The effects of tentoxin on the energy-dependent exchange of the tightly bound adenine nucleotides of chloroplast thylakoid membranes have been studied.

1) The light-induced binding of adenine nucleotides to thylakoid membranes in the dark is strongly inhibited by tentoxin. Fifty per cent inhibition of the binding with ADP, ATP, and adenyl-5'-yl imidodiphosphate (AMP-P(NH)P) is obtained between 2 to 5 μM tentoxin. The tentoxin inhibition of adenine nucleotide binding in the dark is light-dependent, and tentoxin must be present during the illumination of the thylakoid membranes.

2) The rate and extent of the light-induced release of the tightly bound adenine nucleotides is inhibited by tentoxin.

3) The effect of tentoxin on the binding of ADP in the light is a function of the light intensity, whereas tentoxin stimulates the binding of ATP 2- to 4-fold at all light intensities. Half-maximal stimulation of ATP binding occurs at 2 μM tentoxin, and the stimulation saturates at 10 μM. Tentoxin also increases the binding of [γ-32P]ATP and AMP-P(NH)P but less than that obtained with [3H]ATP.

4) The interaction of tentoxin with the membrane-bound chloroplast Coupling Factor 1 does not change the apparent dissociation constant (Kd) for ATP in the light (1.8 μM); however, the maximal binding increases from 0.18 to 0.40 nmol adenine nucleotide bound/mg of chlorophyll. In addition, tentoxin markedly increases the initial rate of binding with ATP.

Adenine nucleotides tightly bound to membrane-bound CF1 undergo energy-dependent exchange with free adenine nucleotides (1-6). Both the exchange and phosphorylation of tightly bound ADP are inhibited by uncouplers (2, 4, 5, 7-9) and energy transfer inhibitors of the type that block proton flux through the hydrophobic proton channel part of the ATPase complex (e.g. dicyclohexylcarbodiimide and triphenyltin chloride) (10). However, light-dependent exchange is apparently not inhibited by energy transfer inhibitors that directly block the catalytic activity of CF1 such as Dio-9 (2), phlorizin (4, 7), and tentoxin (10). Although the function of the tightly bound exchangeable adenine nucleotides is not clear, it has been suggested that they are important for the regulation of the "latent" ATPase activity of CF1, by occupying either the active site or a regulatory site (11). If this suggestion is correct, one might expect CF1-specific energy transfer inhibitors to affect either the extent or the rate of exchange of the tightly bound adenine nucleotides, or both.

Although low concentrations of tentoxin inhibit photophosphorylation and the ATPase activity of both the membrane-bound and soluble CF1 (12-14), higher concentrations of tentoxin stimulate the ATPase activity of both the latent and activated ATPases (15). High concentrations of tentoxin also stimulate an energy-independent adenine nucleotide exchange with the membrane-bound CF1 (10, 16) and induce the binding of adenine nucleotides to soluble CF1, presumably by inducing an exchange of the tightly bound ATP for medium ATP (17). However, the low levels of tentoxin needed to inhibit energy transfer with membrane-bound CF1 have not yet been correlated with any effect on the light-dependent adenine nucleotide exchange.

In this communication, we report the effects of CF1-specific energy transfer inhibitors on light-dependent adenine nucleotide exchange. Energy transfer inhibitors markedly change the binding of adenine nucleotides to chloroplast membranes, however, not all energy transfer inhibitors behave similarly.

In the light, tentoxin increases the steady state level of bound adenine nucleotides. This is related to an inhibition by tentoxin of the release of adenine nucleotides from the adenine nucleotide tight binding site. On the other hand, the binding of adenine nucleotides to thylakoid membranes in the dark (following preillumination) is inhibited by tentoxin. The inhibitory effect of tentoxin on the binding of adenine nucleotides in the dark is light-dependent and is a result of (i) an inhibition of the light-induced release of tightly bound adenine nucleotides and (ii) a destabilization of the loosely bound adenine nucleotide CF1 complex formed in the light.

**EXPERIMENTAL PROCEDURES**

[3,32P]ATP and [8-3H]AMP-P(NH)P were purchased from New England Nuclear and [2,2-3H]ATP from ICN. [γ-32P]ATP was prepared from ADP and [3H]phosphate by photophosphorylation and was purified as described (18) ADP, ATP, AMP-P(NH)P, CCCP, phosphoenolpyruvate, pyruvate kinase (type III), quercetin, and phlorizin were obtained from Sigma. Dithiothreitol was obtained from Aldrich, and tentoxin was prepared as previously described (19).

Chloroplast membranes were isolated from spinach leaves as described (11), and the chlorophyll was determined according to Arnon (19). Light-induced incorporation of adenine nucleotides into mem-

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‡ To whom all correspondence should be addressed.

The abbreviations used are: CF1, chloroplast Coupling Factor 1; Chl, chlorophyll; CCCP, carbonylcyanide-p-trichloromethoxyphenyl hydrazone; tricine, N-[Tris(hydroxymethyl)methyl]glycine; AMP-P(NH)P, adenyl 5'-yl imidodiphosphate; nE, nanoEinstein.
brane-bound CF, was assayed using the methods described by Strotmann et al. (20). For details, see Ref. 11. The incubation medium contained, in a final volume of 0.5 ml, 25 mM tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl2, 20 μM phenazine methosulfate, 10 μM [3H] or [γ-32P]adenine nucleotide (containing approximately 3 to 10 × 104 cpm/nmol), and chloroplasts equivalent to 0.2 mg of chlorophyll/ml. The reaction mixtures were stirred at 22°C for 10 s and then illuminated for the indicated times with white light from a heat-filtered 250-watt tungsten halogen lamp held 16 cm from the sample.

Under those conditions, the maximal light intensity was 620 nano-Einstein cm−2 s−1. The light-induced exchange was quenched by the addition of 0.2 ml of a solution containing 50 μM CCCP and 20 mM ATP or ADP (pH 8.0). The H- or γ-32P-contents of the chloroplast membranes were measured as in Ref. 11.

ATPase activity and ATP formation were measured under the same conditions described for light-induced adenine nucleotide exchange except that the reactions were stopped by addition of trichloroacetic acid at a final concentration of 3%. The released [32P]phosphate was extracted into isobutanyl alcohol/benzene (1:1, v/v) as described (21) and measured by liquid scintillation.

RESULTS

Tentoxin Inhibition of the Light-induced Binding of Adenine Nucleotides to Thylakoid Membranes in the Dark—
Reimer and Selman (10) have reported that tentoxin, at low concentrations (<5 μM), does not inhibit the energy-dependent exchange of ADP, whereas high concentrations (200 to 400 μM) of tentoxin stimulate an energy-independent adenine nucleotide exchange. Fig. 1 shows the effect of tentoxin on the steady state binding of ADP to thylakoid membranes in the light and on the net exchange of ADP in the subsequent dark period. These results confirm that low concentrations of tentoxin (<10 μM) do not inhibit the binding of ADP in the light, although at higher concentrations of tentoxin (about 100 μM) an inhibition (33%) is observed. The binding of adenine nucleotides to thylakoid membranes in the dark (following a 30-s preillumination), however, is severely inhibited by tentoxin. As seen in Fig. 1, the binding of ADP is inhibited 67% and 80% at 10 and 100 μM tentoxin, respectively. Table I shows that 10 μM tentoxin also inhibits the binding of adenine nucleotide in the dark with ATP 70% and AMP-P(NH)P 58% (also see Fig. 6).

The kinetics of the binding of ADP to thylakoid membranes are shown in Fig. 2. Upon illuminating the membranes, the level of bound (labeled) ADP increases presumably by exchanging with the (unlabeled) ADP bound to the membranes prior to illumination. Turning off the light results in a biphasic increase in the amount of bound ADP with a rapid phase (t1/2 ~ 20 ms) (20) and a slow phase (t1/2 > 5 s). The slow phase of binding is completed in about 2 min (data not shown). Depending on the light intensity, the binding in the dark increases about 2- to 3-fold over the steady state level reached in the light (11, 20). Tentoxin (50 μM) inhibits the binding of ADP in the light about 20% and completely inhibits the rapid phase of binding in the dark. The slow phase in the dark is less affected by tentoxin.

Tentoxin Inhibition of Adenine Nucleotide Binding in the Dark: Requirement for a Preillumination in the Presence of Tentoxin—In order to determine if tentoxin must be present during the preillumination to inhibit the binding of adenine nucleotides in the dark, tentoxin was added either prior to the preillumination or concomitantly upon extinguishing the light. Fig. 3 shows the results for the binding in the dark with ATP. Whereas 10 μM tentoxin inhibits the dark binding of ATP 64% when added before the illumination of the chloroplasts, the addition of tentoxin in the dark after preillumination has no
effects on the binding. Identical results have been obtained for the binding of ADP (data not shown). These results suggest that tentoxin either interacts with a light-induced conformation of CF₁ which inhibits the rebinding of adenine nucleotides to the adenine nucleotide depleted form of the enzyme or tentoxin (in the light) prevents the formation of the conformation of CF₁ that binds adenine nucleotides.

Fig. 4 compares the effect of the preillumination light intensity on the magnitude of the binding of ADP in the dark in the presence and absence of tentoxin. As expected for the control, the binding of ADP in the dark increases with increasing preillumination light intensity. This merely reflects the fact that light is needed to drive the exchange reaction and that the steady state level of tightly bound ADP in the light decreases with increasing light intensity allowing more ADP to bind in the subsequent dark period (20). However, the inhibition of the binding by tentoxin is maximal at low preillumination light intensities (where complete inhibition is observed until about 20% of the maximal light intensity) and decreases with increasing light intensity. At the highest light intensity available, the binding in the control saturated at 0.86 nmol of ADP/mg of chloroplasts and was inhibited 50% by 25 μM tentoxin. Because the inhibitory effect of tentoxin on ADP binding in the dark can be partially overcome by increasing the preillumination light intensity, at least one effect of tentoxin appears to be an inhibition of the light induced release of bound ADP.

Tentoxin Inhibition of the Light-dependent Release of Bound ADP—In order to directly determine the effect of tentoxin on the light-dependent release of bound ADP, chloroplasts were illuminated in the presence of [³H]ADP in strong light, incubated for 1 min in the dark (during which tentoxin could be added), and illuminated again for various times. The rate of release of bound ADP is a function of the light intensity, and at high light intensities the release is very rapid (t½ < 1 s) (data not shown). In order to resolve the kinetics for the release of ADP, it is necessary to reduce the light intensity during the second illumination period. The time course for the release of bound ADP is shown in Fig. 5. The release appears to be monophasic and, at the low light intensity used, the rate of the release of ADP was 72 pmol of ADP/mg of Chl/s. The final level reached in the light was 0.4 nmol/mg of Chl, essentially identical to that obtained when the chloroplasts were illuminated for 30 s at the same low light intensity. Tentoxin decreases the initial rate of adenine nucleotide release and the amount released. In the experiment shown in Fig. 5, 50 μM tentoxin inhibited the rate of release 56%.

Tentoxin Stimulation of Adenine Nucleotide Binding to Thylakoid Membranes in the Light—Although Figs. 1 and 2 show that tentoxin inhibits ADP binding to thylakoid membranes in the light, this inhibition is a function of the light intensity. These results are shown in Table II. The inhibition by tentoxin of the binding of ADP in the light is highest at low light intensities and decreases with increasing light intensity. At high light intensity, where the level of tightly bound

<table>
<thead>
<tr>
<th>Light intensity</th>
<th>nmol of ADP bound/mg of Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.208</td>
</tr>
<tr>
<td>160</td>
<td>0.403</td>
</tr>
<tr>
<td>620</td>
<td>0.340</td>
</tr>
</tbody>
</table>
ADP is minimal, tentoxin actually stimulates the binding of ADP (at maximal light intensity, the stimulation is 40%). The stimulatory effect of tentoxin on the binding of adenine nucleotides in the light is even more pronounced with ATP. Fig. 6 shows a concentration curve for the tentoxin stimulation of the binding of adenine nucleotides with ATP. Half-maximal stimulation occurs at about 2 μM tentoxin, whereas the stimulation (70%) saturates at 10 μM. Above 10 μM tentoxin, a slight inhibition of the binding is observed. Table I also compares the stimulatory effect of tentoxin on the binding of ATP and AMP-P(NH)P. At 10 μM tentoxin, the binding with ATP increases 125%, whereas the binding of AMP-P(NH)P increases 45%.

Table III compares the effect of tentoxin on the binding of adenine nucleotides with [3H]ATP and [γ-32P]ATP in the light and dark (following a 30-s preillumination). In the absence of tentoxin, both nucleotides are bound to the thylakoid membranes in the light to the same extent. In the presence of 10 μM tentoxin, however, the binding of [3H]ATP increases 2-fold, whereas the binding of [γ-32P]ATP only increases about 20%. The small increase in the binding of [γ-32P]ATP is similar to the tentoxin-induced increase in the binding of AMP-P(NH)P (Table I) and suggests that the difference between the binding measured with [3H]ATP and [γ-32P]ATP is due to the hydrolysis of bound ATP.

Tentoxin increases the binding of ATP at all ATP concentrations (Fig. 7). Double reciprocal plots of the data shown in Fig. 7 (inset) demonstrate that the apparent dissociation constant (Ks) for ATP in the light (1.8 μM) is unchanged by 10 μM tentoxin, whereas the maximal binding increases from 0.18 in the absence of tentoxin to 0.40 nmol of AdN bound/mg of Chl in the presence of tentoxin. Therefore, the increased binding of ATP is not due to tentoxin altering the affinity of CF1, for ATP, but rather by increasing the steady state concentration of bound adenine nucleotide. In addition, tentoxin increases adenine nucleotide binding in the light even in the presence of dithiothreitol, which stimulates the ATPase activity (11) (Fig. 8). Fig. 8 also shows that tentoxin not only increases the extent of the light-dependent binding but the initial rate of binding as well. A 5-fold stimulation of binding is obtained within the first second of illumination. Thus, tentoxin affects both the rate and extent of the binding of ATP to CF1 in the light.

**Fig. 6.** Tentoxin stimulation of light-dependent ATP binding. Conditions as in Fig. 1 except that [3H]ATP (7 × 10⁶ cpm/nmol) replaced [3H]ADP and the reaction mixtures contained 2 mM phosphoenolpyruvate, 20 units/ml of pyruvate kinase, and the indicated concentrations of tentoxin. The chloroplasts were illuminated for 30 s and quenched by the addition of ATP and CCCP either in the light (●) or 1 min after the light was turned off (○).

**Fig. 7.** Adenine nucleotide binding in the light in the presence of tentoxin as a function of ATP. The binding of [3H]ATP was determined after 30 s as in Fig. 6 except that the concentration of [3H]ATP was varied and, where indicated, 10 μM tentoxin was included in the reaction mixtures. The dark controls for the various ATP concentrations were determined and were subtracted from the light values. Control (○), + Tentoxin (○).

<table>
<thead>
<tr>
<th>Conditions</th>
<th>[3H]ATP</th>
<th>[γ-32P]ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>0.139</td>
<td>0.146</td>
</tr>
<tr>
<td>Light + tentoxin</td>
<td>0.283</td>
<td>0.172</td>
</tr>
<tr>
<td>Dark</td>
<td>0.710</td>
<td>0.263</td>
</tr>
<tr>
<td>Dark + tentoxin</td>
<td>0.510</td>
<td>0.301</td>
</tr>
</tbody>
</table>

**Table III**


The experiments were performed as described in Fig. 6 except that, where indicated, 10 μM [γ-32P]ATP replaced the [3H]ATP. Chloroplasts were illuminated in the presence or absence of 10 μM tentoxin for 30 s and then the reactions quenched in the light (Light) or after 1 min in the dark (Dark).

**The Effect of Other Energy Transfer Inhibitors on Adenine Nucleotide Binding and the ATPase Activity of Thylakoid Membranes**—If the tentoxin-induced increase in the binding of adenine nucleotides to thylakoid membranes in the light is a general property of energy transfer inhibitors, it might be expected that other energy transfer inhibitors would behave similarly. Table IV shows that quercetin (0.2 mM) also increases the light-induced binding with ATP, whereas phlorizin, up to 1.4 mM, does not. Table IV also summarizes the effects of tentoxin, phlorizin, and quercetin on the ATPase activity in the light and dark. Whereas tentoxin (10 μM) apparently stimulates the ATPase activity in the light about 2-fold, in the dark, tentoxin inhibits the (light-triggered) ATPase about 80%. On the other hand, both quercetin and phlorizin inhibit the ATPase activity both in the light (60 and 65%,...
was turned off (Dark).

Procedures" after 30-s illumination (Light) or 1 min after the light.

MgATPase activity was assayed (as described under "Experimental Procedures") after 30-s illumination (Light) or 1 min after the light was turned off (Dark).

The effect of other energy transfer inhibitors on the light-dependent adenine nucleotide binding and on the ATPase activity in the light and dark

Conditions as in Fig. 6, except that the reaction mixtures contained the inhibitors at the concentrations indicated and [γ-32P]ATP (6 × 10^{6} cpnm/mmol) replaced [3H]ATP for the ATPase assay. The MgATPase activity was assayed (as described under "Experimental Procedures") after 30-s illumination (Light) or 1 min after the light was turned off (Dark).

The binding of ATP is the result of increased ADP binding (Table III), we suggest that the immediate effect of tentoxin is to increase the binding of ATP and not to stimulate the hydrolysis of ATP. The hydrolysis probably occurs during the time necessary to recover the chloroplasts from the incubation medium and wash them free of nonbound labeled cofactor. This conclusion is based on several observations. (i) Both tentoxin and quercetin (Table IV) stimulate the binding of ATP. Whereas the net ATPase activity is stimulated by tentoxin in the light, it is inhibited by quercetin. (ii) In the experiments reported here, the binding of ATP was always measured in the presence of an ATP-regenerating system which maintained a very low concentration of free ADP in the medium (11). Furthermore, the stimulation of binding is due to tentoxin increasing the maximal amount of ATP bound rather than by changing the affinity of CF1 for ATP (Fig. 7). (iii) Tentoxin increases the binding of ATP even under conditions where the ATPase activity is activated (light plus dithiothreitol). In addition, we have previously shown that activating the ATPase activity leads to a decrease, not an increase, of the steady state level of bound ATP (but not ADP) in the light (11).

The Interaction of Tentoxin with CF1.—In Fig. 9, we suggest a model to explain the effects of tentoxin on the light-induced exchange of adenine nucleotides with membrane-bound CF1. State I is the conformation of CF1 normally found in the dark with the tight adenine nucleotide binding sites filled. State I is converted to State II by the input of energy (the formation of a protonmotive force). In this conformation, the adenine nucleotide tight binding sites are converted to "loose" binding sites. In State III, the nucleotide binding sites are empty. Because tentoxin decreases the rate of release of bound adenine nucleotides (Fig. 5), we postulate that tentoxin decreases the rate of conversion of State I to State II. This is analogous to the rate of conversion of State II to State III. The Interaction of Tentoxin with CF1 in the light.

The binding of ATP is the result of increased ADP binding (Table III), we suggest that the immediate effect of tentoxin is to increase the binding of ATP and not to stimulate the hydrolysis of ATP. The hydrolysis probably occurs during the time necessary to recover the chloroplasts from the incubation medium and wash them free of nonbound labeled cofactor. This conclusion is based on several observations. (i) Both tentoxin and quercetin (Table IV) stimulate the binding of ATP. Whereas the net ATPase activity is stimulated by tentoxin in the light, it is inhibited by quercetin. (ii) In the experiments reported here, the binding of ATP was always measured in the presence of an ATP-regenerating system which maintained a very low concentration of free ADP in the medium (11). Furthermore, the stimulation of binding is due to tentoxin increasing the maximal amount of ATP bound rather than by changing the affinity of CF1 for ATP (Fig. 7). (iii) Tentoxin increases the binding of ATP even under conditions where the ATPase activity is activated (light plus dithiothreitol). In addition, we have previously shown that activating the ATPase activity leads to a decrease, not an increase, of the steady state level of bound ATP (but not ADP) in the light (11).

**DISCUSSION**

Very little information is available on the mechanism by which energy transduction is disrupted by energy transfer inhibitors that interact specifically with CF1. Our experimental results on the effects of tentoxin on the light-induced adenine nucleotide exchange and ATPase activity with membrane-bound CF1 may provide a possible mechanism of action for tentoxin.

**Tentoxin Stimulation of ATP Binding in the Light**—In the light, the amount of ATP found tightly bound to CF1 is usually substantially lower than ADP (11). However, tentoxin induces a marked increase in the amount of bound adenine nucleotide in the light with ATP. Tentoxin also increases the amount of bound ADP in the light; however, this effect of tentoxin is a function of the light intensity and is observed best at high light intensities where the amount of bound ADP is suppressed. Although a comparison of the amount of bound [3H]ATP to bound [γ-32P]ATP would suggest that the tentoxin-induced increase in bound adenine nucleotides with ATP is the result of increased ADP binding (Table III), we suggest that the immediate effect of tentoxin is to increase the binding of ATP and not to stimulate the hydrolysis of ATP. The hydrolysis probably occurs during the time necessary to recover the chloroplasts from the incubation medium and wash them free of nonbound labeled cofactor. This conclusion is based on several observations. (i) Both tentoxin and quercetin (Table IV) stimulate the binding of ATP. Whereas the net ATPase activity is stimulated by tentoxin in the light, it is inhibited by quercetin. (ii) In the experiments reported here, the binding of ATP was always measured in the presence of an ATP-regenerating system which maintained a very low concentration of free ADP in the medium (11). Furthermore, the stimulation of binding is due to tentoxin increasing the maximal amount of ATP bound rather than by changing the affinity of CF1 for ATP (Fig. 7). (iii) Tentoxin increases the binding of ATP even under conditions where the ATPase activity is activated (light plus dithiothreitol). In addition, we have previously shown that activating the ATPase activity leads to a decrease, not an increase, of the steady state level of bound ATP (but not ADP) in the light (11).

**TABLE IV**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>AdN binding (nmol/mg Chl)</th>
<th>ATPase activity Light (nmol of phosphate released/mg of Chl)</th>
<th>Dark (nmol of phosphate released/mg of Chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tentoxin</td>
<td>10</td>
<td>0.149</td>
<td>8.7</td>
<td>75.2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50</td>
<td>N.D.</td>
<td>3.3</td>
<td>47.2</td>
</tr>
<tr>
<td>Phlorizin</td>
<td>140</td>
<td>0.136</td>
<td>3.1</td>
<td>21.2</td>
</tr>
</tbody>
</table>

"N.D., not determined.

respectively) and in the dark (90 and 70%, respectively). Thus, although quercetin resembles phlorizin with respect to its effect on the ATPase activity, it more closely resembles tentoxin with respect to adenine nucleotide exchange.

FIG. 8. The time course of the tentoxin stimulation of ATP binding in the light. Chloroplasts, in reaction mixtures described in Fig. 6, were illuminated for the indicated periods of time in the absence or presence of 10 mM dithiothreitol, or 10 μM tentoxin or both.

**FIG. 9. Model for the effects of tentoxin on adenine nucleotide exchange with membrane-bound CF1.** k1 is the rate constant for the formation of State II and is proportional to the absorbed light intensity, k2 is the rate constant for the decay of State II to State I; k3 is the rate constant for the formation of State III; k-1 is the rate constant for the decay of State III to State II; k-2 is the rate constant for the decay of State II to State V; k-3 is the rate constant for the decay of State III to State II; k-3 is the rate constant for the decay of State II to State V. k4 is the rate constant for the energy-dependent conversion of State V to State III and is proportional to the absorbed light intensity. CF1 is an inactive conformation of CF1 formed in the light in the presence of tentoxin. En, energy. See "Discussion" for details of the model.

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\(^{2}\) V. Shoshan and H. Strotmann, submitted to J. Biol. Chem.
to decreasing the light intensity and would result in an increase in the steady state concentration of State I in the light. This would explain the tentoxin stimulation of adenine nucleotide binding in the light (Fig. 6 and Table II) and the observation that the inhibition of the binding in the dark decreases with increasing light intensity (Fig. 4).

The effect of tentoxin on CF₁, however, is more complex than simply decreasing the efficiency of the conversion of State I to State II. Tentoxin markedly decreases the magnitude of the rapid phase (Δ₁/₂ₙ ≈ 20 ms) of binding in the dark associated with the back reaction of State II to State I (Fig. 2) and decreases the total amount of adenine nucleotides that bind in the dark. This implies that tentoxin destabilizes State II leading to a form of CF₁ that does not bind adenine nucleotides, State V, and is catalytically inactive. State V represents a stable conformation of CF₁, formed only in the presence of tentoxin in the light. On the other hand, if there were no way for State V to be dissipated, after a short time of illumination, all of the CF₁ would be found in State V and there would be no CF₁ available to bind ADP. At high concentrations of tentoxin (≈100 μM) this is indeed what is found (Fig. 1); however, at lower concentrations of tentoxin (<10 μM) the system is in equilibrium after a few seconds of illumination. We propose, therefore, that State V is converted back to State III in an energy-dependent reaction. In this regard, it is important to note that, (i) tentoxin does not alter the apparent Kₑ for ATP in the light (Fig. 7); (ii) tentoxin, in contrast to phlorizin and quercitin, inhibits the ATPase activity of CF, more in the dark than in the light (Table IV); and (iii) the tentoxin inhibition of adenine nucleotide binding decreases with increasing light intensity (Fig. 4).

Previously, we have suggested that the tightly bound adenine nucleotides occupy either the catalytic site of the ATPase or a regulatory site near the catalytic site and that removal of the tightly bound adenine nucleotides is a prerequisite to the activation of the protein (11). There is abundant evidence in the literature to support either a direct or indirect involvement of the nucleotide tight binding site(s) in ATP synthesis (5, 9, 23-25). Therefore, we suggest that tentoxin acts as an energy transfer inhibitor by (i) slowing down the conformational change of CF₁ associated with the transition from State I to State II, and therefore to State III, and (ii) destabilizing State II, forming an inactive adenine nucleotide depleted CF₁.

It has been well established that electron transfer is coupled via the proton motive force to changes in the conformation of CF₁ (25-29). Strotmann and Bickel-Sandkotter (6) and Graber et al. (30) have also attributed light-dependent adenine nucleotide exchange to conformational changes in the ATPase, and Harris and Crofts (31) have interpreted the activation of the ATP synthetase and ATPase in short saturating light flashes as a rapid displacement of the ε-subunit away from the active site of CF₁, i.e., a conformational change. Our results are consistent with the interpretation that the tightly bound adenine nucleotides are released upon an energy-dependent conformational change of the protein (although energy is not a necessary prerequisite (10, 17)). It has been proposed that ATP formation may involve, in part, conformational changes in CF₁ that lead to changes in the properties of the adenine nucleotide binding site which releases the newly formed ATP. Our observations that tentoxin and quercetin inhibit the rate of the release of the bound adenine nucleotides would support this supposition; however, we cannot rule out the possibility that the adenine nucleotides are bound to a "regulatory" site and must be displaced before CF₁ can synthesize ATP.

Because of the differences in the effect of various energy transfer inhibitors on the adenine nucleotide exchange in both the light and dark, it is probable that the mechanism by which energy conservation is disrupted by the inhibitors is not exactly the same for all the inhibitors. Studies on the possible mechanisms by which other energy transfer inhibitors affect phosphorylation and ATPase activity are in progress.

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V Shoshan and B R Selman


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