Chloroplast coupling factor 1: A species-specific receptor for tentoxin
(cyclic peptide/phytotoxin/disease resistance)

JOHN A. STEELE*, THOMAS F. UCHYTIL*, RICHARD D. DURBIN*, PRADIP BHATNAGAR†, AND DANIEL H. RICH‡

* Plant Disease Research Unit, Agricultural Research Service, U.S. Department of Agriculture, and Department of Plant Pathology, University of Wisconsin, Madison, Wisc.; † School of Pharmacy, University of Wisconsin, Madison, Wisc., 53706

Communicated by John C. Walker, April 14, 1976

ABSTRACT  Tentoxin, a cyclic tetrapeptide, induces chlorosis in certain plant species. It inactivated photophosphorylation and coupling factor 1 (CF1) ATPase in lettuce, a sensitive species. This effect was due to binding of tentoxin with CF1 at a single site (affinity constant 1.3 to 20 x 10⁷ M⁻¹). Neither AMP nor adenylyl-imidodiphosphate appeared to bind to this site. In radish, an insensitive species, 20 times more tentoxin was required for 50% inhibition of photophosphorylation. In this species CF1 ATPase was unaffected by tentoxin, and its CF1 bound tentoxin only weakly (affinity constant less than 1 x 10⁴ M⁻¹). Sensitivity of photophosphorylation to tentoxin was correlated with chlorosis sensitivity in six other species examined.

Evidence that supports a receptor site hypothesis has been developed during our investigations of the mode of action of tentoxin (cyclo(-L-leucyl-N-methyl-(Z)-dehydrophenylalanylglycyl-N-methyl-L-alanyl-), molecular weight, 414) (9). Produced by the phytopathogenic fungus Alternaria tenuis, it induces chlorosis in many plants, including lettuce, potato, cucumber, and spinach, but has little or no effect on others such as radish, tobacco, and corn (4, 5). The chlorosis results from a selective disruption of chloroplast function that in the amounts of lipids (6) and proteins (7) specific to chloroplasts are reduced and the ultrastructural alterations found are confined to the chloroplast (8). The sharp demarcation between sensitive and insensitive species suggested that chloroplasts from sensitive species might possess a specific receptor site for tentoxin. Because preliminary studies have shown that tentoxin inhibits phosphorylating electron transport in lettuce chloroplasts (9) and chloroplast coupling factor 1 (CF1) is directly associated with phosphorylaphoresis (10), we investigated it as a potential receptor molecule for tentoxin.

This paper presents evidence that CF1 from lettuce, a tentoxin-sensitive species, has a single binding site for tentoxin and that when tentoxin occupies this site, both CF1 ATPase and phosphorylating electron transport are inactivated. In contrast, CF1 from radish, an insensitive species, has a lower affinity for tentoxin than does lettuce CF1; tentoxin does not inhibit radish ATPase and only slightly inhibits photophosphorylation.

MATERIALS AND METHODS

Chloroplasts were prepared from leaves of lettuce (cv. Romaine) and spinach purchased locally and from seedlings of radish (cv. Comet) and other species grown in controlled environment chambers. One hundred grams of selected leaves were blended for 10 sec at 4° in 250 ml of a 0.4 M sucrose 0.05 M N-[tris(hydroxymethyl)methyl]glycine (Tricine) buffer, pH 8.0, (ST). The homogenate was filtered through eight layers of cheesecloth, and the chloroplasts were collected by centrifugation (1000 x g for 10 min). After washing in 40 ml of ST and an additional centrifugation, the chloroplast pellet was suspended in 10 ml of ST and adjusted to 1 mg of chlorophyll per ml (11).

Electron transport was measured with an oxygen electrode using methods modified from those of Arntzen (9). Chloroplasts (35 µg of chlorophyll) in ST were added to 3 ml of stirred reaction mixture at 28° followed by the addition of tentoxin. Next, the electrode was inserted during a brief cessation of stirring. Two minutes after the addition of tentoxin, the reaction chamber was illuminated with a tungsten lamp (15 klm/m²). The course of oxygen uptake was followed during the subsequent 1 min. Basal rates were measured in the presence of 3 mM ADP; complete rates were determined in the added presence of 5 mM NaH₂PO₄. Only preparations having a complete rate more than twice the basal rate were used.

Coupling factor 1 was prepared from chloroplasts by the methods of Lien and Racker (12). Lettuce CF1 was judged to be pure by gel electrophoresis (13, 14) after chromatography on DEAE-Sephadex A-50 using a linear (NH₄)₂SO₄ gradient (Step 3 of ref. 12). Radish CF1 of comparable purity was obtained after initial concentration (Step 2 of ref. 12). Both preparations were similar with respect to electrophoretic mobility, specific activity, and activation by heat or trypsin. Their ATPase activity was abolished by reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and restored by dithiothreitol (15).

Tentoxin was prepared as previously reported (6). Tritium-labeled tentoxin was prepared as follows: L-[2,3,3-H]Ala (2.5 mCi, specific activity, 31 Ci/mmol) was mixed with L-Ala (4.6 mg) and dissolved in water (85 µl). Triethylamine (32 µl) and 4-butylazidofomate (10 µl in 85 µl of dioxane) were added (16). After 16 hr the yield of Boc-L-[2,3,3-H]Ala was 80%.

The Boc-L-[2,3,3-H]Ala was dissolved in tetrahydrofuran (1 ml) and cooled to -70 °C. Sodium hydride (20 mg, 50% dispersion) and methyl iodide (300 µl) were added under N₂. The mixture was warmed to 25° and stirred for 40 hr. The solvent was evaporated and the residue was suspended in 1 M NaHCO₃.
and washed with hexane. The aqueous layer was acidified using 0.33 M citric acid and the product was extracted with ether, dried, and concentrated. Boc-L-[2,3-3H]Me Ala was obtained in 88% yield.

Boc-L-[2,3-3H]Me Ala was dissolved in methylene chloride (600 μl) and cooled to 0°C. The hydrochloride of L-Leu-MeAPhe-Gly-OEt (ΔPhe is dehydrophenylalanine) (21.2 mg), triethylamine (7.2 mg), and dicyclohexylcarbodiimide (10.3 mg) were added. The mixture was stirred at 2°C for 15 hr, then filtered. The resulting tetrapeptide (50% yield) was purified by preparative thin-layer chromatography (silica gel F254, ethyl acetate:methanol, 95:5) and isolated in 30% yield (specific activity, 47 mCi/mmol).

Inhibition curves were analyzed assuming that coupled electron transport and CF1 ATPase were completely inhibited by the binding of tentoxin. Under this assumption, the total inhibitor present may be partitioned into bound and free portions as follows: I = I1 + I2 where I1 = total inhibitor, I2 = bound inhibitor, and I3 = free inhibitor. If the fraction of active protein bound to tentoxin is η, then I1 = I3 where β1 is the binding capacity of the system. The term i/(1 - i) is equal to the bound/free ratio and β2 is equal to the free inhibitor, where β0 is the reciprocal of the affinity constant. If β0 is introduced as a constant for statistical purposes, then I1 = β1 + β2i/(1 - i) + β3. Experimental inhibition curves were fitted to this model using standard multiple linear regression techniques (18).

The affinity of CF1 for tentoxin was measured using continuous ultrafiltration. First, CF1, recovered from a suspension in 2 M (NH4)2SO4, was dissolved in 5 ml of 50 mM Tris-HCl, pH 8.0, containing 5 mM CaCl2. After clarification by centrifugation (15,000 × g for 10 min), excess salts and nucleotides were removed by continuous ultrafiltration with 20 volumes of buffer. The protein concentration was determined spectrophotometrically (ε280 = 0.476 ml·mg⁻¹ cm⁻¹, molecular weight, 325,000) (19). The amount of [3H]MeAla-tentoxin (0.3–1 μg), protein (0.4–1 mg), and, in some cases, ADP or adeny1-5′-yl imidodiphosphate, in a total volume of 4 ml were equilibrated for 1 hr. This solution was transferred to a stirred ultrafiltration cell (Amicon Model 12) equipped with a XM-50 membrane that retains solutes above 5 X 104 molecular weight. The chamber was pressurized to 1400 kg/m² (13.7 kPa) with N2 and then connected to a buffer reservoir at the same pressure. Fractions (1.8–3.6 ml) were collected in previously weighed tubes until a total of 40 ml was collected. These tubes were weighed to determine the volume of each fraction. Tentoxin was determined as [3H] by scintillation counting in Bray’s solution (20). Initial experiments established that: (a) this system was near equilibrium at the flow rate used (0.05 ml/min), (b) tentoxin was not bound by the membrane, and (c) CF1 was completely retained.

The relationship between the proportion of added tentoxin recovered and total filtrate volume was used to estimate the numbers and kinds of binding sites and their affinity constants. The nonlinear equations of Feldman et al. (21) describing the interaction of binding sites and ligands were solved by Newton's method (22) to calculate the free equilibrium concentration of each ligand. The integral of the free concentration with respect to collected volume is the total quantity of ligand collected. This integration was achieved using a second-order Runge-Kutta method (22). To estimate affinity constants and numbers of binding sites from experimental results, this algorithm was coupled to an iterative least-squares program based on the methods of Marquardt (23). Systems including a maximum of two ligands and two classes of sites were investigated. Iteration was continued until the parameters being estimated ceased to change by more than one part per thousand. A complete description of this method is in preparation (Steele and Durbin).

RESULTS

The inhibitory effect of tentoxin on photophosphorylation by lettuce chloroplasts was linear with respect to concentration until the basal rate was reached (Fig. 1). This could be interpreted to mean that a large proportion of the tentoxin was bound by the chloroplasts. However, when twice the amount of chlorophyll was used, the response of the system to tentoxin was unchanged. This indicated that most of the tentoxin remained free and that the observed inhibition was limited by the rate of tentoxin uptake. When coupled electron transport measurements were made using chloroplasts from other species, the amounts of tentoxin (in μg/3 ml) required for 50% inhibition of coupled electron transport in sensitive species were: lettuce, 1; spinach, 1; potato, 1; and eggplant, 1.5. For chlorosis-insensitive species, the values were: turnip, 5.5; tobacco, 17; cabbage, 19; and radish, 23.

When lettuce chloroplasts (1 mg of chlorophyll per ml) were incubated in ST containing 2.4 μM tentoxin for variable lengths of time at 4°C, inhibition was first-order with respect to time. Maximum inhibition was reached in 1 hr at 4°C, with 50% of the maximum being reached in 10 min.

Lettuce chloroplasts (2 mg of chlorophyll) were incubated with various amounts of tentoxin in ST (2 ml) at 4°C for 2 hr, and
The coupled electron transport rates were determined. Unlike the response obtained at disequilibrium, the resulting curve was nonlinear (Fig. 1). When this inhibition curve was analyzed by multiple linear regression, an affinity constant of $1.3 \times 10^7 \text{ M}^{-1}$ ($28^\circ$) and a binding capacity of 2.09 to 2.65 $\times 10^{-9}$ mol of tentoxin bound per mg of chlorophyll were obtained (Table 1).

In order to estimate the affinity constant for solubilized lettuce CF$_1$, an inhibition curve for trypsin-activated ATPase activity was obtained (Table 2). The affinity constant was again estimated to be $1.3 \times 10^7 \text{ M}^{-1}$ ($37^\circ$). A similar value was estimated for heat-activated lettuce CF$_1$ (12). Since under the conditions of the ATPase assay only a small fraction of the added tentoxin is bound, binding capacities cannot be precisely estimated. No inhibition of the heat- or trypsin-activated enzyme from radish was detected at tentoxin concentrations as great as 10 $\mu$g/ml, which is 200-fold greater than that required for 50% inhibition of the lettuce enzyme.

In direct binding studies, unactivated lettuce CF$_1$ was found to have 0.81 tentoxin-binding sites per molecule of CF$_1$, with an affinity constant of $2 \times 10^8 \text{ M}^{-1}$ (20). The binding of tentoxin was not altered by the addition of 11.5 mM ADP or 0.75 mM adenylyl-5'-yl imidodiphosphate. Figure 2 illustrates the binding of tentoxin to heat-activated lettuce CF$_1$ at 20$^\circ$. The estimated number of sites was 0.85/CF$_1$ molecule and the estimated affinity constant was $3.1 \times 10^8 \text{ M}^{-1}$. The number of binding sites on radish CF$_1$ could not be reasonably estimated; however, the affinity constant was less than $1 \times 10^4 \text{ M}^{-1}$. More reliable values are difficult to obtain because the solubility of CF$_1$ is only 0.4 mM (19).

**DISCUSSION**

Our results demonstrate that tentoxin is a species-specific inhibitor of photophosphorylation. This inhibition in lettuce results from the binding of about one molecule of tentoxin per 500 chlorophylls. Such a binding capacity is consistent with reported values of the CF$_1$ : chlorophyll ratio, which generally range from 1/500 to 1/2000 (24), suggesting that the site of tentoxin action is associated with CF$_1$. This conclusion is strengthened by results showing that lettuce CF$_1$ has a single site with an affinity constant of $2 \times 10^8 \text{ M}^{-1}$ for tentoxin and that CF$_1$ ATPase is inactivated when tentoxin is bound. Neither ADP nor adenylyl-5'-yl imidodiphosphate were observed to interact with the tentoxin binding site, indicating that this site may be different from the nucleotide binding sites (19).

The CF$_1$ of radish bound tentoxin weakly and was not inhibited. These results demonstrate the CF$_1$ from radish is different from that of lettuce and suggest that some inhibitors used

---

**Table 1.** Inhibition of coupled electron transport in lettuce chloroplasts equilibrated with tentoxin for 2 hr at 4$^\circ$.

<table>
<thead>
<tr>
<th>Tentoxin (µg/2 ml)</th>
<th>$i^*$</th>
<th>$i/(1 - i)$</th>
<th>$T_x^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.030</td>
</tr>
<tr>
<td>0.16</td>
<td>0.064</td>
<td>0.068</td>
<td>0.096</td>
</tr>
<tr>
<td>0.25</td>
<td>0.239</td>
<td>0.314</td>
<td>0.277</td>
</tr>
<tr>
<td>0.50</td>
<td>0.437</td>
<td>0.777</td>
<td>0.487</td>
</tr>
<tr>
<td>0.75</td>
<td>0.644</td>
<td>1.818</td>
<td>0.723</td>
</tr>
<tr>
<td>1.00</td>
<td>0.851</td>
<td>5.78</td>
<td>1.048</td>
</tr>
<tr>
<td>1.50</td>
<td>0.942</td>
<td>16.66</td>
<td>1.471</td>
</tr>
<tr>
<td>2.00</td>
<td>0.971</td>
<td>34.40</td>
<td>2.041</td>
</tr>
</tbody>
</table>

* Fractional inhibition.
† Tentoxin (µg/2 ml) estimated from linear least squares fit: $T_x = 0.9896 i + 0.03051 (i/(1 - i)) + 0.0307$. Coefficient of determination = 0.997.

---

**Table 2.** Inhibition of trypsin-activated, lettuce CF$_1$, Ca$^{2+}$-dependent ATPase by tentoxin$^*$.

<table>
<thead>
<tr>
<th>Tentoxin (ng/ml)</th>
<th>$i^*$</th>
<th>$i/(1 - i)$</th>
<th>$T_x^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.9</td>
</tr>
<tr>
<td>8</td>
<td>0.049</td>
<td>0.052</td>
<td>4.3</td>
</tr>
<tr>
<td>20</td>
<td>0.343</td>
<td>0.523</td>
<td>23.9</td>
</tr>
<tr>
<td>32</td>
<td>0.429</td>
<td>0.754</td>
<td>32.5</td>
</tr>
<tr>
<td>40</td>
<td>0.492</td>
<td>0.969</td>
<td>40.3</td>
</tr>
<tr>
<td>48</td>
<td>0.534</td>
<td>1.145</td>
<td>46.5</td>
</tr>
<tr>
<td>60</td>
<td>0.596</td>
<td>1.476</td>
<td>57.7</td>
</tr>
<tr>
<td>80</td>
<td>0.682</td>
<td>2.149</td>
<td>79.8</td>
</tr>
<tr>
<td>100</td>
<td>0.735</td>
<td>2.776</td>
<td>100</td>
</tr>
<tr>
<td>120</td>
<td>0.775</td>
<td>3.456</td>
<td>121</td>
</tr>
<tr>
<td>166</td>
<td>0.824</td>
<td>4.655</td>
<td>159</td>
</tr>
<tr>
<td>200</td>
<td>0.851</td>
<td>5.729</td>
<td>192</td>
</tr>
</tbody>
</table>

$^*$ 3.11 mg of CF$_1$ assayed by the methods of Lien and Racker (12) with 2 mM ATP. Incubation was at 37$^\circ$ for 10 min.
† Fractional inhibition.
in the study of photosynthesis may differ in their effect from species to species. Tentoxin inhibited photophosphorylation by 50% in lettuce and other sensitive species at concentrations of 0.4–0.6 μM; 5- to 20-fold greater concentrations were required for equal inhibition of insensitive species. Thus, a causal relationship may exist between the inhibition of photophosphorylation and chlorosis. Although the details of such a linkage have not been elucidated, Bennett (25) has shown that tentoxin inhibits light-driven, but not ATP-driven, protein and RNA synthesis in isolated chloroplasts. Assuming then that chlorosis is caused by an inhibition of phosphorylating electron transport, a low affinity of CF₁ for tentoxin, as with radish, may account for insensitivity to chlorosis. However, tentoxin-transforming enzymes or permeability barriers also may prevent the toxin from reaching CF₁ and mask sensitivity at the molecular level. Such mechanisms may account for the observation that corn and lettuce chloroplasts are equally sensitive to tentoxin (9) whereas intact corn plants are insensitive (5).

The receptor site concept offers a general model to explain the sensitivity of plants to toxins, especially if multiple sites are allowed. However, mechanisms other than the absence or decreased affinity of a receptor should be considered in studies of insensitivity to toxins.

Research was cooperative with the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and the Agricultural Research Service, U.S. Department of Agriculture. The research was supported in part by a grant (GM 19311) from the National Institute of General Medical Sciences. Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.