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# Involvement of Thylakoid Overenergization in Tentoxin-Induced Chlorosis in *Nicotiana* spp.[1]

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**The purpose of this work was to clarify the mechanism of tentoxin-induced chlorosis in *Nicotiana* spp. seedlings. We found that chlorosis does not correlate with the inhibition of chloroplast ATP synthesis in vivo, since it occurs at tentoxin concentrations far higher than that required for the inhibition of photophosphorylation measured in the same seedlings. However, tentoxin-induced chlorosis does correlate with in vivo overenergization of thylakoids. We show that tentoxin induces overenergization in intact plants and isolated thylakoids, probably via multiple interactions with ATP synthase. Furthermore, gramicidin D, a protonophore that relieves overenergization, also relieves chlorosis. Two lines of evidence suggest that reactive oxygen species may be involved in the process of chlorosis: ascorbate, a quencher of oxygen radicals, significantly protects against chlorosis, whereas transgenic *Nicotiana* spp. mutants overexpressing chloroplast superoxide dismutase are partially resistant to tentoxin-induced chlorosis. It is proposed that chlorosis in developing seedlings results from overenergization of thylakoids, which leads to the generation of oxygen radicals.**

Tentoxin is a cyclotetrapeptide produced by the pathogenic fungus *Alternaria alternata*. Early observations demonstrated that tentoxin causes chlorosis in germinating seedlings of sensitive species (Templeton et al., 1967), whereas in isolated thylakoids, it inhibits ATP synthesis at micromolar concentrations (Arntzen, 1972). Most plants with tentoxin-sensitive ATP synthase also became chlorotic under tentoxin treatment (Steele et al., 1978a). Thus, tentoxin-sensitive plant species can be defined as plants possessing a tentoxin-sensitive ATP synthase. The site of tentoxin action is localized to the catalytic $\alpha$- and $\beta$-subunits of $CF_1$, and its mechanism of inhibition is defined as an energy transfer inhibitor (Steele et al., 1977). Recently, a more precise definition of the site of interaction of tentoxin was proposed, i.e. a single amino acid substitution within the $\beta$-subunit (Glu to Asp at position 83) changes the plant from tentoxin-resistant to tentoxin-sensitive (Avni et al., 1992).

Another effect of tentoxin in intact plants is the inhibition of the processing of polyphenol oxidase and its energy-dependent import into the chloroplast (Vaughn and Duke, 1984; Sommer et al., 1994). However, it was shown that the chloroplastic ATP pool is obligatory for processing of proteins imported into the chloroplast (Olsen et al., 1989; Theg et al., 1989). Since the tentoxin-induced inhibition of polyphenol oxidase is overcome only by the combination of exogenous ATP and increased cyclic electron flow mediators (Sommer et al., 1994), it is likely that tentoxin-induced depletion of chloroplastic ATP is the reason for the inhibition of polyphenol oxidase. It was also reported that tentoxin affects the light-induced P515 electrochromic absorbance changes and variable Chl fluorescence in tentoxin-sensitive plants, implying that it increases the level of thylakoid membrane energization and inhibits electron transport, which is similar to other energy-transfer inhibitors (Duke, 1993). Finally, it was suggested that tentoxin may have secondary effects that cannot be accounted for by depletion of ATP (Lax and Vaughn, 1986; Duke, 1993). An example of the complex interactions of tentoxin with chloroplasts is its dual effect on ATP hydrolysis by $CF_1$: strong inhibition at submicromolar concentrations and large stimulation at higher concentrations (Steele et al., 1978b). Thus, it is conceivable that tentoxin affects different reactions in chloroplasts through multiple effects on the chloroplastic ATP pool, the energization level, and electron transport.

The purpose of this work was to elucidate the mechanism of tentoxin-induced chlorosis by comparing the in vivo effect of tentoxin on seedlings with the in vitro effect on thylakoid membranes isolated from the same plant. No strict comparison has been made between tentoxin-induced chlorosis, which is scored in the intact plant, and inhibition of photophosphorylation, measured in isolated thylakoids. Such a comparison is complicated by the permeability barriers of specific plant tissues, leading to uncertainty regarding the internal tentoxin concentration at a given externally applied inhibitor concentration. To be able to compare chlorosis and inhibition of ATP synthesis in the same plant,

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Abbreviations: 9-AA, 9-aminoacridine; $CF_1$, coupling factor of the chloroplast ATP synthase; Chl, chlorophyll; SOD, superoxide dismutase.

we utilized the observation that tentoxin is, practically speaking, an irreversible inhibitor, since it binds with very high affinity and does not come off of the ATP synthase even after repeated washings (Steele et al., 1978a). A controlled comparison of the effects of tentoxin in vivo (chlorosis) and in vitro (photophosphorylation) revealed that tentoxin-induced chlorosis in *Nicotiana* spp. cannot be directly accounted for through inhibition of ATP synthesis. Rather, it correlates with an increased in vivo thylakoid energization level and free-radical formation that apparently results from multiple interactions of the toxin with the ATP synthase.

## MATERIALS AND METHODS

*Nicotiana tabacum* line 92 is a tentoxin-sensitive, male-sterile line that contains *Nicotiana undulata* cytoplasm in a nuclear background of *N. tabacum* var *Xanthi* (Fluhr et al., 1984). *Nicotiana plumbaginifolia* wild-type and transgenic seeds were obtained from Dr. D. Aviv and Professor E. Galun (Weizmann Institute of Science, Rehovot, Israel). *N. tabacum* var *Xanthi* and line 92 seeds were surface-sterilized with 3.5% sodium hypochlorite, washed with sterile, distilled water, and placed on Nitsch agar medium (Nitsch, 1969) without Suc. Seeds were germinated under continuous white light (50 $\mu$E $m^{-2}$ $s^{-1}$) in a $CO_2$-rich (3%) atmosphere at 24°C for the times indicated.

### Inhibitor Treatments

Tentoxin was obtained from BioMakor (Jerusalem, Israel) and was added to the plant germination medium as indicated. Other chemicals were obtained from Sigma.

### Chl Fluorescence Measurements

Chl fluorescence of 7-d-old seedlings was measured in a pulse fluorometer (PAM, Walz, Effeltrich, Germany) by modulated fluorometry (Schreiber et al., 1986). The following light sources were provided: modulated light (1.6 kHz, 0.07 $\mu$mol $m^{-2}$ $s^{-1}$, 650 nm), actinic light (6 $\mu$mol $m^{-2}$ $s^{-1}$, 650 nm), and saturating white light (1600 $\mu$mol $m^{-2}$ $s^{-1}$). Minimal, maximal, and steady-state fluorescence were measured according to the method of Van Kooten and Snel (1990). The parameter $(F_m - F_{vs})/F_m$, where $F_m$ is the maximum fluorescence in dark-adapted seedlings and $F_{vs}$ is the maximum fluorescence in energized seedlings after 3 min of variable fluorescence decrease, was utilized to estimate the nonphotochemical quenching and to express the extent of thylakoid membrane energization.

### Starch Determination

Seven-day-old seedlings were transferred to a higher light intensity (150 $\mu$E $m^{-2}$ $s^{-1}$ white light) for 24 h. Seedlings were processed and starch was determined using a UV test kit (207748, Boehringer Mannheim).

### ATP Synthesis

Eight-day-old seedlings were washed and homogenized, and thylakoids were extracted according to the method of Evron and Avron (1990). No tentoxin was added to the extract. ATP synthesis was measured in vitro either as the alkalization of a weakly buffered solution (Chance and Nishimura, 1967) or as a decrease in Pi (Soteropoulos et al., 1992). Values are averages of three independent experiments and are presented as a percentage of the untreated control (55–220 $\mu$mol ATP $mg^{-1}$ Chl $h^{-1}$).

### In Vitro Energization

Lettuce thylakoids (Evron and Avron, 1990) were pretreated with 1 $\mu$M tentoxin for 30 min on ice (thylakoids, 0.5 mg Chl $mL^{-1}$) and added to a reaction mixture containing 50 mM NaCl, 50 mM sodium-Tricine (pH 8), 3 mM $MgCl_2$, 2 mM Pi, 10 $\mu$M pyocyanine, and 1 $\mu$M 9-AA. The final thylakoid concentration in the reaction mixture was 10 $\mu$g Chl $mL^{-1}$.

Thylakoids from tobacco seedlings, cultured in the presence of various tentoxin concentrations, were isolated as described for lettuce and suspended in a buffer containing 50 mM sodium-Tricine (pH 8), 3 mM $MgCl_2$, 2 mM Pi, 2 mM ADP, 10 $\mu$M pyocyanine, and 1 $\mu$M 9-AA. The thylakoid concentration was 10 $\mu$g Chl $mL^{-1}$. The mixture was placed in a fluorescence spectrometer (MPF 44A, Perkin-Elmer) and stirred continuously. Actinic light was supplied by a homemade halogen projector and filtered through a 640-nm long-pass filter (Schott, Cologne, Germany). The initial quenching of 9-AA fluorescence was measured. After steady-state fluorescence was reached, 1 $\mu$M venturicidin, an ATP synthase-directed energy transfer inhibitor, was added and the system was allowed to reach a new steady-state fluorescence level. The ratio of energization was defined as $F_{phos}/F_{nonphos}$, where $F_{phos}$ is the original steady-state fluorescence, and $F_{nonphos}$ is the steady-state fluorescence after the addition of venturicidin.

## RESULTS

### Tentoxin-Induced Chlorosis Does Not Correlate with Inhibition of Photophosphorylation

Thylakoids were isolated from seedlings treated with various tentoxin concentrations, and their ATP synthesis activity was compared with the level of Chl accumulation in aliquots taken from the same plants. Figure 1 shows that tentoxin concentrations too low to cause chlorosis completely inhibited ATP synthesis; chlorosis was evident only at 10 to 20 $\mu$g $mL^{-1}$, whereas ATP synthesis was inhibited almost completely at 1 to 2 $\mu$g $mL^{-1}$.

Starch accumulation is an additional measure of ATP synthase activity, since ATP is the rate-limiting factor for the incorporation of Glc monomers into starch (Maclachlan and Porter, 1959). As shown in Figure 1, 1 $\mu$g $mL^{-1}$ tentoxin (which is totally ineffective at causing chlorosis) completely inhibited starch accumulation. Thus, our results suggest that inhibition of ATP synthesis cannot be the only cause of chlorosis.

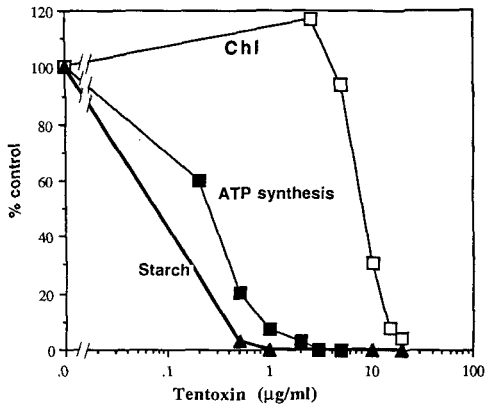


**Figure 1.** Effect of tentoxin on Chl accumulation, ATP synthesis, and starch content in 8-d-old seedlings of *N. tabacum* line 92. Total Chl was extracted from tentoxin-treated or control plants in *N*-*N*'-dimethyl formamide and determined from the $A_{645}$ and $A_{663}$. ATP synthesis was measured in thylakoids isolated from tentoxin-treated or control plants with no further addition of toxin. Values are averages of three independent experiments. Control activities ranged from 55 to 220 μmol ATP $mg^{-1}$ Chl $h^{-1}$. Starch accumulation was determined in 7-d-old seedlings transferred to 150 μE $m^{-2}$ $s^{-1}$ white light for 24 h.

### Effects of Tentoxin on the Thylakoid Energization Level

Energy-transfer inhibitors act by blocking proton translocation through ATP synthase, leading to an increase in the level of thylakoid membrane energization (Bamberger et al., 1973; Davenport and McCarty, 1986). Since tentoxin blocks ATP synthesis and coupled electron flow, it is by definition an energy-transfer inhibitor and therefore can be expected to increase thylakoid energization levels. As shown in Figure 2, addition of tentoxin in the presence of ADP, Pi, and Mg (phosphorylating conditions) to thylakoid membranes indeed led to increased quenching of 9-AA fluorescence, indicating increased energization. The energization level reached under these conditions was higher than under nonphosphorylating conditions (control). However, in contrast to other energy-transfer inhibitors such as dicyclohexylcarbodiimide, tentoxin had only a marginal effect on 9-AA fluorescence in the absence of ADP. Thus, tentoxin appears to overenergize thylakoids only in the presence of ADP. This result is consistent with our earlier observation that tentoxin inhibited electron transport in chloroplasts only in the presence of micromolar concentrations of ADP, which suggested a specific requirement for adenine nucleotides for tentoxin inhibition (Evron and Avron, 1990).

To determine whether overenergization is correlated with tentoxin-induced chlorosis, we measured and expressed the level of energization in vivo and in vitro by two different parameters in seedlings treated with various concentrations of the inhibitor. Energization in vitro was measured in thylakoids isolated from seedlings grown on tentoxin and expressed as the ratio between 9-AA fluorescence quenching under phosphorylating and nonphosphorylating conditions. This ratio gives a measure of how close the thylakoids are to maximal energization due to inhibition of

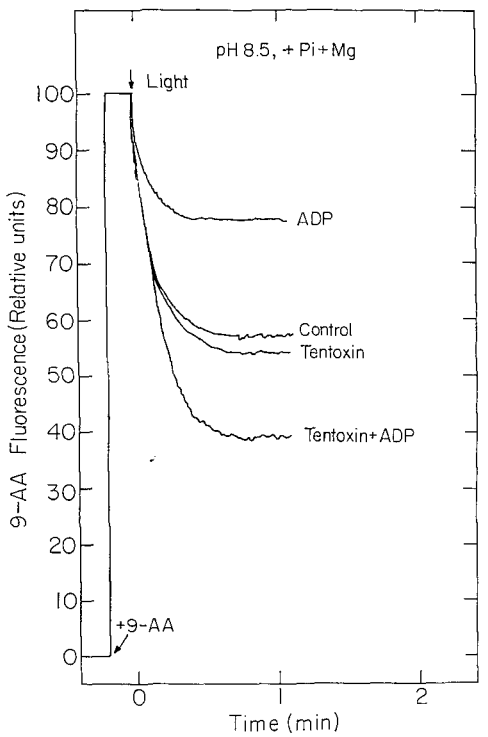


**Figure 2.** Thylakoid energization in lettuce thylakoids was measured using 9-AA. The reaction mixture was as described in "Materials and Methods," except that for phosphorylation conditions it also contained 2 mM ADP. Tentoxin (1 μM) was added where indicated.

ATP synthesis. Nonphosphorylating conditions were created by the addition of the energy-transfer inhibitor venturicidin, which blocks $H^+$ leaks through chloroplast ATP synthase and therefore is expected to yield maximal levels of energization. Figure 3 demonstrates that at 0.5 μg $mL^{-1}$ tentoxin, thylakoids are already fully energized because of inhibition of ATP synthesis.

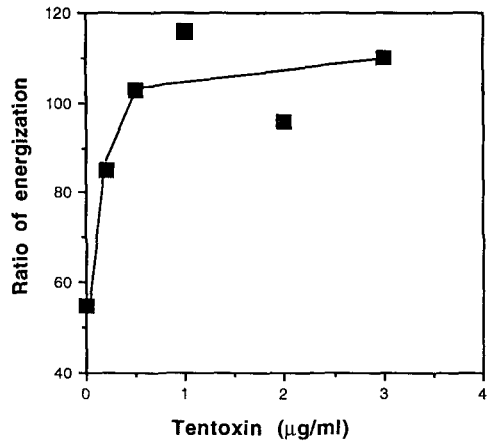


**Figure 3.** Effect of tentoxin on energization in vitro. 9-AA fluorescence was measured in the presence of Mg, Pi, and ADP as in Figure 2. The ratio of energization represents the 9-AA fluorescence to quenching ratio in the absence and presence of the chloroplast ATP synthase blocker venturicidin.

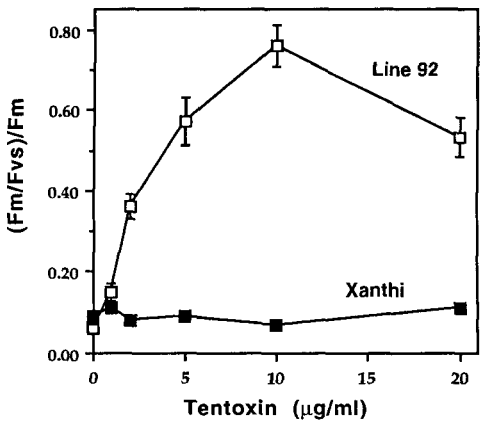


**Figure 4.** Effect of tentoxin on thylakoid energization in vivo. Seedlings of *N. tabacum* line 92 and var *Xanthi* were germinated on Nitsch medium (Nitsch, 1969) containing the indicated concentrations of tentoxin.

Energization in vivo was estimated from the nonphotochemical quenching of Chl fluorescence in 8-d-old seedlings from tentoxin-sensitive line 92 and tentoxin-resistant var *Xanthi* germinated on tentoxin-containing medium. Line 92 seedlings exhibited an increase in thylakoid energization that reached a maximum value at 5 to 10 $\mu$g mL$^{-1}$ tentoxin. Photochemical quenching of Chl fluorescence was not affected by the addition of tentoxin in seedlings from var *Xanthi*. (Fig. 4). A comparison of the data in Figures 1 and 4 clearly shows that inhibition of ATP synthesis alone cannot account for the vast increase in energization obtained in vivo beyond 1 $\mu$g mL$^{-1}$ tentoxin. Our results therefore indicate that chlorosis correlates with tentoxin-induced energization in vivo far better than with inhibition of ATP synthesis.

To test the strength of this correlation, line 92 seeds were germinated in the presence of tentoxin and the protonophore gramicidin, an uncoupler that creates a proton channel across the membrane. The addition of 2 $\mu$M gramicidin partially protected against both chlorosis and thylakoid overenergization measured in vivo in tentoxin-treated seedlings (Table I) without significantly affecting PSII activity (100% at 15 $\mu$g mL$^{-1}$ tentoxin).

**Table II.** *Partial protection against tentoxin-induced chlorosis in a transgenic plant overexpressing SOD*

wt, *N. plumbaginifolia* wild type; T1(23), transgenic *N. plumbaginifolia* line overexpressing SOD (Perl-Trevis, 1990). Seeds were germinated in the presence or absence of 2 $\mu$g mL$^{-1}$ tentoxin. Chl was extracted from 10 mg of fresh tissue.

| Tentoxin | Chl Content | |
|---|---|---|
| | wt | T1(23) |
| $\mu$g mL$^{-1}$ | *Fraction of control* | |
| 0 | 1.00 | 1.00 |
| 2 | 0.26 | 0.82 |
| 4 | 0.13 | 0.37 |

## Chlorosis and Free-Radical Formation

Thylakoid overenergization may lead to oxidative damage by the accumulation of excited triplet-state Chl, which can convert molecular oxygen to singlet oxygen and generate toxic free radicals. We therefore tested whether the chlorotic effect of tentoxin could be overcome in vivo by scavenging free radicals. Seeds of transgenic *N. plumbaginifolia* overexpressing chloroplastic SOD by approximately 2-fold (Perl-Treves, 1990) were germinated and challenged for their ability to accumulate Chl in the presence of tentoxin. As shown in Table II germinating seedlings from wild-type *N. plumbaginifolia* are highly sensitive to tentoxin, becoming chlorotic at 2 $\mu$g mL$^{-1}$, whereas the transgenic line T1(23) (which overexpresses SOD) clearly exhibits an improved capacity to accumulate Chl in the presence of the toxin. Moreover, application of the free-radical scavenger ascorbic acid to the growth medium was found to significantly protect *N. tabacum* line 92 seedlings against tentoxin-induced chlorosis (Table III). These results are consistent with the idea that free radicals generated by thylakoid overenergization are involved in the mechanism of tentoxin-induced chlorosis.

## DISCUSSION

Based on early in vitro studies of Arntzen (1972) and Bennett (1976), it was suggested that tentoxin-induced chlorosis is due to the inhibition of photophosphorylation.

**Table I.** *Comparison between the effect of gramicidin on tentoxin-induced chlorosis and in vivo energization*

Chl content and energization were measured in 8-d-old seedlings of line 92 germinated in the presence of the indicated concentrations of tentoxin with (+Gra) or without (−Gra) 2 $\mu$M gramicidin Chl was extracted from 20 mg of fresh tissue. Chl content for the control (no tentoxin) with and without gramicidin was 31.1 and 23.8 $\mu$g, respectively; energization was 0.075 and 0.062, respectively.

| Tentoxin | Chl Content | | | Energization | | |
|---|---|---|---|---|---|---|
| | −Gra | +Gra | Percentage protection[a] | −Gra | +Gra | Percentage protection[b] |
| $\mu$g mL$^{-1}$ | *Fraction of control* | | | $F_m - F_{vs}/F_m$ | | |
| 0 | 1.00 | 1.00 | 0 | 1.0 | 1.0 | 0 |
| 5 | 0.81 | 0.85 | 21 | 13.7 | 13.8 | 0 |
| 10 | 0.74 | 0.84 | 38 | 15.5 | 8.6 | 47 |
| 20 | 0.13 | 0.54 | 47 | 19.7 | 7.7 | 64 |

[a] ([+Gra] − [−Gra])/(1 − [−Gra]). [b] ([−Gra] − [+Gra])/([−Gra] − 1).

**Table III.** *Ascorbic acid partially protects against tentoxin-induced chlorosis*

Chl content was measured in line 92 seedlings germinated in the presence of the indicated concentrations of tentoxin with (+Asc) or without (−Asc) 0.2% ascorbic acid. Chl was extracted from 20 mg of fresh tissue on the 8th d of germination and measured as in Table I.

| Tentoxin | Chl Content | | |
|---|---|---|---|
| | −Asc | +Asc | Percentage protection[a] |
| $\mu g\ mL^{-1}$ | *Fraction of control* | | |
| 0 | 1.00 | 1.00 | – |
| 5 | 0.93 | 0.89 | 0 |
| 15 | 0.36 | 0.80 | 68 |
| 20 | 0.29 | 0.70 | 58 |

[a] ([+Asc] − [−Asc])/(1 − [−Asc]).

Duke et al. (1982) and Lax and Vaughn (1986) interpreted their own observations as indicating that chlorosis does not result from inhibition of ATP synthase; however, their case was never rigorously established. In the present study, the lack of correlation found between tentoxin-induced chlorosis and inhibition of ATP synthesis in the same tobacco seedlings clearly demonstrates that one is not the direct consequence of the other.

The novel issue in our study is the relationship between tentoxin-induced chlorosis and thylakoid overenergization. A positive correlation is implied by (a) the similar in vivo tentoxin concentration dependence of chlorosis and energization; (b) the fact that tentoxin-insensitive *N. tabacum* line 92, which does not undergo chlorosis, also does not undergo overenergization in the presence of tentoxin; and (c) the fact that the protection against chlorosis was obtained with the uncoupler gramicidin. These data agree with the idea of tentoxin interacting with $CF_1$, resulting in blockage of the dissipation of the electrochemical proton gradient and leading to an increase in energization level (also see Duke, 1993).

How does overenergization induce chlorosis? The possibility that the mechanism involves reactive oxygen species (Duke, 1993) is supported by the protective effect found with ascorbate and by the increased resistance to tentoxin of transgenic *N. plumbaginifolia* overexpressing SOD. Whether free radicals are involved in the inhibition of Chl formation or in enhanced Chl degradation is not known. However, if tentoxin damage does result from overenergization and generation of reactive oxygen species, then decrease in light absorption should lead to protection. One way for the developing seedling to accomplish this would be by a decrease in the Chl level, which would result in lower light absorption and, consequently, a lower energization level. Therefore, a decrease in Chl may temporarily protect the plant against tentoxin-induced damage and help it to survive, although at the expense of a lower photosynthetic capacity.

It is not clear, however, why the effect of tentoxin on the in vitro energization level saturates at 1 $\mu g\ mL^{-1}$ tentoxin, whereas in vivo, maximal energization is reached at approximately 10 $\mu g\ mL^{-1}$. It appears that, whereas in isolated thylakoids overenergization can be well accounted for by the inhibition of phosphorylation alone, some additional effect of tentoxin at higher concentrations in vivo induces a further massive increase in energization. As shown previously, tentoxin has a dual effect on $CF_1$, inhibition of ATP hydrolysis at submicromolar concentrations and stimulation at higher concentrations (Reimer and Selman, 1978; Steele et al., 1978b; Pick et al., 1982). A possible explanation for this seeming contradiction is that massive overenergization in vivo results from the association of tentoxin with the low-affinity-binding site on $CF_1$. This effect would not be observed in isolated thylakoids because tentoxin bound at the low-affinity site will dissociate during isolation. We suggest that the association of tentoxin with its low-affinity site on $CF_1$ induces a conformational change in ATP synthase that leads to a further decrease in proton leaks through the thylakoid membrane, thus increasing the steady-state energization level.

The interrelationship between the high- and low-affinity tentoxin-binding sites of $CF_1$ (Pinet et al., 1996) is not known. However, the fact that tentoxin-dependent stimulation of ATP hydrolysis is observed only in tentoxin-sensitive plants (Steele et al., 1978a; Pick et al., 1982) implies that the dual effect of tentoxin is a reflection of a single genetic element. It is well-established that tentoxin binds to the $\beta$-subunit (Steele et al., 1976; Richter et al., 1986; Avni et al., 1992), which exists in three copies per $CF_1$. We hypothesize that the dual effect of tentoxin results from interactions with distinct subunits in the same enzyme complex, which are constrained in different conformations and exhibit different affinities for the toxin. This idea agrees with the alternating-site hypothesis (Boyer et al., 1977), which predicts different conformational states of $CF_1$ catalytic subunits during catalysis.

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