

Effects of dicyclohexylcarbodiimide (DCCD) treatment on coupled activities of vanadate-sensitive ATPase from plasma membrane of maize roots

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The presence of dicyclohexylcarbodiimide (DCCD) inhibited the activities of vanadate-sensitive H^+ -ATPase in both native and reconstituted plasma membrane of maize (*Zea mays* L. cv. WF9 × Mo17) roots. Concentration dependence of DCCD inhibition on adenosine triphosphate (ATP) hydrolysis of native plasma membrane vesicles suggested that the molar ratio of effective DCCD binding to ATPase was close to 1. The DCCD inhibition of ATP hydrolysis could be slightly reduced by the addition of ATP, Mg:ATP, adenosine monophosphate (AMP), Mg:AMP and adenosine diphosphate (ADP). More hydrophilic derivatives of DCCD such as 1-ethyl-N'-3-trimethyl ammonium carbodiimide (EDAC) or 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) gave no inhibition, indicating that the effective DCCD binding site was located in a hydrophobic region of the protein. The proton transport activity of reconstituted plasma membrane at a temperature below 20°C or above 25°C was much more sensitive to DCCD treatment. Build-up of the proton gradient was analyzed according to a kinetic model, which showed that proton leakage across de-energized reconstituted plasma membranes was not affected by DCCD, but was sensitive to the method employed to quench ATP hydrolysis. Reconstituted plasma membrane vesicles treated with DCCD exhibited a differential inhibition of the coupled H^+ -transport and ATP hydrolysis. The presence of 50 μM DCCD nearly abolished H^+ transport but inhibited less than 50% of ATP hydrolysis. The above results suggest that the link between proton transport and vanadate-sensitive ATP hydrolysis is indirect in nature.

Introduction

The carboxyl group-modifying agent, N,N'-dicyclohexylcarbodiimide (DCCD), has been widely used in the study of membrane proton translocation (Kurtenbach and Verjovski-Almeida 1985). In the case of the F_1 - F_0 ATPase of mitochondria and bacteria, DCCD specifically modifies either an aspartyl or a glutamyl group (Fillingame 1980, Hoppe and Sebald 1981) located in the hydrophobic stretch that forms part of the F_0 portion

of the ATPase complex. As a result, proton movement through the F_0 portion is blocked, and this inhibits adenosine triphosphate (ATP) hydrolysis. In plasma membranes of fungi and plants, the E_1E_2 -type of ATPase (Perlin and Spanswick 1981, Sussman and Slayman 1983) forms a covalent phosphorylated intermediate during the course of ATP hydrolysis on an essential aspartyl residue in the active site of the enzyme. This type of ATPase is also sensitive to DCCD. Using [^{14}C]-

DCCD, Cid et al. (1987) determined that the molar binding ratio of DCCD to enzyme was 1.

In the maize root system, a kinetic model for the description of proton transport by tonoplast ATPase was proposed by Tu et al. (1987). The model quantifies the overall process of proton pumping by simultaneously considering the pumping and the leakage of protons from membrane vesicles. By this model, the differential inhibitions of the tonoplast ATPase by various treatments have been interpreted in terms of an indirect coupling mechanism. Thus, the proton pumping exhibited a greater sensitivity to various treatments than did ATP hydrolysis. Recently, Brauer et al. (1989) have extended the use of this model to characterize the activity of the vanadate-sensitive proton pump in maize microsomal membranes. Furthermore, Brauer et al. (1991) also suggest that the coupling between ATP hydrolysis and proton transport is indirect, reasoning from observations of differential temperature dependence of proton transport and ATP hydrolysis.

In order to examine further the mechanism by which proton transport is coupled to ATP hydrolysis, we have studied the effects of DCCD treatment on the functions of vanadate-sensitive H⁺-ATPase in native and reconstituted plasma membrane vesicles of maize roots. In the present report, we describe the kinetics of DCCD inactivation under various conditions and utilize the established kinetic model to investigate the process of proton transport. The results suggest that the ATP hydrolysis step is linked to proton pumping through a molecular arrangement which is sensitive to DCCD modification. A possible indirect coupling between ATP hydrolysis and proton pumping is proposed.

Abbreviations – AO, acridine orange; BTP, Bis-Tris-Propane; DCCD, N,N'-dicyclohexylcarbodiimide; DOC, deoxycholic acid; EDAC, 1-ethyl-N'-3-trimethyl ammonium carbodiimide; EDC, 1-ethyl-3-3-dimethyl-aminopropyl carbodiimide; k_1 , rate constant for processes that hinder the build-up of a proton gradient; k_2 , rate constant for return of transported protons after the plasma membrane ATPase-induced ATP hydrolysis has been completely inhibited; m , the coupling factor relating the rate of ATP hydrolysis to the rate of proton transport; δ , the number of protons transported; R , rate of ATP hydrolysis; subscript o and s following δ and R denote initial and steady-state values, respectively.

Materials and methods

Preparation of membrane vesicles

Microsomes washed with 0.25 M KI were prepared from roots of 3-day-old maize (*Zea mays* L. cv. WF9 × Mo17) seedlings as described by Brauer et al. (1988). The plasma membrane-enriched fraction was isolated by pelleting microsomes through a layer of 34% (w/w) sucrose concentration (Hsu et al. 1991). To make reconstituted plasma membrane vesicles, 690 μ l of membranes (1.5 mg) and 10 mg asolectin in 250 μ l of reconstitution buffer, 10 mM tris(hydroxymethyl) ami-

nomethane-2-(N-morpholino)ethanesulfonic acid (Tris-MES), pH 6.45, 50 mM potassium acetate and 5 mM sodium nitrate, were combined and dispersed by the addition of 10% (w/v) deoxycholic acid (DOC) to a final concentration of 0.6%. The reconstituted liposomes were recovered in the cloudy void volume of a G-150 column. In general, the specific activity of plasma membrane was 45 μ mol (mg protein)⁻¹ h⁻¹.

Proton transport assay

Proton pumping activity was measured by changes in the absorbance of acridine orange (AO) at 492 nm as described by de Michelis and Spanswick (1986). Typically, 200 μ l of vesicles were diluted with 2 ml of 17.5 mM MES-Bis-Tris-Propane (MES-BTP), pH 6.45, 2.5 mM MgSO₄, 1 mM ethylene glycol-bis (β -aminoethyl ether N,N'-tetraacetic acid; EGTA), 7.5 mM AO and 50 mM KNO₃. After equilibration at room temperature for 10 min, the reaction was initiated by the addition of 20 μ l of 0.2 M ATP (pH adjusted to 6.45 with BTP).

ATP hydrolysis and protein determination

ATP hydrolysis was measured in an aliquot of 100 μ l of reaction medium after the proton transport reached a steady state. ATPase activity was measured by the vanadate-sensitive activity in the presence of azide and molybdate and the amount of inorganic phosphate released was determined by the formation of a Malachite green-molybdate complex as described previously (Tu et al. 1987). Protein concentration was measured by a modified Lowry method using bovine serum albumin as the standard (Bensadoun and Weinstein 1976).

Modification with DCCD and other diimides

Native and reconstituted vesicles were incubated with different concentrations of DCCD or other diimides for various time periods at 25°C. Fresh stock solutions of DCCD, 1-ethyl-N'-3-trimethyl ammonium carbodiimide (EDAC) and 1-ethyl-3-3-dimethyl-aminopropyl carbodiimide (EDC) were prepared daily by dissolving the diimides in methanol. Aliquots of the stock solution were added in the incubation mixture and preincubated with enzyme for 5–10 min. Proton pumping and ATP hydrolysis were then assayed as described above. All the experiments were carried out in at least duplicate trials, and the experimental variation was less than 5%.

Kinetic analysis of proton transport

As previously reported by Tu et al. (1987) and more recently by Brauer et al. (1989), a kinetic model can be used to describe the time course of the formation of a proton gradient driven by a H⁺-translocating ATPase: from this model, k_1 was determined from

$$d\delta/dt = mR_o - k_1\delta \quad (1)$$

$$\ln(1 - \delta/\delta_s) = k_1 t, \quad (2)$$

in which δ is the net proton transported across the membrane at time t after the pump is activated by the addition ATP. R is the rate ATP hydrolysis. k_1 is the rate constant for membrane leakage during pumping and the back pressure effect. k_1 can be conveniently calculated from a plot of $\ln(1 - \delta/\delta_s)$ against t after ATP addition. Additionally,

$$mR_0 = k_1 \delta_s, \quad (3)$$

in which R_0 is the initial rate of ATP hydrolysis, δ_s is the maximum proton transport at steady state and m is the coupling factor (or stoichiometric ratio) relating the ATP hydrolysis to proton transport.

An established proton gradient can be collapsed by the addition of protonophores or inhibitors of ATP hydrolysis. In the case of inhibition of ATP hydrolysis, the decay of proton gradient may be assumed to obey first-order kinetics,

$$d\delta/dt = k_2 \delta \text{ or } \ln(d\delta/\delta_s) = -k_2 t, \quad (4)$$

in which k_2 is the first-order rate constant for membrane leakage when ATPase is inhibited.

Results

Concentration dependence of DCCD inhibition

Treatment of the plasma membrane with DCCD led to a decrease in plasma membrane catalyzed ATP hydrolysis. The effect of DCCD modification was a function of both incubation time and concentration (Fig. 1). After a 15-min preincubation with 100 μM DCCD, ATP hydrolysis decreased by almost 50%. After 30 min, ATP hydrolysis decreased to 20% of the control activity. Fifty percent inhibition of ATP hydrolysis occurred after 12, 15 and 24 min with 100, 60 and 20 μM DCCD, respectively. The inhibition was linear with time up to 90% of inactivation. Prolonged incubation in excess of 30 min also decreased control enzyme activity (without the addition of DCCD). The rate constant of this inhibition, based on the pseudo-first-order kinetics, was estimated to be $3.71 \times 10^{-3} \text{ min}^{-1}$ with 10 μM DCCD, $9.84 \times 10^{-3} \text{ min}^{-1}$ with 20 μM DCCD, $2.37 \times 10^{-2} \text{ min}^{-1}$ with 50 μM DCCD and $2.22 \times 10^{-2} \text{ min}^{-1}$ with 100 μM DCCD. When the logarithm of the rate constant was plotted vs the DCCD concentrations, a straight line with a slope of 0.90 was obtained (Fig. 1B). For reconstituted plasma membrane vesicles, this slope was 0.96 (data not shown). These results suggest that the effective binding which led to the observed inhibition resulted from 1 molecule of DCCD binding to a single site on the ATPase (Cid et al. 1987).

In order to gain information on the physical envi-

ronment of the DCCD-sensitive site on the ATPase, two hydrophilic diimides, EDC and EPAC, were used to treat membrane vesicles. No inhibition was observed even at a concentration of 200 μM (data not shown). Thus, the COOH group assumed to be sensitive to DCCD is likely to reside within a hydrophobic region.

Differential inhibition of DCCD on ATP hydrolysis and proton transport of reconstituted plasma membrane

When reconstituted plasma membrane vesicles were treated with various concentrations of DCCD, proton transport activity showed a greater inhibition than did ATP hydrolysis (Fig. 2). Incubation with 30 μM DCCD for 5 min diminished the transport activity by 70%, while ATP hydrolysis decreased by less than 30%. The ATPase in reconstituted plasma membrane vesicles was

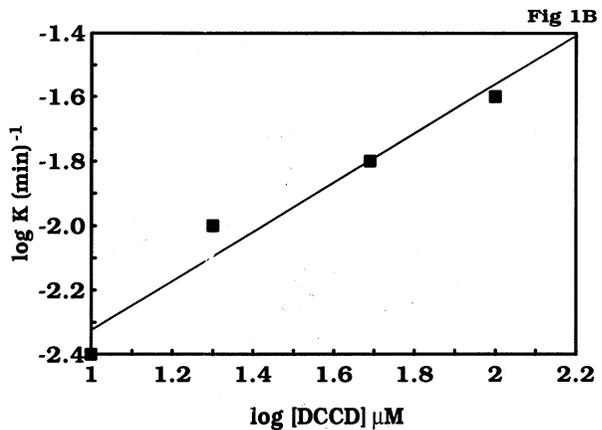
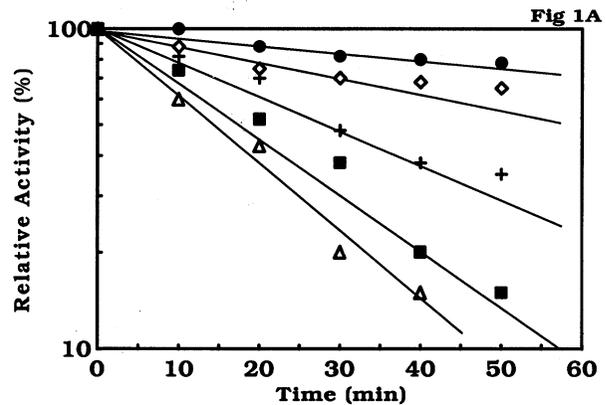


Fig. 1. Inactivation of native plasma membrane ATP hydrolysis by DCCD. Native plasma membrane vesicles (50 μg) preincubated with DCCD (10, 20, 60 or 100 μM) for various times (t) before the addition of (2 mM) ATP to initiate ATP hydrolysis. The rate obtained with DCCD added at $t = 0$ was assigned as 100%. All other ATPase activities were expressed relative to the control activity (\bullet — \bullet , control; \diamond — \diamond , 10; \times — \times , 20; \blacksquare — \blacksquare , 60; \triangle — \triangle 100 μM DCCD). All the lines in Fig. 1A were analyzed by an exponential fit program to determine the pseudo first order rate ($\log \text{ activity} = Kt + C$). Fig. 1B. Plot of logarithm of DCCD concentrations vs the logarithm of the pseudo-first order rate constant determined from the lines in Fig. 1A.

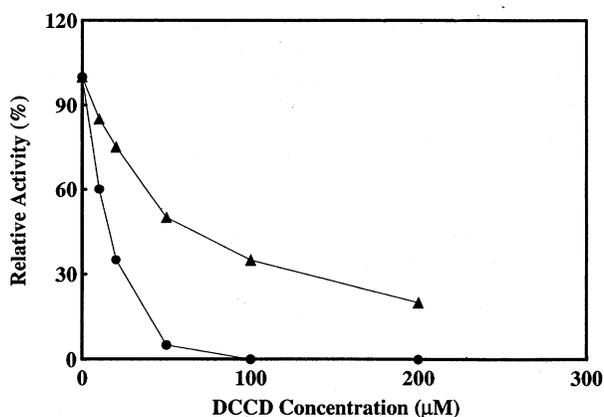


Fig. 2. Differential effect of DCCD inhibition on coupled activities of the ATP in reconstituted plasma membrane. Different concentrations of DCCD were preincubated with reconstituted plasma membrane vesicles for 5 min before proton transport activity was initiated, as described in Materials and methods. When proton pumping reached a steady state (no change in absorbance), 100 μ l of the incubation mixture was assayed for released phosphate. 100% activity represented the ATP hydrolysis or proton transport rate without the addition of DCCD. \blacktriangle — \blacktriangle , ATP hydrolysis; \bullet — \bullet , H⁺-pumping.

more sensitive to DCCD than was that of native vesicles (data not shown).

The effect of DCCD treatment on the temperature dependence of proton transport in reconstituted plasma membrane

Figure 3 shows the effects of temperature on the initial rate of proton transport by reconstituted plasma membrane with and without DCCD modification. At 25°C, proton transport activity exhibited the least inhibition, about 60% compared to membrane incubated without the addition of DCCD. Except for a narrow range between 20 and 25°C, other temperatures of incubation enhanced the inhibition by DCCD (Fig. 3). This result indicated that the plasma membrane modified by DCCD was extremely sensitive to low or high temperature treatment. Apparently, proton pumping became much more sensitive to DCCD inhibition when incubation temperature was below 20°C or above 25°C.

The effect of nucleotides on inhibition of DCCD in native and reconstituted plasma membrane ATPase

Various nucleotides have been shown to protect ATPase in *Vigna radiata* L. (Kasamo 1988) against inhibition by 2,3 butanedione. Similar experiments were conducted in this study to test whether nucleotides and dithiothreitol (DTT) could protect against DCCD inhibition. Plasma membrane vesicles were treated with various nucleotides and DTT for 15 min. Subsequently, ATP hydrolysis was measured. As shown in Tab. 1, all the tested substances except DTT were able partially to reverse DCCD inhibition of ATP hydrolysis. Except for

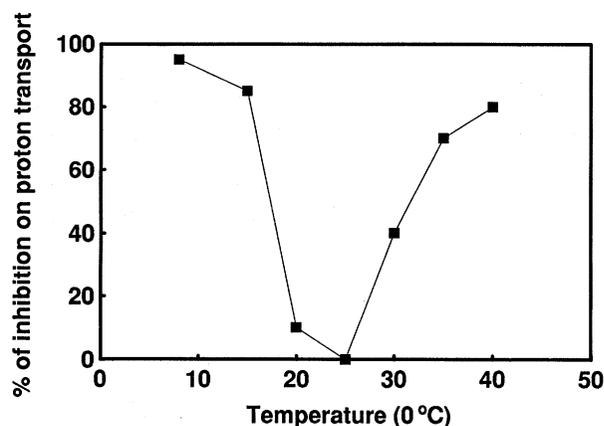


Fig. 3. The effect of temperature on proton transport activity of DCCD-modified plasma membrane. Aliquots (200 μ l) of reconstituted plasma membrane vesicles were assayed for proton transport at 8, 15, 20, 25, 30, 35 and 40°C with preincubation of 20 μ M of DCCD at 25°C for 10 min. The percentage of inhibition of proton transport was calculated by comparing the proton transport activity at each temperature to the activity at 25°C. The proton transport activity at 25°C was assigned as 100%.

DTT, all substances provided better protection against DCCD inhibition in reconstituted plasma membrane vesicles.

The effect of DCCD treatment on proton leakage of reconstituted plasma membrane

The vanadate-sensitive H⁺-ATPase-induced proton pumping by reconstituted plasma membranes was analyzed according to a steady-state kinetic model (Tu et al. 1987). This model was applied to analyze the effect of DCCD modification on kinetic parameters of the transport process (Tab. 2). The proton leakage of de-energized membrane was measured as k_2 , and was affected by the quenching method employed as previously reported (Brauer et al. 1989). Three different quenchers

Tab. 1. The effect of nucleotides and DTT on DCCD inhibition of ATP hydrolysis in native and reconstituted plasma membrane. Control experiment: Details of ATP hydrolysis and plasma membrane reconstitution were measured as described in Materials and methods. Experiment 2 was carried out the same as the control with the addition of 200 μ M DCCD. Experiments 3 to 7 were carried out in the presence of DCCD with the addition of various substances. All the experiments were carried out in duplicate trials.

Experiment	Native plasma membrane	Reconstituted plasma membrane
1 Control	100 \pm 5.0	100 \pm 5.0
2 DCCD (200 μ M)	20 \pm 1.0	25 \pm 1.0
3 ADP (5 mM)	24 \pm 1.0	29 \pm 1.5
4 Mg:ATP (5 mM)	31 \pm 1.5	29 \pm 1.5
5 AMP (5 mM)	30 \pm 1.5	40 \pm 2.0
6 Mg:AMP (5 mM)	35 \pm 2.0	45 \pm 2.0
7 DTT	18 \pm 1.0	17 \pm 1.0

Tab. 2. Effect of DCCD modification on the proton transport in reconstituted plasma membrane. The amount of protein concentration in each experiment was 500 μg . ATP hydrolysis and proton transport assays are described in Materials and methods. The control experiment was performed without DCCD. k_2 measured as collapsed by 0.2 mM vanadate.

Assay conditions	δ_s (mg protein) ⁻¹	k_2 , min ⁻¹
Control	0.43	0.58
10 μM DCCD	0.23	0.56
20 μM DCCD	0.18	0.58
40 μM DCCD	0.07	0.58

(EDTA, vanadate and hexokinase) were used in this study. Values of k_2 were 0.70, 0.58 and 0.64 min⁻¹ as collapsed by hexokinase, 0.2 mM vanadate and 10 mM EDTA, respectively (data not shown). The DCCD treatment did not affect the passive proton leakage (k_2) of de-energized membrane but decreased the values of δ_s . In the presence of 0, 10, 20 and 40 μM DCCD, k_2 was 0.58, 0.56, 0.58 and 0.58 min⁻¹ and δ_s was 0.43, 0.23, 0.18 and 0.07 absorbance units (mg protein)⁻¹, respectively.

Discussion

The carboxylic group-modifying reagent DCCD was used in this study to test a hypothetical indirect coupling mechanism between ATP hydrolysis and proton pumping in maize plasma membrane. We first determined that the effective binding of DCCD per mol of enzyme (Fig. 1) was close to 1. The value is similar to the effective binding of N-ethylmaleimide to cysteine of the plasma membrane ATPase of *Avena sativa* (Katz and Sussman 1987) and arginine on the enzyme from mung bean (*Vigna radiata* L.; Kasamo 1988). We have also shown that the DCCD binding site is most likely located in a hydrophobic region of the protein, since EDC and EDAC (hydrophilic carbodiimides) are not inhibitory.

The indirect relationship between ATP hydrolysis and proton translocation associated with the ATPases of maize tonoplast (Tu et al. 1987) and microsomal membrane (Brauer et al. 1989) was previously suggested by a steady-state analysis. Applying the same model in the present study, we found that the coupled activities of plasma membrane ATPase also showed a differential sensitivity, suggesting that the coupling between ATP hydrolysis and proton pumping is indirect. Thus the primary energy release step of the ATPase reaction may be only linked conformationally to proton translocation.

The differential effect of DCCD in this study is consistent with an indirect coupling between ATP hydrolysis and proton pumping, as suggested in a two-domain hypothesis between ATP hydrolysis and proton pumping, as suggested by Tu et al. (1990) on tonoplast H⁺-ATPase and Brauer et al. (1991) for the plasma mem-

brane ATPase. Accordingly, the DCCD modification may result in a weakened response of the protogenic domain to the events occurred in the catalytic domain.

Significant nucleotide protection of plasma membrane ATPase against inhibition by DCCD was observed in fungi (Sussman and Slayman 1983), mung bean root (Kasamo 1988) and *Avena sativa* roots (Katz and Sussman 1987). An analogous protection against DCCD inhibition of ATP hydrolysis by maize root plasma membrane was not observed (Tab. 1). The partial reversal of DCCD inhibition of ATP hydrolysis caused by the addition of nucleotides suggests that the binding sites of DCCD and nucleotide are close but different.

While DCCD modification decreased the proton pumping rate in general, a temperature dependence study (Fig. 3) revealed that the decrease reached a minimum between 20 and 25°C. It appears that the molecular event(s) leading to proton pumping are more resistant to DCCD in this temperature range. The change in resistance brought about by temperature suggests that the molecule affected by DCCD may not be the only one involved in proton pumping, i.e. the proton pumping requires the involvement of multiple molecular events.

To characterize the kinetic origin of DCCD inhibition on the proton transport associated with plasma membrane ATPase, the value of δ_s was analyzed by the steady-state kinetic model. When the proton gradient was discharged by different quenchers, such as hexokinase, vanadate or EDTA, different basal (de-energized) membrane leakage constants (k_2) were obtained. The k_2 values in this report were higher than those of reconstituted microsomes (Brauer et al. 1989). As shown in Tab. 2, DCCD treatment resulted in a decrease in δ_s , which is inversely related to the membrane leakage in the presence of ATP hydrolysis, but had no significant effect on basal membrane leakage (k_2). This result is consistent with the assumption that the structure of the plasma membrane is influenced by the energetic status. Thus, membrane proton leakage in the presence and absence of an energy input (ATP hydrolysis) may exhibit a different response to DCCD treatment.

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