

***N*-Cyclo-*N'*-(4-Dimethylamino- α -Naphthyl)Carbodiimide Inhibits Membrane-Bound and Partially Purified Tonoplast ATPase from Maize Roots**

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ABSTRACT

Certain carboxylic acid groups within the primary structure of proton translocating proteins are thought to be involved in the proton pathway. In this report, the effects of a lipophilic carboxylic acid reactive reagent, *N*-cyclo-*N'*-(4-dimethylamino- α -naphthyl)carbodiimide (NCD-4), on the two types of proton pumps in maize (*Zea mays* L.) root microsomes were investigated. NCD-4 was found to inhibit the vacuolar-type H⁺-ATPase in microsomal preparations; however, the plasma membrane-type H⁺-ATPase was unaffected. The H⁺-ATPase in highly purified tonoplast vesicles was also inhibited by NCD-4. Inhibition was dependent on the concentration and length of exposure to the reagent. However, there was little, if any, increase in the fluorescence of treated vesicles, indicating few carboxylic acid residues were reacting. Inhibition of the tonoplast H⁺-ATPase by NCD-4 was examined further with a partially purified preparation. The partially purified H⁺-ATPase also showed sensitivity to the NCD-4, supporting the hypothesis that this carboxylic acid reagent is an inhibitor of the tonoplast ATPase from maize roots.

Eukaryotic cells, including plants, are thought to have two predominant classes of transport ATPases which convert chemical energy from the hydrolysis of ATP to ADP into an electrochemical gradient, which is then utilized in the transport of inorganic and organic solutes (14, 21). The two classes have been referred to as the vacuolar- and plasma membrane-type ATPase based on their relative enrichment in vacuolar-type membranes (*i.e.* tonoplast and lysosomes) and plasma membranes (14). The plasma membrane-type ATPase is also called E1-E2 type ATPase because these transporters form two distinct conformations designated as E1 and E2 (14). However, both types of ATPase can be found on the same membrane. For example, clathrin-coated vesicles can contain both types of ATPases although the activity of the vacuolar-type predominates (26). The two classes of ATPase can be distinguished biochemically by their differential sensitivity to nitrate and vanadate, the formation of a covalent intermediate and polypeptide composition (14). The two types of transport ATPases are also distinct from the mitochondrial and chloroplast ATPase involved in ATP synthesis (14, 21).

Although the exact mechanism of proton transport by the two types of ATPases is unknown, differences in biochemical properties strongly suggests that the two enzyme have different mechanisms. Despite this, the general nature of the proton

pathway may be similar, involving transfer of protons from one carboxylic acid residue to another within the membrane spanning part of the ATPase (10). Consistent with such a hypothesis, the activities of both types of proton pumps are sensitive to the hydrophobic carbodiimide DCCD¹ (2, 7, 9, 12, 15, 19, 20, 25). Plasma membrane-type transport ATPase which do not usually catalyze the movement of protons like the Na, K-ATPase, and the Ca-ATPase of the sarcoplasmic reticulum are also sensitive to DCCD (9, 19). Therefore, inhibition by DCCD is not unique to proton translocating enzymes.

There are only slight differences in the nature of DCCD inhibition between the two types of transport ATPases. The tonoplast enzyme appears to be a bit more sensitive to DCCD with half-maximal inhibition occurring at 10 to 20 μ M (2, 7, 15, 25) *versus* about 50 μ M for the plasma membrane ATPase (2, 7). Mitochondrial ATPase from oat roots had an even greater sensitivity to DCCD with half-maximal inhibition occurring at submicromolar levels (25). The ATP binding site and the DCCD reactive component of the vacuolar-type ATPase are on separate polypeptides (12). Although both the DCCD reactive component and the ATP binding site are on the same polypeptide in the plasma membrane ATPase (9, 20), DCCD is not thought to react near the ATP hydrolyzing moiety (9, 20).

Recently, carbodiimides which form fluorescent products have been found to inhibit the sarcoplasmic reticulum Ca-ATPase (5, 6). The benefits of these derivatives include following the reaction by nondestructive means (*i.e.* fluorescence) and incorporation of a probe to study relationships between different catalytic properties of the protein by fluorescence energy transfer. We report here that one of these carbodiimides, NCD-4, is an effective inhibitor of the vacuolar-type ATPase in maize roots while having little effect on the plasma membrane-type ATPase.

MATERIALS AND METHODS

Preparation of Membrane Vesicles and Partial Purification of Tonoplast ATPase

Corn (*Zea mays*) seeds (cv WF9 \times Mol7² and FRB73) were germinated on filter paper moistened with 0.1 mM CaCl₂ for

¹ Abbreviations: DCCD, dicyclohexylcarbodiimide; BTP, Bis-Tris-propane; NCD-4, *N*-cyclo-*N'*-(4-dimethylamino- α -naphthyl) carbodiimide, OG, octylglucoside.

3 d at 28 to 30°C and harvested as described previously (13). Crude microsomes with high rates of proton transport by both the tonoplast and plasma membrane ATPases were isolated from roots of WF9 × Mol7 by a modification of the protocol of de Michelis and Spanswick (8), as described previously (3).

Highly purified tonoplast vesicles were isolated from roots of FRB 73 essentially as described previously (24). Based on inhibition to nitrate and vanadate, more than 95% of the proton transport and ATP hydrolysis by the tonoplast vesicles could be attributed to the vacuolar H⁺-ATPase (24). Fractions from the sucrose density gradient were diluted with 5 mM Hepes (pH 7.8) to 10% (w/w) sucrose and then collected by centrifugation at 50,000 rpm in a Ti 70 rotor for 75 min at 4°C. The pellet was resuspended at 1 mg/mL of protein in 5 mM Hepes (pH 7.5) containing 0.25 M sucrose, 10% (w/v) glycerol, and 2 mM DTT. The suspension was adjusted to 20 mM OG by the addition of resuspension buffer containing 400 mM OG. After 10 min at 0 to 4°C, the detergent treated vesicles were centrifuged at 20,000g for 15 min. The resulting supernatant was diluted with an equal volume of 5 mM Hepes (pH 7.8) containing 20 mM OG and 10% (w/v) glycerol, and layered over a 34 mL linear gradient of 15 to 35% (w/v) glycerol in 5 mM Hepes (pH 7.8) containing 20 mM OG and 2 mM DTT. The glycerol gradient was centrifuged in a SW 28 rotor at 26,000 rpm at 4°C. After centrifuging for 20 h, the gradient was fractionated into 1.5 mL aliquots.

Assays for Proton Pumping and ATP Hydrolysis

ATP catalyzed proton transport was assayed by changes in acridine orange absorbance at 492.5 nm as described previously (3). Vesicles were diluted to 2.2 mL with a assay medium containing 17.5 mM Mes titrated to pH 6.45 with BTP, 1 mM EGTA, 2.5 mM MgSO₄, 7.5 μM acridine orange, and 50 mM KCl or KNO₃. The KCl assay medium also contained 0.2 mM vanadate when crude microsomes were used so that the activity could be attributed to the vacuolar proton pump (4, 8, 16). Activity in the presence of KNO₃ was attributed to the vanadate-sensitive, plasma membrane proton pump (4, 8, 16). After incubation at 18 to 22°C for 5 min, proton transport was initiated by the addition of 20 μL of 0.2 M ATP titrated to pH 6.45 with BTP. The initial rate of proton transport was determined analyzing the time course of the quenching of acridine orange absorbance as described previously (4, 23). ATP hydrolysis catalyzed by the tonoplast ATPase was assayed in the KCl medium described above by the direct measurement of inorganic phosphate released (23).

NCD-4 Modification

NCD-4 was dissolved in tetrahydrofuran as either a 10 or 20 mM stock solution and stored up to 2 weeks at -20°C in the dark. The NCD-4 stock was added to either membranes or the partially-purified ATPase, and incubated in the dark on ice. The volume of tetrahydrofuran added to the enzyme preparation never exceeded 2%. Controls contained a corre-

sponding volume of solvent without the reagent. The addition of the tetrahydrofuran had no effect on the ATPase of either the plasma membrane or vacuolar-type ATPase (data not shown). After incubation, the enzyme preparation was diluted 20- to 30-fold with room temperature assay buffer to determine proton transport and ATP hydrolysis as described above.

Protein Determination

The protein concentration of membrane fractions routinely were determined after precipitation by TCA in the presence of deoxycholate by the Lowry method (1). Samples containing octylglucoside could not be analyzed by this method, because of high background absorbance.

The protein concentration of samples containing octylglucoside were determined by a modification of a recently published protocol described by Rodriguez-Vico *et al.* (18). For samples containing OG, up to 0.5 mL of aqueous solutions were diluted with 10 mL of 3:2 (v:v) hexane:isopropanol. The sample was then centrifuged at 3,000g for 10 min. The solvent was decanted off and the remaining solvent in the centrifuge tube was removed under a stream of N₂ gas. The pellet was resuspended in 200 μL of 0.1% (w/v) SDS, 1% (w/v) deoxycholate and 0.5 N NaOH, and diluted with 1.5 mL of 100:1:1 (v:v:v) Lowry reagents A:B:C (1, 18). Next, 0.15 mL of 1:1 (v:v) Folin phenol reagent:water was added. The color was allowed to develop for 60 min at room temperature and absorbance at 660 nm was determined. Standards were prepared with an equivalent amount of OG.

Protein concentrations of soluble proteins and membrane fractions as determined by this method were comparable to those determined by the deoxycholate-TCA precipitation method above (data not shown). The addition of up to 20 mM to these samples had no effect on the determined protein concentration (data not shown). The detection limits and response of the hexane-isopropanol precipitation method were comparable to those of the deoxycholate-TCA precipitation method (data not shown).

RESULTS AND DISCUSSION

Partial Purification of the Tonoplast H⁺-ATPase

Our attempts to purify the tonoplast ATPase from maize roots by the protocol developed by Mandala and Taiz (11) were unsuccessful. Our results suggested that the maize root tonoplast ATPase was unstable in the presence of OG in the absence of glycerol (data not shown). Therefore, a protocol utilizing glycerol containing buffers and glycerol density centrifugation was devised. Treating tonoplast vesicles with 20 mM OG solubilized virtually all of the nitrate-sensitive ATPase activity and 90% of the protein (Table I). A minimum of 20 mM OG was required for optimal solubilization (data not shown). An OG concentration in excess of 30 mM was undesirable because the ATPase activity declined rather rapidly with incubation at 0 to 4°C (data not shown).

A significant increase in the specific activity was achieved by glycerol gradient centrifugation. After centrifuging, there was a large UV absorbing peak at the top of the gradient, containing most of the protein (data not shown). Just beyond this protein peak, the nitrate-sensitive ATPase activity sedi-

Table I. Summary of the Partial Purification of Tonoplast ATPase from 100 g of Corn Roots, Cultivar FRB73

Fraction	Protein	Total Activity	Specific Activity
	mg	nmol Pi/min	nmol/min/mg protein
Tonoplast vesicles	2.9	1100	380
Octylglucoside-soluble	2.6	1090 (0.99) ^a	420 (1.1) ^a
Pooled gradient fractions	0.25	660 (0.60)	2,600 (6.8)

^a Relative to that of tonoplast vesicles.

mented (data not shown). The greatest levels of ATPase activity were found in fractions with 25 to 27% (w/v) glycerol. Because the ATPase activity was removed from the bulk of the protein, a purification of almost sevenfold was achieved (Table I). The specific activity varied between 2 and 3 $\mu\text{mol}/\text{min}/\text{mg}$ protein in the pooled fractions, which is comparable to highly purified vacuolar-type ATPase from both plant and animal sources (16, 17, 24, 26). The ATPase activity in the partially purified preparation was rather stable at 0 to 4°C retaining over 65% of its activity after 20 h at 0 to 4°C (data not shown).

Effects of NCD-4

The effects of NCD-4 on the proton pumping by the vanadate-sensitive and nitrate-sensitive ATPases were compared using microsomes from roots of WF9 \times Mo17. Roots of this cultivar were chosen because microsomes isolated in the presence of glycerol, ATP and other protectants have high rates of proton pumping attributed to the two different types of ATPases (3, 4, 8). Microsomes incubated with NCD-4 had reduced rates of nitrate-sensitive proton pumping (Fig. 1). In the presence of 0.4 mM NCD-4, nitrate-sensitive proton pumping decreased by more than 50% during the 240 min incubation on ice. The effect of NCD-4 increased with increasing incubation. In addition, inhibition was dependent on the concentration of NCD-4 (data not shown). However, nitrate-insensitive, vanadate-sensitive proton transport was essentially unaffected by 0.4 mM NCD-4 (Fig. 1). NCD-4 had no significant effect on the vanadate-sensitive proton pump even when the concentration was increased to 2 mM (data not shown). These results indicate that the nitrate-sensitive proton pump is much more susceptible to inhibition by NCD-4.

Proton pumping by highly purified tonoplast vesicles from roots of cultivar FRB73 was dramatically inhibited by NCD-4 (Fig. 1). The degree of inhibition by NCD-4 was both time- and concentration-dependent. When the logarithm of the pseudo first-rate constants for inhibition was plotted as a function of the logarithm of NCD-4 concentration, the data fell on a straight line with a slope of 0.6 (data not shown).

Nitrate-sensitive proton pumping of tonoplast vesicles from FRB73 was much more sensitive to NCD-4 than the same activity in microsomes from WF9 \times Mo17, (Fig. 1). Although two different corn cultivars were used in these experiments, the difference between microsomes and isolated tonoplast preparation may not reflect a genetic difference in reactivity of the ATPase toward NCD-4. Another alternative explanation for the difference in sensitivity of the tonoplast H^+ -ATPase in microsomes and isolated tonoplast vesicles is the

effective concentration of NCD-4 was different. NCD-4 is very lipophilic (5, 6), and therefore partitions into the membrane phase. Therefore, it is difficult to assess the effective concentration of NCD-4 able to react with the tonoplast ATPase in the two membrane preparations. The activities of the tonoplast ATPases were not affected by the addition of the product formed between acetic acid and NCD-4 to a concentration of 1 mM (data not shown). This results suggest that inhibition was not due to the addition of the NCD-4 moiety into the membrane phase, but rather due to the reaction of carboxylic acid groups in the vesicles with the NCD-4.

It was not possible to determine the extent of reaction between NCD-4 and the tonoplast vesicles by changes in fluorescence. NCD-4 yields fluorescent products when it reacts with carboxylic acids (5, 6). However, there was only a barely detectable increase in the fluorescence of tonoplast vesicles treated with NCD-4, even under conditions that proton transport was totally inhibited (data not shown). These results suggested that NCD-4 reacted with only a few carboxylic acid groups.

To substantiate that the NCD-4 was reacting with the tonoplast ATPase, the effects of the reagent on partially purified ATPase were sought. There was a time dependent decrease in ATP hydrolysis when the glycerol gradient purified ATPase was treated with NCD-4 at 0 to 4°C (Fig. 1). This loss in activity was also dependent on the concentration of NCD-

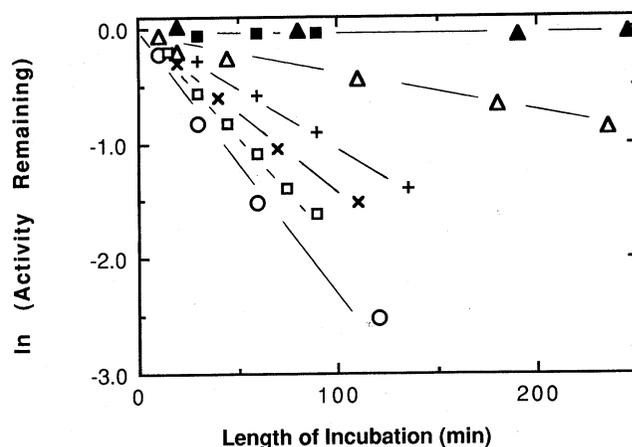


Figure 1. Effects of NCD-4 on the activities of the plasma membrane and tonoplast ATPases. Microsomes were incubated on ice with 0.4 mM NCD-4 for 0 to 240 min and then assayed for proton transport by either the plasma membrane (\blacktriangle) or tonoplast ATPase (\triangle). Initial rates for proton transport from two experiments with two replicates were plotted relative to untreated membranes, which averaged 0.52 and 0.08 $\text{A}/\text{min}/\text{mg}$ protein for the tonoplast and plasma membrane ATPase activities, respectively. Tonoplast vesicles purified from roots of FRB73 were incubated with 0.4 (+), 0.65 (\times), or 1.0 mM (\circ) NCD-4 for up to 180 min on ice and then assayed for proton transport. Data are plotted relative to untreated controls, which averaged 1.8 $\text{A}/\text{min}/\text{mg}$ protein. Glycerol gradient purified ATPase was incubated for 0 to 90 min at 0°C in the presence of 0.4 mM NCD-4 (\square) or an equivalent volume of the solvent tetrahydrofuran (\blacksquare) and then assayed for ATP hydrolysis. The data are plotted relative to untreated controls, which averaged 2750 $\text{nmol Pi}/\text{min}/\text{mg}$ protein.

4 (data not shown). Results from crude membranes, purified tonoplast and partially purified tonoplast ATPase indicated that NCD-4 was an inhibitor of the vacuolar ATPase. Further experimentation is needed to determine the exact nature of inhibition. NCD-4 was an effective inhibitor of both ATP hydrolysis and proton transport catalyzed by the vacuolar ATPase (Fig. 1). Both activities of the ATPase in highly purified tonoplast vesicles from FRB 73 were affected to a similar extent (data not shown). For example, ATP hydrolysis was 55 and 25% of control values when treated with 0.4 and 1 mM NCD-4, respectively, for 60 min at 0 to 4°C. Proton transport after these treatments was 58 and 22% of control, respectively (Fig. 1). Previous data suggested that ATP hydrolysis and proton transport by the maize root vacuolar ATPase are catalyzed by an indirect coupling mechanism (23). Accordingly, the reactions of the ATPase are subdivided into two sets of reactions which share no common steps, one for ATP hydrolysis and the other for proton transport. The two processes are linked by a coupling mechanism. Because NCD-4 affects proton transport and ATP hydrolysis to a similar extent, this reagent is probably reacting either at the ATP binding site or at a site in the coupling mechanism close to the ATP hydrolyzing pathway. It seems unlikely that NCD-4 would react with a component in the ATP hydrolysis pathway, since the ATP binding site is localized on a water soluble part of the ATPase complex spatially removed from the membrane (22), in which the NCD-4 would partition. In addition, the presence of 2 mM Mg-ATP had no effect on the inhibition caused by incubating vesicles with 0.4 mM NCD-4 for 120 min at 0 to 4°C (data not shown). Therefore, the most likely site of reaction for the NCD-4 is some unidentified component in the mechanism coupling between ATP hydrolysis and proton transport. Identification of the coupling mechanism as the site of NCD-4 inhibition requires continued research. The usefulness of NCD-4 as a fluorescent covalent label for some part of the ATPase reaction mechanism may be limited due to the low fluorescent yield associated with inhibition.

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