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.


Tentoxin Sensitivity of Chloroplasts Determined by Codon 83 of β Subunit of Proton-ATPase

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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 atpβ genes from six closely related, tentoxin-sensitive or -resistant N. tabacum 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 atpβ 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-(2)-dehydrophenyl-alanyl-glycoly-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 nucleic-coded proteins into the developing plastid of sensitive, but not resistant, species (5, 6). 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 (CF1) 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 CF1 (12). This binding is uncompetitive with respect to ATP and adenosine di-phosphate (ADP) (13).

We used the β-less Rhodospirillum rubrum chromatophore system (14) to determine which of the two CF1 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 [N. 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 [N. tabacum var. Xanthi (18)] was used (Fig. 1). Thus, the response of the reconstituted chromatophores to tentoxin depended on the source of CF1-β.

The peptide sequence identity among CF1-β subunits from a variety of higher plants is 92 to 95% (19). We therefore reasoned that within a single genus, such as N. tabacum [where out of 40 species tested, 9 are tentoxin-resistant and 31 are tentoxin-sensitive (3, 18)], CF1-β sequence variation might be limited enough that a unique difference could be identified between resistant and sensitive plants. Accordingly, the atpβ coding regions from two resistant (R) and three sensitive (S) N. tabacum 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 CF1-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 Chlamydomonas reinhardtii with the A. thaliana codon 83 of the plastid atpβ gene to determine whether this region of the gene contributed to resistance (22). Chloroplasts were isolated from the transformants and analyzed by immunoblotting with antibodies specific to the CF1-β subunit. The results confirmed that the codon 83 change was responsible for the observed resistance.

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Fig. 1. The effect of tentoxin on ATP hydrolysis in reconstituted Rhodospirillum rubrum chromatophores. Photosynthetic membranes (30) were prepared from Nicotiana and lettuce, and were incubated with 2 M LiCl and 4 mM MgATP (16). CF1 fractions were purified from the LiCl supernatant by anion-exchange chromatography on a fast protein liquid chromatography (FPLC) Mono Q column (16) to yield CF1-β immunologically free of CF1-α (purified-β), and a fraction containing both CF1-α and CF1-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 by lettuce, Line 92 (S). 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).

Fig. 2. Amino acid sequence of codon 83 from N. tabacum var. BY4 (R), N. tabacum var. Xanthi (R), N. rustica (R), N. bigelovii (S), N. plumbaginifolia (S), and N. tabacum line 92 (S).
Chlamydomonas reinhardtii (22) to test the relevance of residue 83 in CF₁-β as a tontoxin target. A chloroplast DNA fragment coding for residues 31 to 322 of C. reinhardtii atpB 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 [VRAVSNPTEGLRGMVEV; (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 tontoxin. Two plasmids, encoding atpB, 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 atpB 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 CF₁-β subunit (29). Thus, photoautotrophy in these lines is due to the reintroduction of CF₁-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 tontoxin. Growth of WT and line 38 (E) was impervious to the highest concentration of tontoxin used (1.8 mM), and growth of line 37 (D) showed a half-maximal inhibition concentration (IC₅₀) at ~250 μM (Fig. 3). The FUD50 mutant was unable to grow photoautotrophically, irrespective of the presence of the inhibitor. The response to tontoxin of 47 tetrads from the cross 37 (D) × WT was analyzed. Their inability to grow at 900 μM of inhib-

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**Fig. 2.** CF₁-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 tontoxin (20 μg/ml) was determined (3, 37). R = tontoxin-resistant; S = tontoxin-sensitive. Arrow indicates codon 83.

**Fig. 3.** Growth of transformed Chlamydomonas in the presence of tontoxin. Two milliliters of 3 × 10⁶ 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 tontoxin as indicated. Appearance of the transformed cultures and cell counts (absorbance at 750 nm (A₇₅₀) 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.

**Fig. 4.** Photophosphorylation in vitro in the presence of tontoxin. 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 Na₃P₂O₇, 2 MgCl₂, 20 glucose, 4 sodium ascorbate, 75 μM phenazine methosulfate, 1 mg of hexokinase, 1 μmol of ³²P (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 tontoxin 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) = tontoxin-resistant; (S) = tontoxin-sensitive.
itor indicates uniparental inheritance of tenotin sensitivity in C. reinhardtii.

Thylakoid membranes were isolated and were tested for ability to carry out light-driven ATP synthesis in the presence of tenotin. 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 back-ground in line 37 (D) extracts was not investigated. Line 38 (E) and var. Xanthi are resistant.

Codon 83 of CF-β is a major plasmodium locus in Nicotiana (31), determining the response of the plant to tenotin: 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 tenotin 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 tenotin 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 F1-β, causing defects in magnesium binding, ATP hydrolysis, catalytic cooperativity, or F1 and CF1 assembly (34, 35). Alternatively, tenotin binding might lead to structural distortion in CF1-β and affect catalytic function only indirectly. Site-directed mutagenesis in the vicinity of codon 83 should help resolve these issues.

REFERENCES AND NOTES

15. Richter et al. (14) found that β-less Rhodospirillum rubrum chromatophores reconstituted with F1-β purified from C. reinhardtii (1) were resistant to tenotin, whereas β-less chromatophores reconstituted with F1-β purified from [a tenotin-resistant species] are resistant to tenotin, whereas β-less chromatophores reconstituted with F1-β purified from [a tenotin-sensitive species] (71) are sensitive. However, Richter et al. (14) noted that the β prepa-
20. EMBS Data Library accession numbers x61316 through x61320.
23. Site-directed mutagenesis was performed as de-
26. EMBS Data Library accession numbers x61316 through x61320.
29. Site-directed mutagenesis was performed as de-
31. Site-directed mutagenesis was performed as de-