HPLC or radioactive tentoxin favour a non-competitive inhibition type [24]. In order to clarify which type of inhibition is present in the mutant E. coli ATP synthase we have analysed steady state kinetic parameters $v_{max}$ and $K_m$ from our ATP hydrolysis measurements. Data are summarized in Table I. The significant decrease in $v_{max}$ observed in the presence of tentoxin is characteristic for both types of inhibition. The apparent Michaelis-Menten constant $K_m$, on the other hand, is not affected by tentoxin which clearly favours a non-competitive rather than an uncompetitive inhibition type where a significant decrease in the apparent $K_m$ in the presence of the inhibitor is found. Thus tentoxin seems to bind to both, enzymes containing no nucleotide at the substrate binding site and to nucleotide-bound enzymes, but not exclusively to the enzyme-substrate complex as for uncompetitive inhibition.

Reactivation of ATP hydrolysis at high concentrations of tentoxin – Binding of tentoxin to a second [5] or third [6] low affinity site on the F$_1$-complex was shown to release the inhibitory effect of the phytotoxin and to reactivate ATP hydrolysis of the chloroplast ATP synthase. This stimulatory effect of tentoxin on the ATPase activity was also observed with the tentoxin-sensitive replacement-mutant βS66A of the TF$_1$-complex at high concentrations of the phytotoxin [12]. As expected a similar reactivation profile was found with our EF$_1$
replacement-mutants. Pre-incubation of mutants αQ49A/L64A/L95A/E96Q/I273M, αL64A-βG58S/S59A/S60T and αQ49A/L64A/L95A/E96Q/I273M-βG58S/S59A/S60T at 1 mM tentoxin resulted in a 15-25 % stimulation of ATP hydrolysis (Fig. 2 B, C). A similar level of restored activity was reported for membrane bound CF₁ [23] while isolated CF₁ shows a much higher reactivation corresponding to a 2-3fold stimulation [24, 25]. In contrast to the inhibitory binding site [7] the location of the reactivating tentoxin site is not known yet. Reactivation of ATP hydrolysis in the TF₁- and EF₁-replacement mutants, however, suggests that inhibitory and stimulatory effects of tentoxin are mediated by the same minor substitutions in the α- and β-subunit. Thus, the low affinity activating binding site is probably located at the same position as the inhibitory high affinity site, but on a second αβ-pair. According to the molecular mechanism suggested for the inhibition of the chloroplast ATPase by tentoxin [7] binding of the inhibitor at two catalytic αβ-interfaces is expected to impair the transition from the closed to the open conformation of the catalytic β-subunit on these sites. The remaining third αβ-interface is the only active catalytic centre left. Whether steric restraints caused by the binding of the phytotoxin arrests this interface in an intermediate conformation allowing high single site turnover or whether opening and closing of this site are initiated by a restricted rotation (wobbling) of the central γ-subunit has to be clarified by single particle studies and/or crystallisation studies on the tentoxin activated F₁-complex.
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REFERENCES


FIGURE LEGENDS

Figure 1: Sequence alignments of the β- and α-subunit of the ATP synthase.

(A) Alignment of the ATP synthase β-subunit from *E. coli* (tentoxin-resistant EF₁) and spinach chloroplasts (tentoxin-sensitive CF₁) at the critical aspartate which is involved in tentoxin binding in the chloroplast enzyme (β₆₁ in EF₁, β₈₃ in CF₁). (B) Sequence alignment of the non catalytic α-subunit from *E.coli* and chloroplasts. Residues forming contacts with the inhibitor in the CF₁-tentoxin crystal structure [7] are indicated.

Figure 2: Effect of tentoxin on ATP hydrolysis in membrane vesicles from *E. coli*.
ATP hydrolysis in wild type and replacement mutants of the *E. coli* ATP synthase after pre-incubation at different tentoxin concentrations. ATPase activity observed in the absence of tentoxin was taken as 100%. (A) ATPase activity of wild type (○) and replacement mutant βG58S/S59A/S60T (△). (B) Effect of increasing concentrations of tentoxin on the ATPase activity in wild type (○), replacement mutant αL64A (●) and mutant αQ49A/L64A/L95A/E96Q/I273M (●). (C) Inhibition of ATPase hydrolysis by tentoxin in wild type (○), mutant αL64A/βG58S/S59A/S60T (△) and mutant αQ49A/L64A/L95A/E96Q/I273M-βG58S/S59A/S60T (▲). Breaks in abscissa axis indicate change in scale.

Figure 3: Superimposition of a homology model of EF₁ on the tentoxin binding site of CF₁.
A homology model of the EF₁-complex [19] was superimposed on the tentoxin binding site of the CF₁-tentoxin crystal structure [7]. Residues in the EF₁-model that form potential steric conflicts with the inhibitor are indicated. The backbone of the *E. coli* α-subunit is shown in blue, the β-subunit and the critical Asp₆¹ are coloured in red. The tentoxin molecule bound to
the site is shown in ball-and-stick representation with transparent van-der-Waals radii. Molecular graphic software PyMol [26] was used for illustration.

**Figure 4: ATP-dependent proton translocation by EF1-mutants αL64A (A), αL64A-βG58S/S59A/S60T (B), αQ49A/L64A/L95A/E96Q/I273M (C), and αQ49A/L64A/L95A//E96Q/I273-βG58S/S59A/S60T (D) in the absence and the presence of tentoxin.**

Proton gradient formation in membrane vesicles is indicated by quenching of the fluorescent dye 9-amino-3-chloro-7-methoxyacridine, as described under Experimental Procedures. ATP (1mM) and the uncoupler nigericin (1 µM) were added at the times indicated.

**Figure 5: Effect of tentoxin on the rate of ATP synthesis in EF1 substitution mutants.**

ATP synthesis in mutants αL64A (A), αL64A-βG58S/S59A/S60T (B), αQ49A/L64A/L95A/E96Q/I273M (C), and αQ49A/L64A/L95A/E96Q/I273-βG58S/S59A/S60T (D) was monitored by the luciferin/luciferase system. Activities measured in the presence of 50 µM tentoxin are indicated by closed symbols. ATP synthesis in the absence of the inhibitor is represented by open symbols. The activity of wild type membrane vesicles was 80.3 nmol ATP / mg / min in the absence of tentoxin and 78.3 nmol ATP / mg / min at 50 µM tentoxin.
### TABLE I

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$K_i$ (TTX) [µM]</th>
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<th>+ TTX [µM]</th>
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<td>α L64A</td>
<td>23.3 ± 5.7</td>
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<td>αQ49A/L64A/L95A/E96Q/I273M</td>
<td>5.4 ± 0.2</td>
<td>232 ± 52</td>
<td>257 ± 50</td>
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<tr>
<td>βG58S/S59A/S60T</td>
<td>5.4 ± 0.2</td>
<td>232 ± 52</td>
<td>257 ± 50</td>
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**Effect of mutations in the α- and β-subunit of the E. coli ATP synthase on kinetic parameters $K_i$ and $K_m$**

$^a$ ATPase activities were measured in triplicate in the absence and in presence of tentoxin at a concentration of 2 $K_i$. 
<table>
<thead>
<tr>
<th>Mutation</th>
<th>ATP-driven proton translocation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>relative rate of ATP synthesis&lt;sup&gt;b,c&lt;/sup&gt;</th>
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<td>αL64A</td>
<td>84 ± 4</td>
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<td>64 ± 3</td>
<td>32 ± 5</td>
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**TABLE II**

*ATP-driven proton translocation and ATP synthesis of mutants in the presence of tentoxin*

<sup>a</sup> percent quenching of 9-amino-3-chloro-7-methoxyacridine fluorescence (see Material and Methods for assay method)

<sup>b</sup> relative rate compared to the activity in the absence of the inhibitor Tentoxin

<sup>c</sup> rates were measured in triplicate in the presence of 50 µM Tentoxin
Figure 1
Figure 2 A-C

A
B
C

rate of ATP hydrolysis [%]
tentoxin [µM]

rate of ATP hydrolysis [%]
tentoxin [µM]

rate of ATP hydrolysis [%]
tentoxin [µM]
Figure 3
Figure 4
Figure 5

ATP synthesis [nmol/mg] vs. time [sec]
Complete inhibition of the tentoxin resistant F1-ATPase from Escherichia coli by the phytopathogenic inhibitor tentoxin after substitution of critical residues in the α- and β-subunit
Claudia Schnick, Nicole Koertgen and Georg Groth

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