Inhibition of Thylakoid ATPase by Venturicidin as an Indicator of CF\textsubscript{1}-CF\textsubscript{o} Interaction\textsuperscript{1}

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Venturicidin inhibits the F\textsubscript{o} portion of membrane-located, H\textsuperscript{+}-pumping ATPases. We find it meets the criteria for an energy transfer inhibitor for spinach (Spinacia oleracea) thylakoids: complete inhibition of photophosphorylation and of photophosphorylation-stimulated and basal electron flow rates, but not of electron flow under uncoupled conditions. The extent of H\textsuperscript{+} uptake in the light is stimulated by venturicidin (vtcd), as expected for a compound blocking H\textsuperscript{+} efflux through CF\textsubscript{o}. Vtcd had no effect on the nonproton pumping, methanol-stimulated ATPase of thylakoids or on soluble CF\textsubscript{1} ATPase. Under totally uncoupled conditions (saturating NH\textsubscript{4}Cl + gramicidin), vtcd can still inhibit sulfite-stimulated thylakoid ATPase completely. The concentration of vtcd needed for inhibition of ATPase was proportional to the concentration of thylakoids present in the assay, with an apparent stoichiometry of about 10 vtcd molecules per CF\textsubscript{1}/CF\textsubscript{o} for 50% inhibition. Vtcd raised the V\textsubscript{m} for ATP somewhat, but had a stronger effect on the V\textsubscript{m} with respect to ATP. Inhibition by saturating vtcd ranged from 50 to 100%, depending on the condition of the thylakoids. Grinding leaves in buffer containing 0.2 M choline chloride (known to provide superior photophosphorylation rates) helped bring on maximum vtcd inhibition; trypsin treatment or aging of thylakoids brought on vtcd-resistant ATPase. We conclude that the extent of inhibition by vtcd can be used as an indicator of the tightness of coupling between CF\textsubscript{1} and CF\textsubscript{o}.

Chloroplast ATP synthase consists of the extrinsic protein complex CF\textsubscript{o}, a reversible ATPase, and the intrinsic membrane protein complex CF\textsubscript{1}, which is the binding site for CF\textsubscript{1} and acts to conduct protons across the membrane (Nelson, 1976; Pick and Racker, 1979). ATP synthesis can be inhibited by compounds that prevent electron flow, by uncouplers, and by compounds that affect either CF\textsubscript{1} or CF\textsubscript{o} directly. The latter two groups are usually called energy transfer inhibitors. Phlorizin and Dio-9 are examples of compounds that affect CF\textsubscript{1} (McCarty et al., 1965; Izawa et al., 1966); DCCD (Sigrist-Nelson et al., 1978; Shoshan and Selman, 1980), triphenyltin (Gould, 1976), and tributyltin (Kahn, 1968) have a primary effect on CF\textsubscript{o} preventing proton flow through the thylakoid membrane. Vtdc is a fungal product that has been known for some time to inhibit mitochondrial ATPase (Walter et al., 1967). Its use with thylakoids has been explored only recently (Lill and Junge, 1989a, 1989b; Schonknecht et al., 1989).

In earlier studies from this laboratory (Larson and Jagendorf, 1989), sulfite was found to replace light in reactivating thylakoids previously reduced in the light. Sulfite seems to substitute for the H\textsuperscript{+} gradient that is needed to keep the ATPase of these thylakoids active (Junge, 1970; Bakker-Grunwald and van Dam, 1973; Gräber et al., 1977). This fact permits measurement of high rates of ATP hydrolysis in the presence of saturating levels of uncouplers. In continuing the study of the sulfite-stimulated ATPase, we have attempted to look more closely at the question of whether it is still coupled to proton pumping as ATP is hydrolyzed. Effective exploration of this issue would be aided by the use of a specific and effective inhibitor that binds to CF\textsubscript{o} and has no direct effect on CF\textsubscript{1}. Although DCCD has been used for this purpose to a considerable extent (McCarty and Racker, 1967; Sigrist-Nelson et al., 1978; Lohe and Strotmann, 1989), the fact that it reacts with CF\textsubscript{1} at higher concentrations (Shoshan and Selman, 1980; Anthon and Jagendorf, 1983) limits its utility for our purposes. Similarly, tributyltin was found to inhibit the ATPase of soluble CF\textsubscript{1} at high concentrations, and also was shown to have an undesirable uncoupling effect in the light (Kahn, 1968; Gould, 1976). Triphenyltin was considered to be a better energy transfer inhibitor than the others (Gould, 1976), but it failed to inhibit basal electron flow even at pH 8.0 and had an anomalous effect on H\textsuperscript{+} uptake at pH 6.7.

Vtdc seemed to be a more reliably specific inhibitor of CF\textsubscript{o}. Molecular analysis of mutations in plant mitochondria demonstrated that the binding site of vtdc on F\textsubscript{o} is on subunit 9 (equivalent to subunit III of CF\textsubscript{o}—see Schonknecht et al., 1989; Pla et al., 1991), on a different domain than the one that binds oligomycin (Galanis et al., 1989; Pla et al., 1991). In this report, we confirm the specificity of vtdc action on CF\textsubscript{o} and explore its effect on sulfite-stimulated ATPase. Our data indicate that ATPase in the presence of sulfite of "healthy" thylakoids involves a close interaction between CF\textsubscript{o} and CF\textsubscript{1}; and the extent of inhibition by vtdc is an indicator of the tightness of the coupling between these two complexes.

MATERIALS AND METHODS

Preparation and Treatment of Thylakoids

Thylakoids were prepared by grinding deveined spinach (Spinacia oleracea) leaves in a Waring Blendor for 30 s with 300 mM sorbitol, 25 mM Tricine-NaOH (pH 8.0), 5 mM MgCl\textsubscript{2}, 2 mg/mL of BSA, and either with or without 200 mM choline chloride. The pellet was washed once and resuspended in the same grinding buffer at a Chl concentration of 1.0

Abbreviations: DCCD, dicyclohexyl carbodiimide; vtcd, venturicidin.

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mg/mL, Chl was determined as described earlier (Wintermans and DeMots, 1965).

Reduced thylakoids were prepared by mixing 2 mL of latent thylakoids with an equal volume of buffer containing 100 mM Tricine-Na (pH 8.0), 100 mM KCl, 4 mM MgCl₂, 100 μM N-methyl phenazonium methosulfate, 20 μM ADP, and 40 mM DTT in a plastic chamber (8 × 8 × 3 cm). The chamber was placed in a 25°C water bath with a glass dish containing water above it, and illuminated from above with 1.1 mmol m⁻² s⁻¹ of white light for 5 min. After illumination, the thylakoids were kept on ice in the dark. Unless aged thylakoids were needed, the reduced thylakoids were prepared freshly and used in approximately 2 to 3 h.

EDTA-washed thylakoids were made based on the procedures described by Neumann and Jagendorf (1964). The thylakoids were washed with 10 mM NaCl, 10 mM Tricine-Na buffer at pH 7.8, and resuspended to 0.1 mg/mL of Chl in 0.75 mM EDTA, 0.5 mM Tricine-Na, pH 7.8, and incubated for 15 min at 0°C. After centrifugation, the thylakoids were resuspended in a small volume of buffer containing 20 mM Tricine-Na, pH 8.0, and 50 mM NaCl.

For trypsin treatment, the reduced thylakoids (0.75 mg/mL of Chl) were incubated with 0.15 mg/mL of trypsin on ice. At different times (see legend to Fig. 8), soybean trypsin inhibitor was added to 10 times the weight of trypsin present. The thylakoids were sedimented in a microcentrifuge at 4°C, then resuspended in 10 mM Tricine-Na (pH 8.0), 50 mM NaCl, 1 mM MgCl₂, 10 mM DTT.

The procedure for uncoupling thylakoids by sulfate + ADP in the light was as described earlier (Grebanier and Jagendorf, 1977). These thylakoids were then washed twice with 50 mM Tricine-Na (pH 8.0), 50 mM NaCl, 2 mM MgCl₂, resuspended in the same buffer to 0.5 mg/mL of Chl, and reduced by incubation with 20 mM DTT for 10 min at room temperature prior to ATPase assay. Control thylakoids were treated identically except for the absence of sulfate and ADP during exposure to light.

**ATP Hydrolysis**

All sulfate-dependent ATPase assays were performed in 96-well microtiter plates. ATPase was initiated by adding 10 mM ATP to 50 μL of reaction mixture containing additions as indicated in the text. The reaction was carried out at 37°C for 3 to 5 min and stopped with 20 μL of 30 mM mercury nitrate in 5 mM acetic acid. The plates were centrifuged and an aliquot of the supernatant was transferred to microtiter strips. To these, 200 μL of the LeBel et al. (1978) reagents for Pi assay were added, followed 5 min later by 50 μL of 30% (w/v) Na-citrate to prevent color development from any slowly breaking down ATP. The optical density was measured at 750 nm.

Methanol-activated ATPase of thylakoids was assayed as described earlier (Anthon and Jagendorf, 1983). The procedures for isolation of CF₁ and the assay conditions for soluble CF₁-ATPase were those described by Binder et al. (1978).

**Ferricyanide Reduction**

Rates of electron transport were measured as ferricyanide reduction. Thylakoids were washed twice with and resuspended in a buffer containing 10 mM Tricine-Na (pH 8.5) and 50 mM NaCl. Thylakoids with 10 μg of Chl were added to 500 μL of a reaction mixture containing additions as indicated in the text, illuminated for 3 min at 3 mmol m⁻² s⁻¹ at 25°C. The reaction was stopped with 500 μL of 2% TCA, and, after centrifugation, absorbance of the supernatant was measured at 420 nm.

**Proton Uptake**

Proton uptake was measured continuously in a 2-mL measuring cell with a pH electrode connected to a Heath/Shlumberger pH/pIOn electrometer (model Eu-200–02) and a recorder. The system was calibrated with standard buffer. The measuring cell contained 100 μg of Chl in a volume of 2 mL, and additions as indicated in the legends to the figures. The reaction mixture at 25°C was illuminated at approximately 3 mmol m⁻² s⁻¹ for 20 s, with recording of the medium pH during and after illumination.

**Materials**

Vtcd was added as a fresh dilution from a 10-mM stock solution in DMSO. The final DMSO concentration was always held below 0.5%. Trials indicated that no preincubation was required for full inhibition. All chemicals, except for common salts, were purchased from Sigma. Spinach leaves were bought from a local supermarket.

**RESULTS**

**Vtcd as Inhibitor of CF₉**

Under our conditions, photophosphorylation is completely sensitive to vtcd, with 0.5 μM serving to inhibit ATP synthesis 50% (data not shown). This result is similar to those recently reported by Bizouarn et al. (1990). The nature of the vtcd effect was checked further by measuring its action on electron transport (Fig. 1). Vtcd inhibited both phosphorylation-stimulated electron flow (with ADP and Pi added) and basal rates between 60 and 75%. To be sure that the inhibition of electron transport was due solely to the blockage of the proton channel of CF₉, uncoupled thylakoids containing NH₄Cl were used under the same conditions. Vtcd had no effect on electron transport in the presence of the uncoupler NH₄Cl (Fig. 1).

To confirm further the functional specificity of vtcd, we tested its effect on proton uptake. As shown in Figure 2, vtcd significantly increased both the rate and extent of proton uptake at pH 8.0, even at low concentrations. Whereas 0.5 μM vtcd stimulated the net extent of proton uptake 2-fold, 2 μM increased it 8-fold. The apparent (net) rates of proton uptake were stimulated to an even greater extent under these conditions.

The stimulation of proton uptake was even more dramatic when the same experiments were conducted with EDTA-washed thylakoids, which are partially CF₁-depleted and, consequently, leakier to protons (Neumann and Jagendorf, 1964). These CF₁-depleted thylakoids at pH 8.0 showed a lag of 4 s in net proton uptake in the light (Fig. 3). Addition of vtcd effectively eliminated this lag and restored the full extent...
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Figure 1. Venturicidin (vtcd) inhibits coupled, but not uncoupled, electron transport. The 0.5-mL illuminated reaction mixture contained 100 mM sorbitol, 50 mM Tricine-Na (pH 8.5), 25 mM NaCl, 2 mM ferricyanide, 2 mM MgCl₂, 0.2 mg/mL of BSA, 10 μg of Chl, and either 1 mM ADP and 5 mM Pi (coupled reaction) or 1 mM NH₄Cl plus the indicated concentration of vtcd. Other details are described in “Materials and Methods.”

Figure 2. Venturicidin stimulates the extent of thylakoid proton uptake in the light at pH 8.0. The illuminated reaction mixture (2 mL) contained 0.5 mM Tricine-Na (pH 8.0), 100 mM sorbitol, 50 mM NaCl, 50 μM pyocyanine, 100 μg of Chl, and the indicated concentrations of vtcd. The change in pH at successive time points, redrawn from original recorder graphs, are shown for vtcd at 0 μM (○), 0.5 μM (●), 1.0 μM (△), and 2.0 μM (▲).

Figure 3. Stimulation by vtcd of proton uptake by CF₁-depleted thylakoids. Conditions and symbols as in Figure 2 except for the use of EDTA-treated thylakoids.

Figure 4. Stimulation by vtcd of sulfite-stimulated ATPase. Conditions and symbols as in Figure 2 except for the use of EDTA-treated thylakoids.

The effect of vtcd on the decay of the proton gradient in the dark following a period of illumination was also investigated in the same experiments. Surprisingly, vtcd slowed down proton decay to only a very small extent (data not shown).

The stimulation of proton uptake in the light by vtcd was found only when the external (medium) pH was higher than 7 (data not shown). Below pH 7.0, the proton uptake was high and there was hardly any further stimulation by vtcd (data not shown). This is consistent with earlier indications of a “slip” of protons through CF₁ at the higher pH only (Evron and Avron, 1990).

All the data above suggest that vtcd indeed acts on the CF₁ as an H⁺ channel. To see if it might have any direct effect on CF₁, we added vtcd to the assay media when measuring the ATPase of isolated (soluble) CF₁ and the nonproton pumping ATPase of thylakoids in the presence of 33% methanol. Vtcd had absolutely no effect on either of these two ATPase activities (data not shown) at concentrations between 1 and 40 μM.

Effects on the Proton-Pumping ATPase

Once we were certain that vtcd is a specific inhibitor of CF₁, we used it to study sulfite-stimulated ATPase. We found it can inhibit this activity completely (Fig. 4). At a Chl concentration of 50 μg/mL, 50% inhibition occurred with 0.5 μM vtcd. This inhibition was the same with or without the addition of 5 mM NH₄Cl + 1 μM gramicidin to provide complete uncoupling. Note the contrast with electron transport (Fig. 1), in which uncouplers removed vtcd inhibition completely.

Kinetic studies of sulfite ATPase showed the major effect
Figure 4. Vtcd inhibition of thylakoid sulfite-stimulated ATPase, with or without uncouplers. The 50-μL reaction mixture contained 50 mM Tricine-Na (pH 8.0), 11 mM MgCl₂, 10 mM ATP, 2.5 μg of Chl, 20 mM DTT, 50 mM sulfite (pH 8.0), and the indicated concentrations of vtcd, with or without the uncouplers (5 mM NH₄Cl + 1 μM gramicidin).

Figure 5. Effect of vtcd on the kinetic constants of ATPase. The reaction mixture was similar to that used in Figure 4 except for the presence of 1 mg/mL of creatine phosphate and 20 units/mL of creatine kinase. Thylakoids were present containing 1 μg of Chl; either with (●) or without (○) 0.4 μM vtcd; and the ATP concentration was varied as shown, with the Mg/ATP ratio at each point held constant at 1.1/1.0. The reaction was started by addition of thylakoids and ran for 2 min at 37°C.

Figure 6. Higher vtcd concentrations are needed to inhibit ATPase at higher thylakoid concentration levels. Conditions are as in Figure 4, with varied Chl concentrations as indicated. The ATPase rates were 290, 286, 248, and 190 pmol mg⁻¹ Chl h⁻¹ for Chl concentrations of 20 (○), 40 (●), 80 (△), and 160 mg/mL (▲), respectively.
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strength because replacing choline with 0.2 M KCl did not produce thylakoids totally inhibitable by vtcd (data not shown).

An increase in thylakoid sulfite-stimulated ATPase resistant to vtcd was also found if thylakoids were aged prior to use (Table I). With 2-d-old thylakoids, for example, the ATPase activity was about 50% of control (fresh thylakoids) and showed 93% inhibition by 2 μM vtcd when choline chloride was used in the isolation and resuspension buffer. For thylakoids prepared without choline, activity at 2 d had dropped to 20%, and vtcd inhibition was only about 50% (data not shown).

Another way to increase the extent of vtcd-resistant ATPase was to treat the thylakoids with trypsin. This raises ATPase rates considerably, especially if the thylakoids were also reduced (Schumann et al., 1985). However, the sulfite-stimulated ATPase is then inhibited only 70 to 80% by vtcd, compared with 100% in the nontrypsin-treated controls (Fig. 8). This was the case for trypsin-treated thylakoids with or without prior reduction by DTT in the light.

In later experiments (fall and winter of 1991–1992), thylakoids tended to be inhibited 100% by vtcd even if prepared without choline in the grinding buffer. However, these thylakoids still lost some of the vtcd inhibition when aged or when treated with trypsin.

Earlier studies had found that photophosphorylation by thylakoids is inhibited by exposure to light in the presence of ADP, Mg²⁺, and SO₄²⁻. This was traced to the displacement of some of the CF₁ from its binding site, leading to uncoupling of the thylakoids (Grebanier and Jagendorf, 1977). However, no overt loss of CF₁ from the membranes could be detected under those conditions. If the displaced but still bound CF₁ is still active in hydrolyzing ATP, that ATPase should not be inhibitable by vtcd. To test this possibility, thylakoids were first exposed to sulfate, Mg²⁺, and ADP in the light, then washed and reduced by DTT in the dark. Sulfite-stimulated ATPase was then measured with and without vtcd. The SO₄²⁻ + ADP-uncoupled thylakoids were inhibited 93% by 2 μM vtcd, which was not significantly different from the 96% inhibition of control thylakoids. This indicates that the displaced CF₁ is almost certainly catalytically inactive.

### DISCUSSION

The electron transport of illuminated thylakoids is coupled to inward pumping of protons (Neumann and Jagendorf, 1964). If the internal protons do not escape, the continuing electron flow is inhibited. Use of the internal protons in photophosphorylation accordingly leads to faster electron

### Table I. Effects of choline and aging on vtcd inhibition of thylakoid sulfite-stimulated ATPase

<table>
<thead>
<tr>
<th>Choline in Grinding Buffer</th>
<th>Thylakoid Storage Time</th>
<th>ATPase Rate</th>
<th>Inhibition</th>
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<tr>
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<td>0</td>
<td>vtcd -</td>
<td>vtcd + vtcd</td>
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<tr>
<td>−</td>
<td>2</td>
<td>130</td>
<td>70</td>
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<td>195</td>
<td>0</td>
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<td></td>
<td>2</td>
<td>140</td>
<td>10</td>
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Figure 7. Apparent stoichiometry of vtcd to CF₁ at 50% inhibition of thylakoid ATPase. Data are taken from Figure 6.

Figure 8. Incomplete vtcd inhibition of the thylakoid trypsin-activated ATPase. The thylakoids were reduced and treated with trypsin (see "Materials and Methods") prior to the ATPase assay. Conditions for measuring ATPase are as in Figure 4. Rates of ATP hydrolysis without vtcd were 374, 911, 1106, and 936 μmol mg⁻¹ Chl h⁻¹, respectively, after incubation with trypsin for 0, 5, 15, and 30 min.
flow rates. However, it has also been known for a long time that the basal, nonphosphorylating electron flow is accelerated at external pH levels above 8.0. This is now considered to be due to a "slip" of protons through the membrane (Evron and Avron, 1990). Because electron flow at the high pH is sensitive to CF₁-directed inhibitors such as DCCD but not to a CF₁-directed inhibitor such as tentoxin (Strotmann et al., 1986; Wagner et al., 1989; Evron and Avron, 1990), it seems likely that the CF₁-proton channel is opened under these conditions and permits leakage of the internal protons. Finding that vtcd inhibits basal electron flow at pH 8.5 but not at pH 6.7 is thus consistent with its role as inhibitor of CF₁. Uncouplers open up alternative ways of disposing of the internal H⁺ stores, and inhibiting CF₁ would not affect their ability to speed up electron flow. Lack of a vtcd effect on uncoupler-stimulated electron flow (Fig. 1) is again consistent with its specific role as inhibitor of CF₁, and especially so since the relationship of thylakoid sulfite-stimulated ATPase (Larson and Jagendorf, 1989) to the proton gradient has not been entirely clear. Stimulation by uncouplers indicated probable inhibition by protons pumped into the lumen during ATP hydrolysis. However, the system was not completely sensitive to DCCD. Our data show that sulfite-stimulated ATPase is completely sensitive to vtcd (Fig. 4) even under uncoupled conditions. Thus, a fruitful interaction with CF₁, probably leading to proton removal to the lumen, must be a required component of the ATPase of CF₁ under these conditions.

For this to work, subunit interactions probably must be tightly controlled. The rigor of these controls, and the firmness of interactions between CF₁ and CF₁, vary with thylakoid conditions. Although the specific events caused by aging are not known, disruption of these tight interactions is likely as the system deteriorates, and this brought on some vtcd-resistant ATPase (Table I). Partial proteolysis of CF₁ subunits by trypsin is known to destroy the tight controls that maintain ATPase in a latent state (Schumann et al., 1985), so it is not a surprise that the ATPase escapes from vtcd inhibition at the same time (Fig. 8).

The role of choline during thylakoid preparation is intriguing. It is known to permit isolation of thylakoids with the highest ATP synthesis rates. It is quite likely that choline at 200 m or above helps to prevent the attack of phospholipase D, which was found to be present in some leaf homogenates and is able to hydrolyze both phosphatidylincholine and phosphatidylethanolamine of various membranes in the homogenate (Scherer and Morrè, 1978). This enzyme also possesses transference activity that catalyzes exchange of the alcohol moiety in phospholipids with free alcohol molecules in solution. Scherer and Morrè (1978) reported that addition of 4% choline (about 285 mm) in plant membrane suspensions could inhibit the phospholipase activity. These considerations make it seem possible that specific boundary lipids may also be required to maintain the CF₁/CF₁ complex in a tightly controlled, completely inhabitable configuration. An earlier indication of the same sort is the report of Pick et al. (1984) that the Mg-ATPase of CF₁/CF₁, reconstituted in liposomes had low activity and was not sensitive to DCCD unless phospholipids were in the membranes.

The absence of the choline effect in our more recent experiments might be due to the relatively low activity of a phospholipase in the homogenate of spinach from particular sources, grown under different conditions, and possibly of
different genetic strains. These concepts for the nature of the choline effect need to be tested further.

In any case, the extent of vtcd inhibition may be useful as an indicator of the state of $CF_{1}$-$CF_{1}$ interaction.

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