Plant Cell Physiol. 34(4): 523–529 (1993)

Tentoxin Inhibits Both Photophosphorylation in Thylakoids and the ATPase Activity of Isolated Coupling Factor F_1 from the Cyanobacterium *Anacystis nidulans*

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Tentoxin strongly inhibited the ATPase activity of isolated coupling factor 1 (AF_1) from the cyanobacterium *Anacystis nidulans*, with 50% inhibition occurring at 0.3 μM. When thylakoids from *A. nidulans* were preincubated with 0.3 μM tentoxin for 30 min, photophosphorylation was inhibited by 50%. Measurements of fluorescence from 9-aminoacridine indicated that tentoxin inhibited the utilization of the proton gradient by ATP formation in thylakoids. These results indicate that tentoxin is a strong energy-transfer inhibitor of photophosphorylation in *A. nidulans*. Tentoxin decreased the level of ATP in intact cells both in the light and in darkness, its effects being much stronger in the dark. Tentoxin at 50 μM strongly inhibited the growth of the cells.

Key words: *Anacystis nidulans* — ATPase — Coupling factor 1 — Energy-transfer inhibitor — Photophosphorylation — Tentoxin.

Tentoxin, a cyclic tetrapeptide produced by the fungus *Alternaria alternata* causes chlorosis of various plant species, apparently by a selective disruption of chloroplast development (for reviews, see Templeton 1972, Lax and Shepherd 1988). Early studies suggested that tentoxin inhibits photophosphorylation, acting as an energy-transfer inhibitor, in chloroplasts of susceptible species but not in chloroplasts of resistant species (Arntzen 1972). Further studies indicated that tentoxin inhibits the ATPase activity of isolated chloroplast coupling factor 1 (CF_1) and the phosphorylation-coupled electron transport in a species-specific manner (Steele et al. 1976). Susceptibility to tentoxin seems to reside in the β-subunit of F_1, because F_1 ATPase from the photosynthetic bacterium *Rhodospirillum rubrum* is insensitive to tentoxin, while after reconstitution of the β-less F_1 with the heterologous β-subunit of CF_1 from spinach which is sensitive to tentoxin, the ATPase was sensitive to tentoxin (Richter et al. 1986). Later studies indicated, however, that tentoxin-mediated chlorosis does not appear to be primarily dependent upon tentoxin's effects on CF_1 because the initial effects of tentoxin occur at an earlier developmental stage than the development of any of the photosynthetic machinary that contributes to greening (Vaughn and Duke 1981, 1984). Vaughn and Duke (1984) suggested that tentoxin induces chlorosis by specifically blocking the import of polyphenol oxidase or of a few, as yet unidentified, proteins into chloroplasts.

Although inhibition by tentoxin of photophosphorylation may not be the direct cause of chlorosis, it is evident that tentoxin inhibits the activity of CF_1 in a species-specific manner, and there seems to be some correlation between the sensitivity of CF_1 and chlorosis (Steele et al. 1976). Because CF_1 plays a key role in the conversion of photosynthetic energy, it is of interest to characterize those features of the structure of CF_1 that confer sensitivity to tentoxin on some forms of CF_1s and insensitivity on others. Thus far, there is no apparent relationship between phylogeny and sensitivity to tentoxin either with respect to

Abbreviations: AF_1, *A. nidulans* coupling factor 1 ATPase; CF_1, chloroplast coupling factor 1 ATPase; F_1, coupling factor 1 ATPase.

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chlorosis (Durbin and Uchytil 1977) or the ATPase activity of CF_1. Even within a single genus, differences between species exist with respect to the sensitivity to tentoxin of CF_1 ATPases. For example, CF_1s from *Nicotiana tabacum* and *N. knightiana* are insensitive to tentoxin, while that from *N. otophora* is sensitive (Selman and Durbin 1978). Among CF_1s from other higher plants, those from spinach and lettuce are sensitive to tentoxin, while that from radish is insensitive (Steele et al. 1976). Among F_1s from lower organisms, CF_1 from the green alga *Chlamydomonas* (Selman-Reimer et al. 1981) and that from a heterotrophic thermophilic bacterium known as PS3 (Bar-Zvi et al. 1985) are insensitive to tentoxin. Studies on the effects of tentoxin at the whole-organism level have revealed that the growth of bacteria, yeast and several filamentous fungi are not affected by tentoxin at concentrations that produce 100% chlorosis in cucumber cotyledons. The alga *Euglena* is not sensitive in broth culture even when bleached by a period of darkness prior to treatment with tentoxin (Templeton 1972).

In contrast to the general view that CF_1s of lower photosynthetic organisms are insensitive to tentoxin, Dahse et al. (1986) presented results from delayed fluorescence measurements that suggest that tentoxin acts as an energy-transfer inhibitor in photophosphorylation in intact cells of the green alga *Chlorella* and the cyanobacterium *Anacystis*. Because CF_1 plays a key role in photosynthetic energy conversion and because cyanobacteria are so suitable for molecular-biological studies, we investigated the effects of tentoxin on *A. nidulans*. We confirmed the observations of Dahse et al. (1986) that tentoxin is an energy-transfer inhibitor of photophosphorylation, and we established that the drug inhibits the ATPase activity of isolated F_1 from *A. nidulans* (AF_1). We also report that tentoxin decreases the level of ATP in intact cells and inhibits the growth of the cyanobacterium.

Materials and Methods

Chemicals—Lysozyme, tentoxin, trypsin (TPCK-treated) and soybean trypsin inhibitor were obtained from Sigma.

Growth of cells and preparation of thylakoids—A stock culture of *A. nidulans* (renamed as *Synechococcus leopoliensis* (Racib.) Komárek, UTEX) was obtained from the Culture Collection of Algae, University of Texas (Austin, TX, U.S.A.). Cells were grown in the medium of Kratz and Myers (1955) at 26°C under white light of 35 μE m^{-2} s^{-1} with bubbling air. The photon flux density was measured with an LI-188B Integrating Quantum/Radiometer/Photometer (Li-cor Co., Lincoln, NB, U.S.A.). Cells at the late logarithmic phase of growth were harvested by centrifugation at 1,300 × *g* for 10 min at 25°C, and washed twice by suspension in 20 mM Tricine-NaOH (pH 8.0) with subsequent centrifugation. The washed cells were suspended in 20 mM Tricine-NaOH (pH 8.0), 0.6 M sucrose and 2 mM EDTA at 0.1 g fr wt ml^{-1} and treated with egg white lysozyme (1 mg ml^{-1}) for 2 h at 30°C under illumination with shaking. The treated cells were collected by centrifugation at 2,000 × *g* for 15 min at 4°C, and suspended in 20 mM Tricine-NaOH (pH 8.0), 0.6 M sucrose and 5 mM $MgCl_2$. The cells were again pelleted by centrifugation as described above. The pelleted cells were then suspended in the above medium at 0.1 g fr wt ml^{-1} and disrupted with a French pressure cell at 80 MPa at 0°C. The homogenate of disrupted cells was centrifuged at 3,000 × *g* for 15 min at 4°C and the supernatant was saved. Thylakoid membranes for measurements of activity were collected from the supernatant by centrifugation at 12,000 × *g* for 15 min at 4°C and the preparation of membranes was suspended in a solution of 20 mM Tricine-NaOH (pH 8.0), 0.6 M sucrose and 5 mM $MgCl_2$ that contained 1 mg bovine serum albumin ml^{-1}. Thylakoid membranes, as starting material for the preparation of AF_1, were collected from the foregoing 3,000 × *g* supernatant by centrifugation at 100,000 × *g* for 30 min at 4°C, and suspended at 0.2 mg Chl ml^{-1} in 20 mM Tricine-NaOH (pH 8.0), 1 mM EDTA and 1 mM ATP.

Extraction and purification of AF_1—Reconstitutively active five-subunit AF_1 was prepared by the method of Nemoto et al. (1990) with slight modifications. Specifically, the above mentioned suspension of thylakoids was gently stirred for 30 min at room temperature and then centrifuged at 100,000 × *g* for 30 min at 20°C. The supernatant was concentrated by ultrafiltration through a PM30 membrane (Amicon) and the concentrate was brought to 15% saturated ammonium sulfate at room temperature by addition of solid ammonium sulfate. The precipitate was removed by centrifugation at 18,000 × *g* for 10 min at 20°C, and the supernatant was brought to 30% saturated ammonium sulfate. The precipitate was again removed by centrifugation at 18,000 × *g* for 10 min at room temperature, and the supernatant was finally brought to 50% saturated ammonium sulfate and then left standing overnight at 4°C. The crude precipitate of AF_1 was collected by centrifugation at 18,000 × *g* for 10 min at 4°C and dissolved in 2–3 ml of Medium A, which consisted of 20 mM Tricine-NaOH (pH 8.0), 2 mM EDTA, 1 mM ATP and 10% (v/v) glycerol. The solution was centrifuged at 18,000 × *g* for 10 min at 20°C in order to remove undissolved particles. The supernatant was applied to a Sepharose CL-6B (Pharmacia) column (1.6 cm × 80 cm) that had been equilibrated and was eluted with Medium A. The fractions from the column were assayed for ATPase activity. The peak fractions were combined, concentrated on a PM30 membrane, applied to a second Sepharose CL-6B column (1.6 cm × 80 cm) and eluted as above. The peak fractions were concentrated on a PM30 membrane and an aliquot of 0.5 ml of the concen-

trate was subjected to centrifugation at 145,000 × *g* for 20 h at 20°C on a linear sucrose density gradient [30 ml, 10–30% (w/v), in Medium A]. The peak fractions were supplemented with glycerol to 50% (v/v) and stored at 4°C until use.

ATPase activity of isolated AF_1—Purified AF_1 (0.025 mg) was treated with trypsin (0.285 mg) in 1 ml of 20 mM Tricine-NaOH (pH 8.0), 0.5 mM EDTA and 5.7 mM ATP for 5 min at 30°C, and the reaction was terminated by the addition of 1 ml of a solution that contained 0.855 mg of soybean trypsin inhibitor. The activated AF_1 was preincubated with various concentrations of tentoxin and the ATPase activity was assayed as described in the legend to Figure 1. The amount of P_i liberated was determined by colorimetry after reduction of phosphomolybdate with $SnCl_2$, essentially according to the methodology exploited for the autoanalyzer of Technicon Co. (1966).

Photophosphorylation—For the assay of photophosphorylation, the reaction mixture (1 ml) containing thylakoids was illuminated at 20°C for 4 min with light that had been filtered through a sheet of red cellophane (1,500 μE m^{-2} s^{-1}) and the reaction was quenched by addition of 1 ml of 8% (w/v) trichloroacetic acid. [^{32}P]ATP was counted in a liquid scintillation counter after removal of [^{32}P]phosphomolybdate with isobutanol-benzene as described by Avron (1960).

Light-induced proton gradient—The light-induced uptake of proton into the thylakoid lumen was measured by monitoring the self-quenching of the fluorescence of 9-aminoacridine (Schuldiner et al. 1972) with a spectrofluorometer (model 1300; Hitachi Co., Tokyo). The photomultiplier was protected from the red actinic light by blue glass filters (5-61, Corning, and C-50S, Toshiba Co., Tokyo) and the fluorescence (excitation at 365 nm, emission at 451 nm) was recorded continuously.

Effects of tentoxin on the level of ATP in intact cells—*A. nidulans* cells (6.6×10^7) in 800 μl of growth medium in a test tube were kept either in the light (53 μE m^{-2} s^{-1}) or in darkness at 26°C without shaking. After 10 min, 16 μl of a tentoxin solution in ethanol were added to the suspension of cells, and incubation was continued either in the light or in darkness. At the times indicated, aliquots of 50 μl of the incubation mixture were withdrawn and 10 μl of 12% (w/v) perchloric acid were added to each aliquot. The supernatant from this mixture was neutralized with K_2CO_3, and ATP was quantified by a standard firefly luciferase-luciferin assay.

Effects of tentoxin on the growth of A. nidulans—Cells were inoculated in 2 ml of growth medium that contained either 0 or 50 μM tentoxin at an initial cell density of 0.8×10^6 or 1.9×10^6 cells ml^{-1} and incubated in loosely stoppered test tubes (inner diameter, 0.9 cm) at 28°C under white light at 35 μE m^{-2} s^{-1} with continuous shaking (3 Hz). Cell growth was followed by measuring A_{750} with a spectrophotometer (model 220A; Hitachi Co., Tokyo).

Results and Discussion

Effects of tentoxin on the ATPase activity of isolated AF_1—The ATPase activity of isolated AF_1 is very low unless the enzyme is appropriately activated. As in the case of isolated spinach CF_1, the ATPase activity of which is enhanced by treatment with trypsin (Vambutas and Racker 1965) or by the presence of methanol (Sakurai et al. 1981), the ATPase activity of isolated AF_1 is also enhanced by trypsin or methanol, but not by dithiothreitol (Nemoto et al. 1990). When AF_1 was activated by trypsin and preincubated for 1 h with tentoxin, its Ca^{2+}-ATPase activity was inhibited by 50% by 0.3 μM tentoxin (Fig. 1). Under similar conditions, trypsin-activated spinach CF_1 was inhibited by 50% by 0.04 μM tentoxin (data not shown). Selman and Durbin (1978) reported that the Ca^{2+}-ATPase activities of trypsin-treated CF_1s from tentoxin-sensitive plants (lettuce, spinach, and *N. otophora*) were inhibited by 50% by 0.01–0.03 μM tentoxin when the duration of preincubation with tentoxin was 1 h. Under similar conditions, CF_1s from insensitive plants (*N. tabacum* and *N. knightiana*) were not affected by 0.24 μM tentoxin. These results indicate that AF_1 is slightly less sensitive to tentoxin than CF_1s from reported tentoxin-susceptible plants and that it is significantly more sensitive than those from reported tentoxin-re-

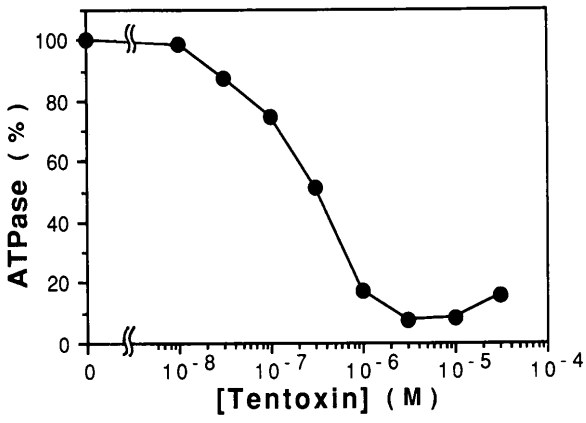

Fig. 1 Effects of tentoxin on the ATPase activity of trypsin-activated AF_1. Purified AF_1 was activated by treatment with trypsin, as described in Materials and Methods, and 5 μg of the activated AF_1 in 200 μl of 50 mM Tricine-NaOH (pH 8.0) were preincubated with tentoxin (at 1.25 times the concentrations indicated) for 1 h at room temperature. The assay was initiated by addition of 50 μl of a solution of ATP and $CaCl_2$ to the preincubation mixture to give final concentrations of 40 mM Tricine-NaOH (pH 8.0), 4 mM ATP, 6 mM $CaCl_2$ and the indicated concentrations of tentoxin. Reactions were terminated, after 15 min at 37°C, by addition of 150 μl of 8% (w/v) trichloroacetic acid. The control ATPase activity was 0.86 μmol P_i liberated (mg protein)$^{-1}$ min^{-1}.

sistant plants.

Steele et al. (1978b) reported that tentoxin is inhibitory at less than 1 μM and markedly stimulatory at 10–1,000 μM when Ca^{2+}-ATPase activities of CF_1s from tentoxin-sensitive plants (romaine lettuce, spinach, and *N. solanifolia*), but not those from insensitive plants (*N. paniculata* and radish), are assayed. We also noticed that when the concentration of tentoxin was increased above 3 μM, the Ca^{2+}-ATPase activity of AF_1 was slightly enhanced (Fig. 1).

Effects of tentoxin on photophosphorylation—Tentoxin inhibited photophosphorylation in thylakoids from *A. nidulans* as it does in those from susceptible higher plants, and the extent of inhibition was dependent on the duration of the preincubation (Fig. 2), as in the case of the ATPase activity of isolated spinach CF_1 (Steele et al. 1978a). In the presence of 4 μM tentoxin, half-maximal inhibition and maximal inhibition of about 95% were observed after 1- and 15-min preincubation, respectively. In the presence of 1 μM tentoxin, half-maximal inhibition and maximal inhibition of about 80% were observed after about 10- and 30-min preincubation, respectively. Selman and Durbin (1978) reported that, after a 1-min preincubation, photophosphorylation in typical sensitive plants (lettuce, spinach and *N. otophora*) was inhibited by 50% by tentoxin at 1–3 μM, while that in *N. tabacum* and *N. knightiana* was inhibited by less than 20% by tentoxin at 6 μM.

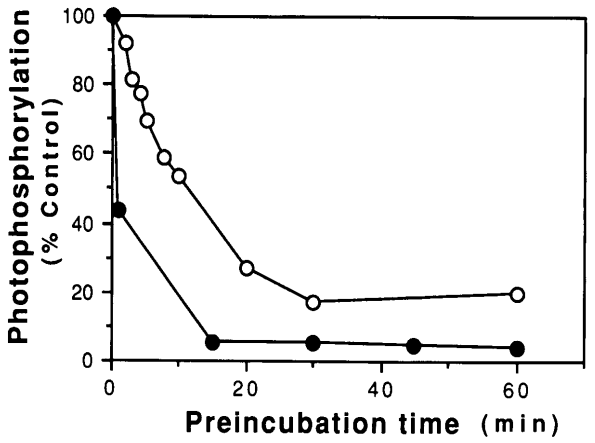

Fig. 2 Effects of the duration of preincubation on inhibition by tentoxin of photophosphorylation. Thylakoid membranes containing 10 μg of Chl were preincubated with tentoxin (1.06 times the final concentrations indicated) in 940 μl of 20 mM Tricine-NaOH (pH 8.0), 5 mM KCl, 5 mM $MgCl_2$ and 5 mM [^{32}P]P_i at room temperature. At the times indicated, photophosphorylation was initiated by addition of 60 μl of a solution that gave final concentrations of 2 mM ADP, 60 μM phenazine methosulfate and 1 mM sodium ascorbate to the preincubation mixture which included 1 (○) or 4 (●) μM (final concentration) tentoxin. Mixing of solutions was followed immediately by illumination for 4 min at 20°C. The control activities were 113 (for 1 μM tentoxin) and 137 (for 4 μM tentoxin) μmol ATP (mg Chl)$^{-1}$ h^{-1}, respectively.

These results indicate that photophosphorylation in *A. nidulans* is sensitive to tentoxin.

When thylakoids from *A. nidulans* were preincubated with tentoxin for 30 min, phenazine methosulfate-supported cyclic photophosphorylation was inhibited by 50% by 0.3 μM tentoxin (Fig. 3). Maximal inhibition of about 95% was observed with 2 μM tentoxin.

Effects of tentoxin on uptake of protons—If tentoxin acts as a typical energy-transfer inhibitor in photophosphorylation in thylakoids of *A. nidulans* as suggested by Dahse et al. (1986) from measurements of delayed fluorescence and as demonstrated in thylakoids from susceptible plants (Arntzen 1972), it can be anticipated that it also inhibits the utilization of the proton gradient under phosphorylating conditions. Using 9-aminoacridine as a fluorescent probe of pH (Schuldiner et al. 1972), we determined the effects of tentoxin on formation of a proton gradient in thylakoids of *A. nidulans* under phosphorylating and non-phosphorylating conditions (Fig. 4). Under non-phosphorylating conditions, illumination caused a quenching of the fluorescence of 9-aminoacridine, which indicated formation of a proton gradient. Under phosphorylating conditions, when ADP and P_i were present, the extent of the light-induced quenching of the fluorescence was smaller, indicating that the size of the steady-state gradient of protons was decreased by utilization of the gradient of protons for ATP formation. Addition of tentoxin to the phosphorylating mixture restored the quenching of fluorescence to a considerable extent, an indication that tentoxin inhibits photophosphorylation not by acting as an uncoupler and collaps-

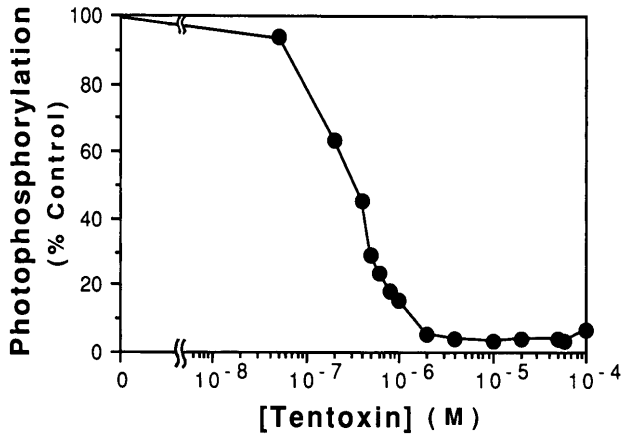

Fig. 3 Effects of the concentration of tentoxin on photophosphorylation. Thylakoid membranes containing 10 μg Chl in 940 μl of medium were preincubated with tentoxin (1.06 times the concentrations indicated) for 30 min at room temperature, and photophosphorylation was initiated by addition of 60 μl of the solution that contained ADP, phenazine methosulfate, and sodium ascorbate, with subsequent immediate illumination, as described in the legend to Fig. 2. The control activity was 113 μmol ATP (mg Chl)$^{-1}$ h^{-1}.

ing the proton gradient, but by acting as an energy-transfer inhibitor and inhibiting the utilization of the proton gradient. The foregoing results (Fig. 1-4) indicate that tentoxin inhibits photophosphorylation in *A. nidulans* by acting on AF_1 as an energy-transfer inhibitor as suggested by Dahse et al. (1986) from measurements of delayed fluorescence with intact cells.

Effects of tentoxin on the level of ATP in intact cells—When tentoxin was added at 200 μM to a suspension of cells (8.3×10^7 ml^{-1}) under air in the light, the intracellular level of ATP decreased gradually by 20–30% in 10–15 min (Fig. 5). With tentoxin at 50 μM, there was a transient decrease of about 10% in the level of ATP, which returned to a level slightly lower than the control in 5 min. In the dark, addition of tentoxin at 50 μM caused a decrease of about 30% in the level of ATP in less than 30 s, and the reduced level was maintained almost unchanged for the subsequent 15 min. Increasing the concentration of tentoxin to 200 μM decreased the level of ATP only slightly further. There were no appreciable changes in levels of ATP in the control cells throughout the course of the above experiments both in light and in darkness (data not shown).

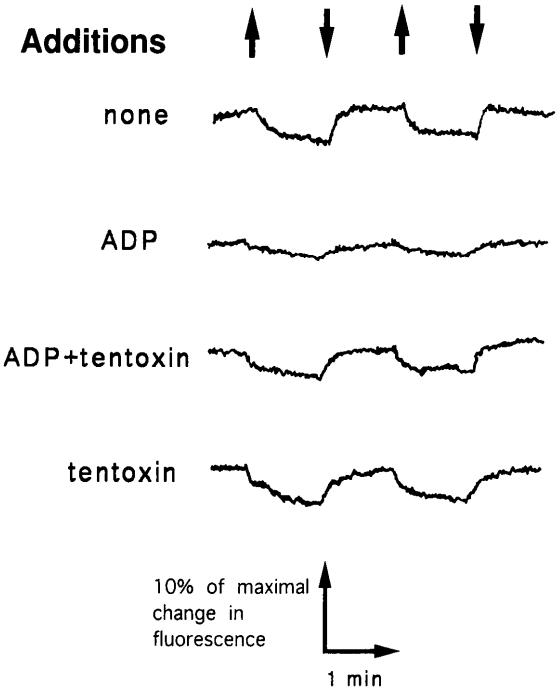

Fig. 4 Effects of tentoxin on fluorescence of 9-aminoacridine. Thylakoid membranes were suspended at 5 μg Chl ml^{-1} in darkness in 10 mM Tricine-NaOH (pH 8.0), 0.4 M sucrose, 5 mM P_i (pH 8.0), 10 mM $MgCl_2$, 5 mM KCl, 30 μM phenazine methosulfate and 1 μM 9-aminoacridine. The mixture in a cell (1 cm × 1 cm) in the fluorometer was illuminated with red actinic light (1,650 μE m^{-2} s^{-1}) at 26°C. Where indicated, ADP and tentoxin were added immediately before measurements to final concentrations of 2 mM and 25 μM, respectively. ↑, Light on; ↓, Light off.

These results suggest that the respiration-linked synthesis of ATP is also catalyzed by AF_1, which is inhibited by tentoxin. We note here, in this connection, that Wastyn et al. (1987) showed that cytochrome oxidase is associated with thylakoid membranes in the cyanobacterium *Synechocystis*. The weaker effects of tentoxin on the level of ATP in the light than in the dark can be explained if we assume that phosphorylating activity is stronger in the light than in the dark so that partial inhibition of phosphorylation has less of an effect in the light than in the dark.

Effects of tentoxin on cell growth—When tentoxin at 50 μM was added to a culture of *A. nidulans* cells, their growth rate decreased considerably but they survived the exposure to tentoxin. In a parallel experiment, although the chlorophyll content (Chl/A_{750}) of the tentoxin-treated cells incubated under white light at 35 μE m^{-2} s^{-1} fell to about 65% of that of the control cells during the period of inhibited growth, the cells were not completely bleached (data not shown). However, when the light intensity was increased to 60 μE m^{-2} s^{-1}, the tentoxin treated cells were bleached and died. We noted that the apparent effectiveness of tentoxin depended on the cell density: the lower was the initial cell density, the greater was the inhibition

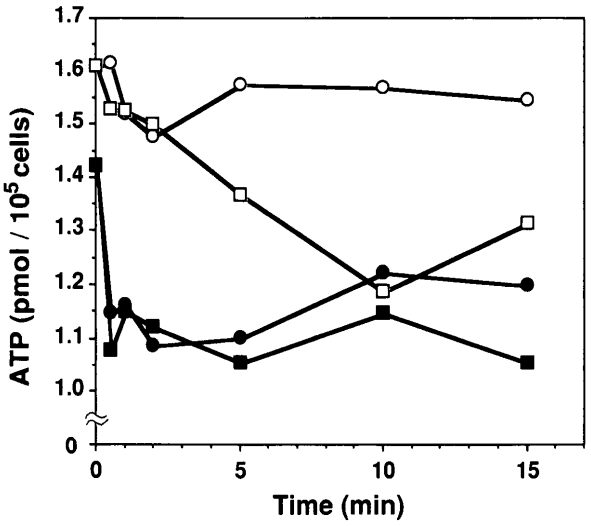

Fig. 5 Effects of tentoxin on levels of ATP in intact cells. *A. nidulans* cells (6.6×10^7) in 800 μl of growth medium were put in a test tube under air and kept either under white light (53 μE m^{-2} s^{-1}) or in darkness at 26°C. After 10 min, 16 μl of a solution of tentoxin in ethanol were added to the cell suspension to give a final concentration of 50 or 200 μM tentoxin, and incubation was continued either in the light or in darkness. At the times indicated, 50 μl of the incubation mixture were withdrawn and added to 10 μl of 12% (w/v) perchloric acid in a plastic tube. ATP in the neutralized supernatant was assayed by a standard firefly luciferase-luciferin assay. ○, Light, 50 μM tentoxin; □, light, 200 μM tentoxin; ●, darkness, 50 μM tentoxin; ■, darkness 200 μM tentoxin.

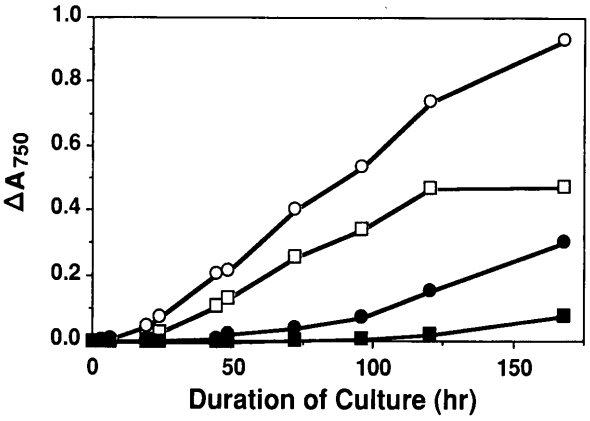

Fig. 6 Effects of tentoxin on cell growth. Cells at 0.8×10^6 ml^{-1} (□, ■) or 1.9×10^6 ml^{-1} (○, ●) in 2 ml of growth medium that contained 0 (□, ○) or 50 μM (■, ●) tentoxin were put in loosely stoppered test tubes and incubated at 28°C under white light at 35 $\mu E\ m^{-2}\ s^{-1}$ with continuous shaking (3 Hz). Cell density was monitored by direct measurements of A_{750} of the suspensions in test tubes with a spectrophotometer. ΔA_{750} represents the average of results from quadruplicates at each concentration of tentoxin.

(Fig. 6). The apparent weaker effect of tentoxin at 50 μM on the level of ATP (Fig. 5) than on cell growth (Fig. 6) can be explained by the difference in the initial cell density between these experiments: 8.3×10^7 in the experiment for which results are shown in Figure 5, and 0.8×10^6 and 1.9×10^6 cells ml^{-1} in that in Figure 6.

Our results indicate that tentoxin acts as an energy-transfer inhibitor on ATP synthase in *A. nidulans* and that it should be useful as a tool for manipulation of levels of ATP in intact cells while the high-energy state of thylakoid membranes is maintained.

This work was supported in part by a Waseda University Grant for Special Research Projects to H.S.

Note

During our preparation of this manuscript, Avni et al. (1992) published a report that the plastid *atp*B genes from six species of *Nicotiana* species differ at the residue encoded by codon 83 according to their response to tentoxin: glutamate at this position is correlated with resistance to tentoxin and aspartate is correlated with sensitivity. The corresponding codon in the cyanobacterium *Synechococcus* 6301 (formerly called *Anacystis nidulans*) encodes aspartate (Cozens and Walker 1987).

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(Received October 5, 1992; Accepted February 22, 1993)