Mn concentration of 93 nM over the shelf, which is never observed. The equation ln Mn = 5.4 − 0.002 X provides a good fit to data from within 500 km of the coast. The offshore transport term calculated from this equation is 0.005 nmol liter^{-1} year^{-1}.
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Tentoxin Sensitivity of Chloroplasts Determined by Codon 83 of β Subunit of Proton-ATPase

Adi Avni,* James D. Anderson,† Neta Holland, Jean-David Rochaix, Zippora Gromet-Elhanan, Marvin Edelman

Tentoxin is a naturally occurring phytotoxic peptide that causes seedling chlorosis and arrests growth in sensitive plants and algae. In vitro, it inhibits activity of the β subunit of the plastid proton–adenosine triphosphatase (ATPase) from sensitive species. Plastid *atp*B genes from six closely related, tentoxin-sensitive or -resistant *Nicotiana* species differ at codon 83, according to their response to the toxin: glutamate correlated with resistance and aspartate correlated with sensitivity. The genetic relevance of this site was confirmed in *Chlamydomonas reinhardtii* by chloroplast transformation. The alga, normally tentoxin-resistant, was rendered tentoxin-sensitive by mutagenesis of its plastid *atp*B gene at codon 83. Codon 83 may represent a critical site on the β subunit that does not compete with nucleotide binding or other catalytic activities.

Tentoxin, a cyclic tetrapeptide [cyclo L-leucyl-*N*-methyl-(Z)-dehydrophenyl-alanyl-glycyl-*N*-methyl-alanyl] (*1*), is a phytotoxin produced by the fungus *Alternaria tenuis*. The diversity of effects in a variety of plants suggests multiple modes and sites of action (*2*). Tentoxin prevents chlorophyll accumulation in germinating seedlings of some, but not all, angiosperms (*3*), an effect termed "chlorosis" (*4*). Chlorosis was claimed to arise from interference with transport or integration of specific nuclear-coded proteins into the developing plastid of sensitive, but not resistant, species (*5, 6*). However, tentoxin is also a potent inhibitor of energy transfer at the terminal step in photophosphorylation in isolated plastid membranes (*7*) and inhibits light-driven, but not adenosine triphosphate (ATP)–driven, protein and RNA synthesis in isolated chloroplasts (*8*). Thus, chlorosis, a cytoplasmically inherited chloroplast character (*9, 10*), may result from inhibition of photophosphorylation by interaction of tentoxin with a specific site on the coupling factor (CF_1) of the plastid proton-ATPase in sensitive species (*11*). Binding studies and ATPase inhibition kinetics show that, in sensitive species, tentoxin binds tightly to a single site on chloroplast-encoded α or β subunits of CF_1 (*12*). This binding is uncompetitive with respect to ATP and adenosine diphosphate (ADP) (*13*).

We used the β-less *Rhodospirillum rubrum* chromatophore system (*14*) to determine which of the two CF_1 subunits interacts with tentoxin (*15*). This system requires addition of β subunit and trace amounts of α subunit for restoration of ATPase activity (*16*). The external source of α subunit was, in all cases, from lettuce, a tentoxin-sensitive species (*17*). Heterologously reconstituted chromatophores proved sensitive to tentoxin when purified β subunit from a tentoxin-sensitive species [*Nicotiana tabacum* line 92 (*18*) or lettuce] was used. However, chromatophores were resistant to >100-fold higher concentrations of tentoxin when purified β subunit from a tentoxin-resistant species [*Nicotiana tabacum* var. *Xanthi* (*18*)] was used (Fig. 1). Thus, the response of the reconstituted chromatophores to tentoxin depended on the source of CF_1-β.

The peptide sequence identity among CF_1-β subunits from a variety of higher plants is 92 to 95% (*19*). We therefore reasoned that within a single genus, such as *Nicotiana* [where out of 40 species tested, 9 are tentoxin-resistant and 31 are tentoxin-sensitive (*3, 18*)], CF_1-β sequence variation might be limited enough that a unique difference could be identified between resistant and sensitive plants. Accordingly, the *atp*B coding regions from two resistant (R) and three sensitive (S) *Nicotiana* species [*N. tabacum* var. *Xanthi* (R), *N. rustica* (R), *N. bigelovii* (S), *N. plumbaginifolia* (S), and *N. tabacum* line 92 (S)] were cloned, were sequenced (*20*), and were compared with that of *N. tabacum* var. BY4 (R) (*21*). Homology among the six genes and among the proteins they encode was >99% (Fig. 2). Between CF_1-β of *N. tabacum* var. *Xanthi* and that of *N. bigelovii*, only a single amino acid difference was found: glutamate (E) or aspartate (D) at residue 83. All three sensitive lines had D at position 83 and all three resistant lines had E.

We transformed chloroplasts of *Chlam-*

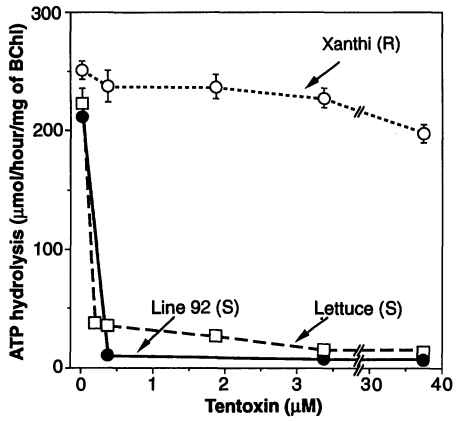


Fig. 1. The effect of tentoxin on ATP hydrolysis in reconstituted *Rhodospirillum rubrum* chromatophores. Photosynthetic membranes (*36*) were prepared from *Nicotiana* and lettuce, and were extracted with 2 M LiCl and 4 mM MgATP (*16*). CF_1 fractions were purified from the LiCl supernatant by anion-exchange chromatography on a fast protein liquid chromatography (FPLC) Mono Q column (*16*) to yield CF_1-β immunologically free of CF_1-α (purified-β), and a fraction containing both CF_1-α and CF_1-β in a ratio of ~1:1 (purified-αβ). Reconstitution of β-less chromatophores was carried out as detailed (*16*), with 3 μg of purified-αβ from lettuce, Lettuce (S); 3 μg of purified-β from *N. tabacum* var. *Xanthi* plus 0.4 μg of purified-αβ from lettuce, Xanthi (R); or 3 μg of purified-β from *N. tabacum line 92* plus 0.4 μg of purified-αβ from lettuce, Line 92 (S) (*30*). Samples were incubated with β-less *R. rubrum* chromatophores (*14*), and the specific rate of ATP hydrolysis determined in the presence of increasing tentoxin concentration. (R) = tentoxin-resistant and (S) = tentoxin-sensitive species as determined by the seedling test (*37*); BChl = bacterial chlorophyll, determined according to Clayton (*38*).

A. Avni, J. D. Anderson, N. Holland, M. Edelman, Department of Plant Genetics, Weizmann Institute of Science, Rehovot 76100, Israel.
J.-D. Rochaix, Department of Molecular Biology, University of Geneva 30, Quai Ernest Ansernet, CH-1211 Geneva, Switzerland.
Z. Gromet-Elhanan, Department of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel.

*Present address and to whom correspondence should be addressed: Plant Molecular Biology Laboratory, U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS) Beltsville Agricultural Research Center, Building 006, Beltsville, MD 20705.
†On leave from Plant Hormone Laboratory, USDA/ARS, Beltsville, and present address: Weed Science Laboratory, USDA/ARS, Beltsville Agricultural Research Center, Beltsville, MD 20705.

ydomonas reinhardtii (*22*) to test the relevance of residue 83 in CF_1-β as a tentoxin target. A chloroplast DNA fragment coding for residues 31 to 322 of *C. reinhardtii atp*B was subjected to site-directed mutagenesis (*23*). Four amino acids were changed around codon 83 such that, on translation, the 18–amino acid stretch from codons 74 to 91 of the *Chlamydomonas* gene [VRAVSMNPTEGLMRGMEV; (*24*)] would be identical to that of *Nicotiana* (Fig. 2). This was to minimize any putative conformational effects around residue 83 in the *C. reinhardtii* protein that might render an Asp at this position inaccessible to tentoxin. Two plasmids, encoding *atp*B, one with the codon for Glu at position 83 (p113-E) and the other, for Asp (p113-D) (*25*), were used to transform (*26*) *C. reinhardtii* mutant FUD50 (*27*). This mutant lacks an intact *atp*B gene, does not assemble chloroplast ATPase, and requires organic carbon to grow (*28*). Presumptive transformant colonies were selected by growth on photoautotrophic medium. Nine colonies were isolated after transformation with p113-E vector and six with p113-D. Immunoblotting after SDS–polyacrylamide gel electrophoresis (PAGE) confirmed the presence and accumulation of the CF_1-β subunit (*29*). Thus, photoautotrophy in these lines is due to the reintroduction of CF_1-β.

Chlamydomonas reinhardtii wild type (WT), FUD50 mutant, and transformed lines 38 (E) and 37 (D) were grown in liquid photoautotrophic medium with or without tentoxin. Growth of WT and line 38 (E) was impervious to the highest concentration of tentoxin used (1.8 mM), and growth of line 37 (D) showed a halfway inhibition concentration (IC_{50}) at ~250 μM (Fig. 3). The FUD50 mutant was unable to grow photoautotrophically, irrespective of the presence of the inhibitor. The response to tentoxin of 47 tetrads from the cross 37 $(D)^+ \times WT^-$ was analyzed. Their inability to grow at 900 μM of inhib-

Fig. 2. CF_1-β amino acid sequences from six *Nicotiana* species (*20*). Chloroplast DNA from all *Nicotiana* strains was isolated (*39*) and was sequenced (*41*). The deduced amino acid sequences are shown. The chlorosis response of *Nicotiana* seedlings, from all six species, to tentoxin (20 μg/ml) was determined (*3, 37*). R = tentoxin-resistant; S = tentoxin-sensitive. Arrow indicates codon 83.

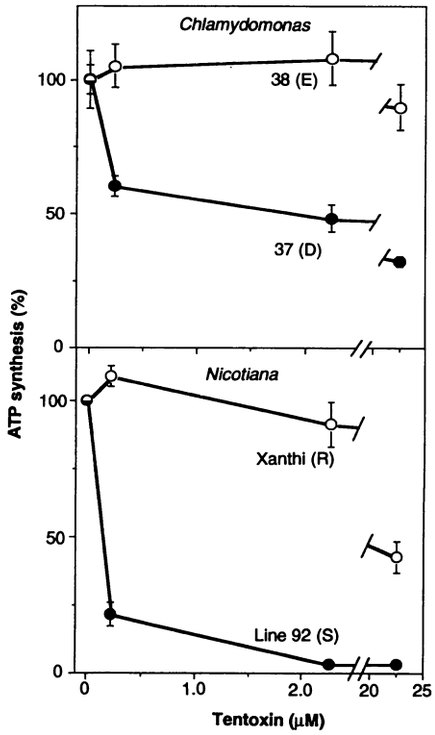
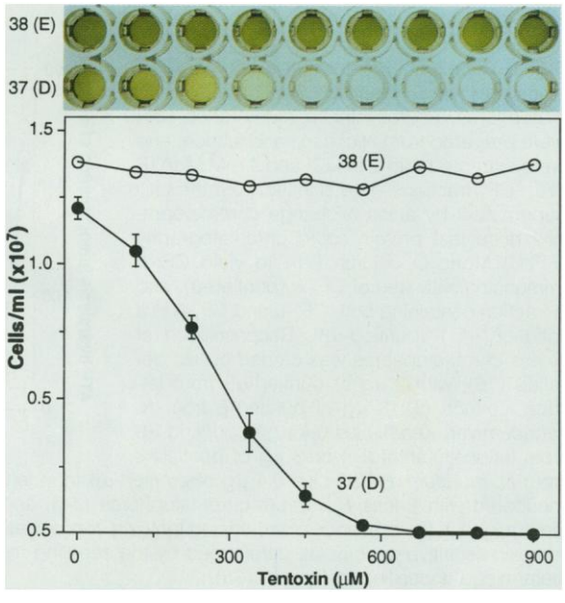


Fig. 4. Photophosphorylation in vitro in the presence of tentoxin. Light-driven ATP synthesis was carried out as described (*42*). Thylakoid membranes (30 μg of chlorophyll) from *Chlamydomonas* (*43*) or *Nicotiana* (*36*) were incubated in (in mM) 60 tricine (pH 8.0), 10 NaCl, 2 adenosine diphosphate, 4 Na_2HPO_4, 2 $MgCl_2$, 20 glucose, 4 sodium ascorbate, 75 μM phenazine methosulfate, 1 mg of hexokinase, 1 μl of ^{32}P (36 Ci/mmol) for 5 min in the dark and then for 3 min at 600 μE m^{-2} s^{-1} light. We stopped ATP synthesis by adding 100 μl of 50% trichloroacetic acid. Radiolabeled ATP was determined as described (*36*). Values were normalized to an activity of 100% in the absence of inhibitor. Bars indicate standard errors calculated from three repeats. The rates of ATP synthesis (micromoles per hour per milligram of chlorophyll) in the absence of tentoxin were as follows: line 38 (E) = 468; line 37 (D) = 431; *N. tabacum* var. *Xanthi* = 666, Xanthi (R); *N. tabacum* line 92 = 605, line 92 (S). (R) = tentoxin-resistant; (S) = tentoxin-sensitive.

Fig. 3. Growth of transformed *Chlamydomonas* in the presence of tentoxin. Two milliliters of 3 × 10^5 cells per milliliter of transformed lines 38 (E) and 37 (D) were grown at 25°C and 40 μE m^{-2} s^{-1} light in minimal medium supplied with different concentrations of tentoxin as indicated. Appearance of the transformed cultures and cell numbers [absorbance at 750 nm (A_{750}) turbidity measurements standardized to cell counts in a counting chamber] were scored after 3 days of growth. Bars indicate standard errors calculated from three repeats.



itor indicates uniparental inheritance of tentoxin sensitivity in *C. reinhardtii*.

Thylakoid membranes were isolated and were tested for ability to carry out light-driven ATP synthesis in the presence of tentoxin. Membranes of *C. reinhardtii line 37* (D) and *N. tabacum* line 92 are inhibited by the same submicromolar concentrations of toxin, although not to the same extent (Fig. 4) (*30*). The origin of the high background in line 37 (D) extracts was not investigated. Line 38 (E) and var. *Xanthi* are resistant.

Codon 83 of CF_1-β is a major plastome locus in *Nicotiana* (*31*), determining the response of the plant to tentoxin: at the level of chlorosis in vivo and at the level of photophosphorylation in vitro. The codon 83 region either is, or interacts with, the tentoxin receptor of the chloroplasts. More than 30 DNA sequences are published for β subunit of proton-ATPase from bacteria, chloroplasts, and mitochondria (*32*). A negatively charged residue (Glu or Asp) at codon 83 (*Nicotiana* numbering) is present in all of them. This observation, together with inhibition of catalysis by tentoxin in sensitive species (*9, 17*), suggests that the codon 83 region directly participates in a critical ATPase function. If so, codon 83 specifies a new functional region of β subunit, which lies outside of the nucleotide and nucleotide-analog (*33, 34*) binding sites and outside of known mutation sites on F_1-β, causing defects in magnesium binding, ATP hydrolysis, catalytic cooperativity, or F_1 and CF_1 assembly (*34, 35*). Alternatively, tentoxin binding might lead to structural distortion in CF_1-β and affect catalytic function only indirectly. Site-directed mutagenesis in the vicinity of codon 83 should help resolve these issues.

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25. A silent Hha I restriction site was introduced during mutagenesis at codons 80 to 81 to enable rapid identification of successful transformants. The mutated DNA fragments were cloned into vector p113, a 2.9-kb Eco RI–Kpn I fragment containing the *C. reinhardtii atp*B gene and flanking regions ligated into pUC18 (*24*). The presence of the Hha I site was confirmed by digestion of total *C. reinhardtii* DNA [J.-D. Rochaix, S. Mayfield, M. Goldschmidt-Clermont, J. Erickson, in *Plant Molecular Biology, A Practical Approach*, C. H. Shaw, Ed. (IRL Press, New York, 1988), pp. 253–275] and amplification of *atp*B nucleotides 91 to 705 by polymerase chain reaction.
26. Transformation of *C. reinhardtii* chloroplast DNA was performed according to Boynton *et al.* (*22*) with a locally built particle gun modified from G. Zumbrunn, M. Schneider, and J.-D. Rochaix [*Technique* **1**, 204 (1989)]. The acetate-requiring FUD50 mutant strain, harboring a deletion in the *atp*B gene (*27*), was grown under low-light intensity (6 μE m^2 s^{-1}) to stationary phase (1 × 10^7 cells per milliliter). Culture (0.5 ml) was spread over nitrocellulose filters that were placed on agar plates containing growth medium plus acetate [I. Ohad, P. Siekevitz, G. E. Palade, *J. Cell Biol.* **35**, 521 (1967)]. The cells were further grown for 24 hours and then bombarded, under a vacuum of 40 mmHg, with tungsten particles coated with DNA. After bombardment, cultures were incubated for 24 hours in the dark on acetate-containing medium and then transferred to selective plates without acetate. Cultures were grown at 24°C and 60 μE m^{-2} s^{-1} light for 2 weeks. Green transformed colonies were identified against a background of dying, untransformed cells on the nitrocellulose filters.
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30. Three micrograms of purified β from either *N. tabacum* var. *Xanthi* or line 92 did not form active hybrid F_1-ATPase with 5 μg (bacterial chlorophyll) of β-less *R. rubrum* chromatophores unless activated by 0.4 μg of purified-αβ from lettuce. Three micrograms of purified-αβ from lettuce formed active hybrid F_1-ATPase when reconstituted with β-less chromatophores, but 0.4 μg of the lettuce preparation alone were below the threshold level for hybrid formation.
31. Besides *Nicotiana*, comparative data for, respectively, tentoxin response and codon 83 composition exist for: spinach (*3, 32*); pea (*3, 32*); maize (*3, 32*); wheat (*3, 32*); barley (*3, 32*); *Chlamydomonas reinhardtii* (this work, *24*); *Anabaena* sp. strain PCC 7120 [N. Holland, unpublished work (*32*)]; *Synechococcus 6301* [A. Avni, unpublished work (*32*)]; *Rhodospirillum rubrum* (*14, 32*). Tentoxin response correlates with codon 83 composition in all cases except that of wheat and barley (aspartate at codon 83 but chlorosis-resistant), where detoxifying enzymes or permeability barriers may prevent tentoxin from reaching its receptor site (*9*); or where deviation from the *Nicotiana* sequence in the tentoxin binding niche may impose structural changes overriding the codon 83 correlation; and that of maize [glutamate at codon 83 but photophosphorylation-sensitive in vitro (*6, 7*)], where a combination of such events could explain the anomalous behavior.
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41. A 3.4-kb Eco RI fragment within the X8 fragment (*37*) was cloned into KS vector (Stratagene). DNA sequencing was performed by dideoxy chain termination (*36*) with α-^{35}S-labeled deoxyadenosine triphosphate. Oligonucleotide primers (17-mer) complementary to the *atp*B sequence from chloroplast DNA of *N. tabacum* var. BY4 (*21*) were prepared. DNA was separated by electrophoresis on 5 to 6% polyacrylamide, 7 M urea gels. Sequencing was in one orientation and was verified by at least two independent experiments.
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