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Anion-Sensitive, H^+-Pumping ATPase of Oat Roots[1]

DIRECT EFFECTS OF Cl^-, NO_3^-, AND A DISULFONIC STILBENE

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ABSTRACT

To understand the mechanism and molecular properties of the tonoplast-type H^+-translocating ATPase, we have studied the effect of Cl^-, NO_3^-, and 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) on the activity of the electrogenic H^+-ATPase associated with low-density microsomal vesicles from oat roots (*Avena sativa* cv Lang). The H^+-pumping ATPase generates a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH) that make up two interconvertible components of the proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$). A permeant anion (*e.g.* Cl^-), unlike an impermeant anion (*e.g.* iminodiacetate), dissipated the membrane potential ([^{14}C]thiocyanate distribution) and stimulated formation of a pH gradient ([^{14}C]methylamine distribution). However, Cl^--stimulated ATPase activity was about 75% caused by a direct stimulation of the ATPase by Cl^- independent of the proton electrochemical gradient. Unlike the plasma membrane H^+-ATPase, the Cl^--stimulated ATPase was inhibited by NO_3^- (a permeant anion) and by DIDS. In the absence of Cl^-, NO_3^- decreased membrane potential formation and did not stimulate pH gradient formation. The inhibition by NO_3^- of Cl^--stimulated pH gradient formation and Cl^--stimulated ATPase activity was noncompetitive. In the absence of Cl^-, DIDS inhibited the basal Mg,ATPase activity and membrane potential formation. DIDS also inhibited the Cl^--stimulated ATPase activity and pH gradient formation. Direct inhibition of the electrogenic H^+-ATPase by NO_3^- or DIDS suggest that the vanadate-insensitive H^+-pumping ATPase has anion-sensitive site(s) that regulate the catalytic and vectorial activity. Whether the anion-sensitive H^+-ATPase has channels that conduct anions is yet to be established.

We have distinguished two types of electrogenic, H^+-pumping ATPases in nonmitochondrial membrane vesicles from oat roots (9, 39) and tobacco callus (35, 36). One type is vanadate-insensitive and enriched in low-density membranes, such as the tonoplast, and one type is vanadate-inhibited and enriched in the plasma membrane fraction (9, 39, 40). Several other laboratories initially could demonstrate only a vanadate-resistant proton-pumping ATPase in microsomal vesicles (2, 14, 16, 24, 25, 30). However, within the last year, the vanadate-sensitive H^+-pumping ATPase has also been demonstrated in various tissues (1, 13). The properties of the two H^+-ATPases are summarized in a minireview (37).

The differential sensitivity to vanadate suggests that the mechanism of ATP hydrolysis, H^+ pumping, and the molecular properties of the two ATPases may differ. For example, the plasma membrane ATPase from corn roots forms a phosphorylated enzyme complex. Since vanadate inhibits formation of a phosphoenzyme intermediate (7, 23), the reaction mechanism of the vanadate-insensitive ATPase is apparently different from the plasma membrane enzyme. Furthermore, unlike the vanadate-sensitive ATPase, the vanadate-insensitive ATPase is stimulated by Cl^- and inhibited by nitrate (2, 3, 11, 14, 16, 25, 27, 30, 32, 33). Our preliminary studies also showed that DIDS[3] preferentially inhibited the anion-sensitive ATPase (9).

To understand the mechanism and molecular properties of the vanadate-insensitive H^+-pumping ATPase, we have examined in more detail the effect of Cl^-, nitrate, and DIDS on three activities: (a) ATPase activity in the presence and absence of gramicidin; (b) ATP-generated membrane potential; and (c) ATP-dependent pH gradient formation. We concluded previously that the vanadate-insensitive ATPase, ΔpH, and $\Delta\psi$ activities are manifestations of an electrogenic H^+-pumping ATPase based on the parallel distributions of these activities on a linear dextran gradient (9). The present results suggest that the H^+-ATPase has anion-sensitive site(s) which regulate its catalytic and vectorial activities. Preliminary results of this study have been reported (10, 39).

MATERIALS AND METHODS

Plant Material. Oat (*Avena sativa* L. var Lang) seedlings were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$. After 5 to 6 d of growth, the apical tips (3–4 cm) of the roots were harvested. Lang oats were generously provided by the Agronomy Department of the University of Maryland (College Park) and Kansas State University (Manhattan).

Preparation of Sealed Microsomal Vesicles. Sealed microsomal vesicles were prepared as described by Churchill and Sze (11) using a 6% dextran (mol wt 69,000, Sigma) cushion. Vesicles containing the vanadate-insensitive ATPase are enriched in the 0/6% interface (9).

Determination of ATP-Generated ΔpH and $\Delta\psi$. A relative measure of the pH gradient (ΔpH) generated by inward H^+ pumping into vesicles was determined as [^{14}C]methylamine (New England Nuclear) accumulation using a filtration procedure (11). Membrane potential ($\Delta\psi$) generation (inside positive) was determined as ATP-dependent SCN^- (^{14}C from Amersham or ^{35}S from New England Nuclear) uptake into vesicles using a filtration procedure described previously (38). We have shown that [^{14}C]

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[3] Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; $\Delta\psi$, membrane potential; ΔpH, pH gradient; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical gradient; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; KIDA, potassium iminodiacetate; BTP, bis-tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane).

methylamine accumulation is completely vanadate-resistant and thus it reflects H⁺-pumping activity entirely from the vanadate-insensitive (or tonoplast-type) ATPase (9, 11, 24, 25).

ATPase Activity. ATPase activity was determined by measuring Pi release at 36°C according to the method described by Hodges and Leonard (19) with some modifications (35). To determine vanadate-insensitive ATPase activity specifically, reaction mixtures included 100 μM Na orthovanadate, 0.1 mM ammonium molybdate, and 0.2 mM Na azide to inhibit the plasma membrane ATPase, unspecific phosphatase, and mitochondrial ATPase, respectively (9, 15). The plasma membrane ATPase activity could be specifically determined as either K⁺-stimulated or vanadate-inhibited ATPase activity (9). K⁺ stimulation was determined as the difference in activity in the presence and absence of 50 mM KIDA. Vanadate-sensitive ATPase activity was determined as the difference between total (no vanadate) and vanadate-resistant (plus 100 μM vanadate) ATPase activities, both of which were determined in the presence of 0.1 mM ammonium molybdate and 0.2 mM azide. Protein concentration was estimated by the Lowry method (22) after precipitation with 10% TCA using BSA as a standard.

Chemicals. Sodium ATP was purchased from Boehringer-Mannheim and converted by Dowex ion exchange and bis-tris propane titration to ATP-BTP at pH 6.8. DIDS, CCCP, and gramicidin were obtained from Sigma.

RESULTS

Effect of Cl^- on ATPase Activity, ΔpH, and Δψ Generation. *(a) Cl^- Collapses Δψ.* Chloride stimulated formation of an ATP-dependent pH gradient, as measured by [¹⁴C]methylamine accumulation (11) while IDA had no effect (Fig. 1a). In contrast membrane potential generation as measured by ATP-dependent SCN^- uptake, was maximum in the absence of Cl^- (37). ATP-dependent SCN^- uptake was decreased by increasing concentrations of Cl^- (Fig. 1b). Thiocyanate uptake was decreased 78% by 25 mM Cl^-, while iminodiacetate at 10 or 25 mM had no effect.

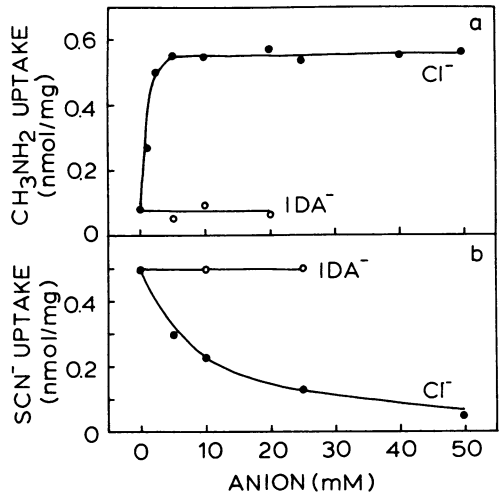

FIG. 1. Effect of Cl^- or IDA (iminodiacetate) concentration on ATP-dependent uptake of [¹⁴C]methylamine or [¹⁴C]thiocyanate into low-density vesicles from oat roots. (a) Methylamine uptake (pH gradient generation). Reaction mixture contained 175 mM mannitol, 25 mM Hepes-BTP at pH 7.5, 1 mM $MgSO_4$, 5 μg oligomycin, 20 μM [¹⁴C] methylamine, and ± 1 mM ATP-Tris. (b) SCN^- uptake (membrane potential generation). Reaction mixture consisted of 175 mM mannitol, 25 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 16 μM $S^{14}CN^-$, 0.2 mM Na orthovanadate, 5 μg oligomycin with or without 3 mM ATP-Tris. BTP-Cl or BTP-IDA were prepared by titration of HCl or iminodiacetic acid with BTP. Vesicles (0.2–0.3 mg protein/ml) from a 0/6% dextran interface were used to start the reaction. Triplicate samples (usually 100 μl each) were taken after 10 min incubation at 21°C. Results are the mean of at least two experiments.

One interpretation for Cl^- stimulation of ΔpH formation is that Cl^-, being a permeant anion, moved into the vesicles down an electrical gradient generated by H^+ pumping (interior positive). Collapse of the electrical gradient by Cl^- entry permitted more H^+ pumping by the ATPase resulting in formation of a steep pH gradient (2, 11, 38). In the presence of IDA, an impermeant anion, the electrical gradient was not dissipated and thus the pH gradient remained low. Sulfate also behaved like an impermeant anion. Sulfate did not dissipate the electrical potential (38) and did not stimulate formation of a pH gradient (11).

Although this interpretation is consistent with the idea that the total free energy from a H^+-pumping ATPase is made up of two interconvertible components (Δψ and ΔpH) (26), the kinetics of Cl^- concentration dependence between ΔpH stimulation and Δψ decrease were not similar (Fig. 1, a and b). At 5 mM Cl^-, when the membrane potential was partially (40%) dissipated, ΔpH formation was maximal. These results suggested that stimulation of ΔpH generation by Cl^- was not solely dependent on dissipation of a membrane potential.

(b) Direct Cl^- Effect on ATPase. ATPase activity was also differentially sensitive to anions. Chloride stimulated ATP hydrolysis in a concentration-dependent manner while impermeant anions, such as sulfate or IDA (not shown), had a small stimulatory effect on Mg,ATPase activity (Fig. 2). However, the differential effect of Cl^- and IDA on membrane potential collapse (Fig. 1b) was only partially responsible for Cl^- stimulation of a H^+-pumping ATPase as indicated by other results.

It is important to recall that only tightly sealed vesicles can show ΔpH or Δψ generation while both sealed and leaky vesicles hydrolyze ATP. Though 40% or less of the total vesicle population is tightly sealed to K^+ and perhaps H^+ (34; KA Churchill, unpublished), ATPase activities in the absence of gramicidin may originate mostly from leaky vesicles as the enzymes are uncoupled. ATPase activities in the presence of gramicidin reflects uncoupled activity from the total vesicle population. So total ATPase activities cannot be compared quantitatively to either ΔpH or Δψ generation. Gramicidin-stimulated ATPase activity (calculated from the difference in activity in the presence and absence of gramicidin) more closely represents ATPase activity from the sealed vesicles if they were made leaky.

Cl^- stimulation of ATPase activity in the presence of gramicidin (Fig. 3a) suggests that Cl^- directly increases the rate of ATP hydrolysis independent of the electrochemical gradient, as shown previously (2, 33, 34). Gramicidin-stimulated ATPase activity was also stimulated by Cl^- (Fig. 3b). The increase in Mg,ATPase

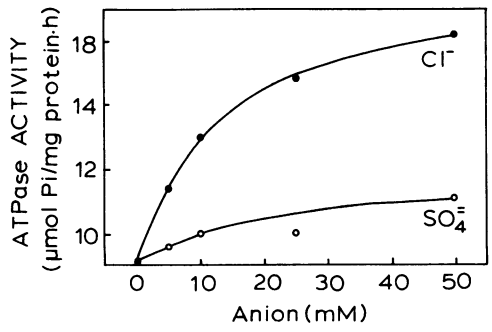

FIG. 2. Effect of Cl^- or SO_4^{2-} concentration on Mg,ATPase activity in low-density vesicles from oat roots. Reaction mixture consisted of 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-Tris, 5 μg oligomycin, 0.2 mM Na orthovanadate, and vesicles from a 0/6% dextran interface. ATPase activity that was stimulated by the presence of 5 to 50 mM KCl or K_2SO_4 is shown. Results are the mean of two experiments.

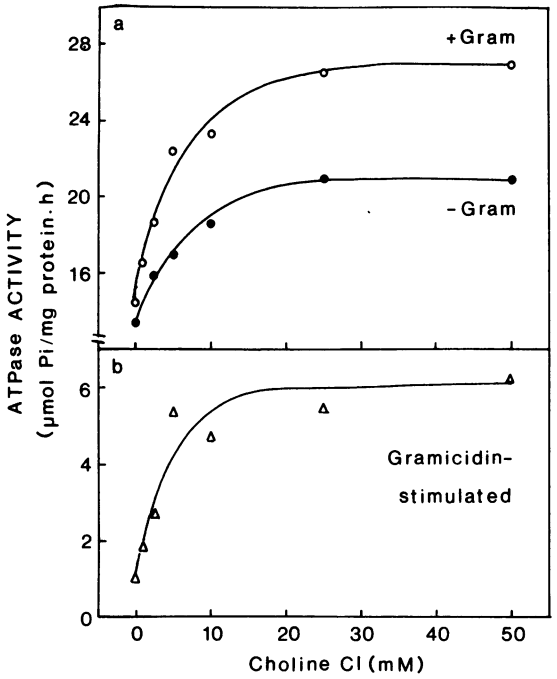

FIG. 3. Effect of Cl^- concentration and gramicidin on ATPase activity in low-density vesicles from oat roots. (a) ATPase activity in the presence (O) or absence (●) of gramicidin. (b) Gramicidin-stimulated ATPase activity calculated from the difference in activity in the presence and absence of gramicidin. Reaction mixture included 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-Tris, 0.2 mM Na azide, 0.2 mM Na orthovanadate, 0.1 mM ammonium molybdate, vesicles from a 0/6% dextran interface, ±2.5 μM gramicidin, and 1 to 50 mM choline chloride. Mean result of two experiments shown.

activity of 1.1 (from 13.4 to 14.5) μmol Pi/mg protein·h caused by gramicidin in the absence of Cl^- could be interpreted as stimulation of the basal ATP hydrolysis due to membrane potential and ΔpH collapse. Additional increase of gramicidin-stimulated ATPase activity from 1.2 to 6.3 μmol Pi/mg protein·h was dependent on an increase of Cl^- concentration from 1 to 50 mM (Fig. 3b) which could be interpreted as a direct Cl^- activation of ATPase associated with sealed vesicles.

Direct stimulation of ATPase activity by Cl^- appeared to be related to Cl^- stimulation of ΔpH generation. Since ionophore-stimulated ATPase activity reflects the uncoupled H^+ pumping activity of the enzyme associated with sealed vesicles, the kinetics of Cl^- stimulation of H^+ pumping and of gramicidin-stimulated ATPase activities were compared directly (Figs. 1a and 3b). The kinetics between Cl^- stimulation of methylamine uptake (apparent K_m for Cl^- = 1.5 mM) (Fig. 1a) and gramicidin-stimulated ATPase activity (K_m = 2.0 mM) (Fig. 3b) were strikingly similar. These results suggested that Cl^- stimulation of ΔpH generation could be due to specific Cl^- stimulation of the enzyme, in addition to dissipation of the membrane potential.

From the ATPase activities (Fig. 3), one can estimate that Cl^--stimulated ΔpH formation was about 20% or less (1.1 out of 6.3 μmol Pi/mg protein·h) due to dissipation of the membrane potential and about 80% due to a direct Cl^- stimulation of ATPase activity. In other experiments, gramicidin or CCCP has little or no stimulatory effect on Mg,ATPase activity from oat roots or tobacco callus (8, 9, 34), suggesting that collapse of a membrane potential alone does not significantly stimulate ATP hydrolysis. Other laboratories have also concluded that chloride stimulated an ATPase (2, 14, 16, 25, 33), but no attempt was made to separate the relative effect of Cl^- on membrane potential collapse and direct enzyme activation.

Table I. *Anion Effect on ATPase Activity Associated with Low-Density Vesicles of Oat Roots*

Reaction mixtures were as described in Figure 6. The pH of the reaction mixtures was maintained at pH 6.8 by 30 mM Hepes-BTP buffer. Most salt solutions did not alter the pH of the reaction mixture. KIDA, BTP-Cl, or BTP-NO_3 were buffered to pH 7.0. Hepes did not stimulate the ATPase activity significantly. Salt-sensitive ATPase activity is the difference between total and basal Mg,ATPase activity in the absence of gramicidin. Gramicidin-stimulated ATPase is the difference between activity in the presence and absence of gramicidin (Gram). Results are the average of two to six experiments.

Salt	ATPase Activity			
	−Gram	+Gram	Salt-sensitive (no Gram)	Gram-stimulated
	μmol Pi/mg protein·h			
None	10.5			
10 mM KCl	14.6	21.4	4.1	6.8
KBr	15.4	21.7	4.8	6.3
KI	12.4	18.6	1.9	6.2
$KHCO_3$	11.0	17.2	0.4	6.2
KIDA	11.3	14.6	0.7	3.3
K_2SO_4	9.6	13.6	−0.9	4.0
KSCN	11.6	15.0	1.1	3.4
KNO_3	7.0	6.9	−3.5	−0.1
BTP-NO_3	8.2	8.4	−2.4	0.3
NH_4Cl	23.5	23.3	13.0	−0.2
$(NH_4)_2SO_4$	13.7	13.5	3.2	−0.2
50 mM KCl	18.5	26.6	8.0	8.1
KBr	19.5	25.6	8.9	6.2
KI	16.2	22.2	5.7	6.0
$KHCO_3$	15.2	23.4	4.6	8.2
KIDA	14.0	18.7	3.5	4.7
K_2SO_4	12.1	15.7	1.6	3.6
KSCN	14.1	14.9	3.6	0.8
KNO_3	8.0	9.2	−2.5	1.2
BTP-NO_3	7.6	7.9	−3.0	0.3
NH_4Cl	27.5	28.6	17.0	1.1
$(NH_4)_2SO_4$	19.1	18.2	8.6	−0.9

Effect of Various Cations and Anions. To determine whether other ions directly altered H^+ pumping, the effect of several cations and anions on gramicidin-stimulated ATPase activity was examined and, when possible, compared to their effect on membrane potential or pH gradient generation.

Using K^+ as the cation, we found that Cl^-, Br^-, I^-, and HCO_3^- were similarly effective in stimulating gramicidin-dependent ATPase activity (Table I). As with Cl^-, Br^- appeared to stimulate the ATPase directly, in addition to dissipating the membrane potential. Bromide at 10 mM partially decreased the ATP-generated membrane potential (8) yet maximally stimulated H^+ pumping (11). In the presence of IDA or sulfate, gramicidin-stimulated ATPase activity was relatively low not because these anions inhibited H^+ pumping or generation of a membrane potential (Fig. 1; Ref. 11). Rather IDA or sulfate did not stimulate the ATPase activity. Thus, the ATPase is mainly stimulated by anions, such as Cl^-, Br^-, or HCO_3^-. Apart from a few exceptions, these results are similar to those observed in corn roots (27).

In the presence of NO_3^-, there was little or no gramicidin-stimulated ATPase activity (Table I). These results are consistent with the observations that NO_3^- inhibited ATPase activity (Table I) and H^+ pumping as shown below. SCN^-, being a permeant anion, will completely (98%) dissipate the membrane potential at 10 mM (8) without apparently affecting gramicidin-stimulated

Table II. *Cation Effect on ATPase Activity Associated with Low-Density Vesicles from Oat Roots*

Reaction mixtures were as described in Table I. Results are the average of two to six experiments.

Cation-Cl	ATPase Activity			
	−Gram	+Gram	Salt-sensitive (no Gram)	Gram-stimulated
	μmol Pi/mg protein·h			
None	10.5			
10 mM Choline-Cl	15.8	21.7	5.3	5.9
KCl	14.6	21.3	4.1	6.8
RbCl	14.5	21.4	4.0	7.0
CsCl	17.0	21.8	6.5	4.8
NaCl	16.6	22.1	6.1	5.5
LiCl	16.4	21.1	5.9	4.7
BTP-Cl	16.7	22.3	6.1	5.6
NH_4Cl	23.5	23.3	13.0	−0.2
50 mM Choline-Cl	20.1	26.9	9.6	6.8
KCl	18.5	26.6	8.0	8.1
RbCl	19.6	26.2	9.1	6.6
CsCl	20.9	26.2	10.4	5.3
NaCl	22.7	27.6	12.2	4.9
LiCl	23.4	27.9	12.8	4.5
BTP-Cl	21.4	26.8	10.8	5.4
NH_4Cl	27.5	28.6	17.0	1.1

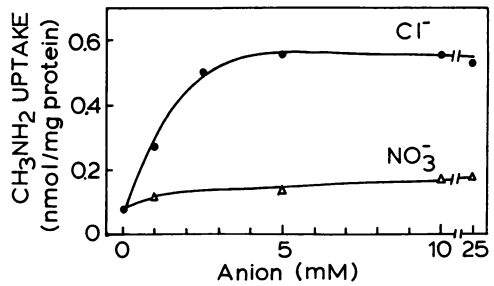

FIG. 4. Effect of Cl^- or NO_3^- concentration on ATP-dependent methylamine uptake into low-density vesicles from oat roots. Reaction mixture was as described in legend to Figure 1a except BTP-Cl or BTP-NO_3 concentrations ranged between 0.5 and 25 mM. Triplicate samples were taken after 10 min incubation at 21°C. Results are the mean of five experiments.

ATPase activity. However, 50 mM SCN^- appeared to inhibit ATPase activity as gramicidin-stimulated activity was eliminated (Table I).

Chloride was effective in stimulating gramicidin-dependent ATPase activity regardless of whether the counterions were alkali-cations or organic cations (choline, bis tris propane) (Table II). Though the ATPase was clearly more sensitive to anions than cations, alkali-cations appeared to have little effect on the vanadate-insensitive ATPase. It is not clear whether less stimulation by K^+ than Li^+ of ATPase activity (in the absence of gramicidin) was significant or not. Gramicidin-stimulated ATPase activity was frequently higher in the presence of K^+ or Rb^+ than with Choline, BTP, Cs^+, Li^+, or Na^+ (Table II), probably because of the specificity of gramicidin for K^+ and Rb^+.

One can estimate the relative contribution of direct Cl^- stimulation of the ATPase from the differences between activities in the presence of NH_4Cl and $(NH_4)_2SO_4$ (Table I). As NH_4^+ dissipates the pH gradient (11) and probably the membrane potential, gramicidin had no stimulatory effect on ATPase activity as previously reported with tobacco callus (35). In the absence

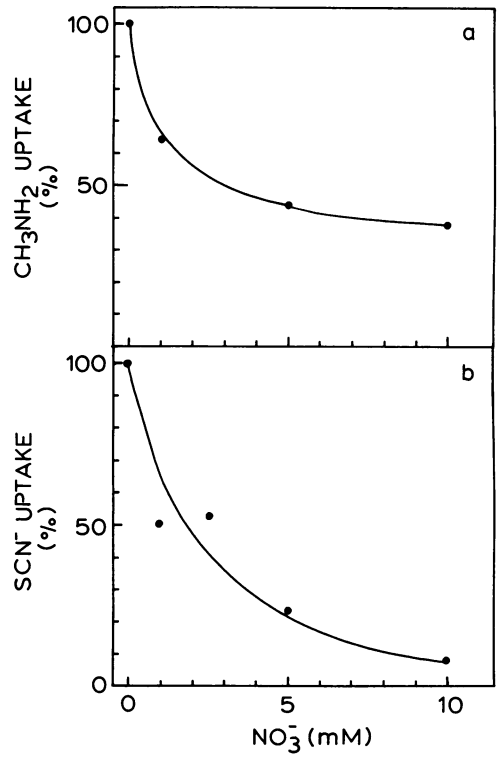

FIG. 5. Effect of NO_3^- concentration on ATP-dependent generation of a pH gradient and membrane potential in low-density microsomal vesicles from oat roots. (a) ATP-dependent methylamine uptake. Reaction conditions were as described in legend to Figure 1a except BTP-Cl was 10 mM and BTP-NO_3 ranged from 1.0 to 10 mM. Methylamine uptake in the absence of NO_3^- (100%) was 585 pmol CH_3NH_2/mg protein. (b) ATP-dependent SCN^- uptake. Reaction conditions were as described in legend to Figure 1b with 1 to 10 mM BTP-NO_3. In the absence of NO_3^-, 178 pmol SCN^- was taken up per mg protein (100%).

of a proton electrochemical gradient, ATPase activity in the presence of 10 mM NH_4Cl was 9.8 μmol Pi/mg protein·h higher than activity in the presence of $(NH_4)_2SO_4$. pH gradient collapse by $(NH_4)_2SO_4$ accounted for an increase in ATP hydrolysis of 3.2 μmol Pi/mg protein·h. Thus, NH_4Cl stimulation of the ATPase activity was approximately 75% (9.8 of 13) due to a direct Cl^- effect rather than dissipation of an $\Delta\bar{\mu}_{H^+}$.

Inhibition by NO_3^-. Although NO_3^- is frequently used as a permeant anion and in several systems can dissipate membrane potential and increase ΔpH more effectively than Cl^- (29, 40), NO_3^- alone had little or no effect on ATP-dependent methylamine uptake (Fig. 4). Increasing NO_3^- concentrations (from 1 to 25 mM) slightly increased the level of methylamine accumulation; however, at 25 mM, NO_3^--dependent methylamine accumulation was only 20% of that dependent on Cl^- (Fig. 4).

We had previously reported that NO_3^- inhibited ΔpH formation and ATPase activities (11) but had not examined the concentration dependence. A few millimolar NO_3^- (1 and 5 mM) not only inhibited Cl^--stimulated methylamine uptake (by 35 and 55%) (Fig. 5a), but also was more effective than Cl^- in decreasing SCN^- uptake (membrane potential formation) (Fig. 5b and 1b). Though the decrease in membrane potential could be interpreted as collapse of a membrane potential by a permeant anion (NO_3^-), the inhibitory effects of NO_3^- on Mg,ATPase activity (Fig. 6) would suggest that NO_3^- both dissipated and inhibited formation of a membrane potential. In agreement with this idea, we found that NO_3^- also inhibited Cl^--stimulated ATPase activity in the presence or absence of gramicidin (Fig. 6b) and inhibited gramicidin-stimulated ATPase (Fig. 6c). Fur-

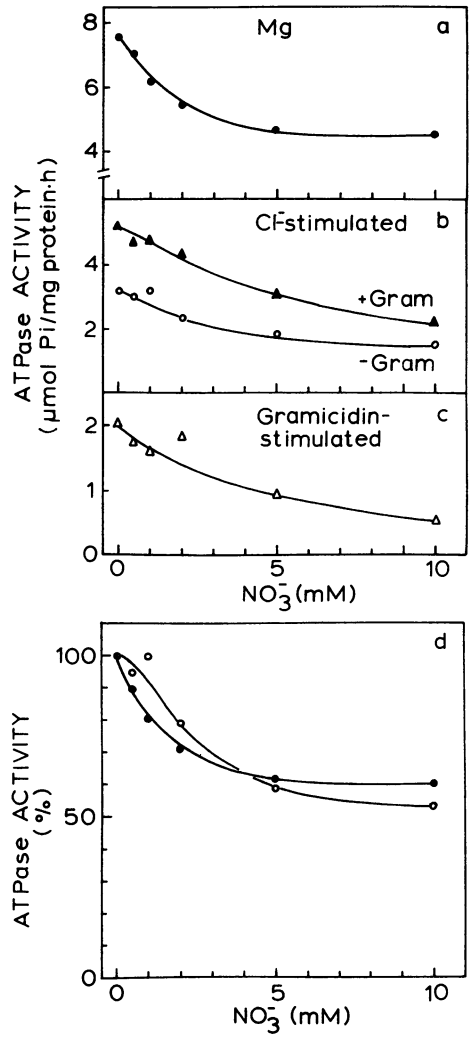

FIG. 6. Effect of NO_3^- concentration on ATPase activity in low-density microsomal vesicles from oat roots. Results are the mean of four experiments. (a) Mg,ATPase activity. Reaction mixtures were as described in legend to Figure 3 with 0 to 10 mM BTP-NO_3 (in the absence of choline Cl and gramicidin). (b) Cl^--stimulated ATPase activity is the increase in activity due to 50 mM BTP-Cl assayed in the presence (▲) or absence (O) of 2.5 μM gramicidin. (c) Gramicidin-stimulated Cl^--Mg,ATPase activity calculated from (b) as the difference in activity in the presence and absence of gramicidin. (d) Relative Mg,ATPase (●) and Cl^--stimulated ATPase (O) activities in per cent from data in (a) and (b).

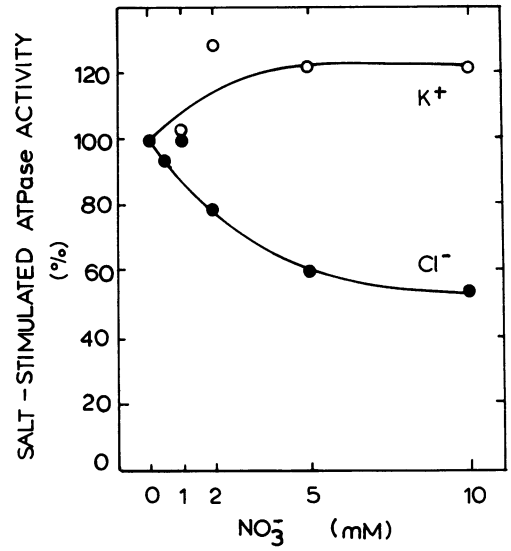

FIG. 7. Effect of NO_3^- concentration on K^+-stimulated or Cl^--stimulated ATPase activity in low-density microsomal vesicles from oat roots. Assay conditions were as described in legend to Figure 3 except Na orthovanadate was excluded and 50 mM KIDA (O) or BTP-Cl (●) was added. BTP-NO_3 concentration was varied from 0.5 to 10 mM. Activity in the absence of NO_3^- was set to 100%.

thermore, 1.0 mM NO_3^- effectively inhibited Cl^--stimulated H^+ pumping (Fig. 8a). The relative inhibition (%) of the various ATPase activities by different NO_3^- concentrations were similar, suggesting that all these enzyme activities are properties of one enzyme which is directly affected by NO_3^-.

We have previously shown that NO_3^- inhibited ATPase activity associated wth low-density vesicles (1–4% dextran) but NO_3^- had little or no effect on ATPase collected at 4 to 7% dextran (9). The latter vesicles were enriched in plasma membrane-type ATPase as the enzyme was vanadate-sensitive and showed K^+ stimulation. Figure 7 shows clearly that K^+-stimulated ATPase activity was not inhibited by 1 to 10 mM NO_3^-. In fact, NO_3^- appeared to slightly stimulate the ATPase activity, supporting the idea that NO_3^- can act as a permeant anion that would dissipate the membrane potential in plasma membrane-type vesicles (29, 40).

We tested the possibility that NO_3^- might be competing for a common anion site with Cl^-. Figure 8a shows the effect of NO_3^- on pH gradient formation measured with various concentrations of Cl^-. A Lineweaver-Burk plot (Fig. 8b) or a direct linear plot of Eisenthal and Cornish-Bowden (12) (not shown) of Figure 8a, indicated that NO_3^- was a noncompetitive inhibitor of the Cl^--stimulated H^+ pump. Though careful kinetic studies have not been conducted on the ATPase activity, Figure 6c shows that Cl^- may have a slight protective effect at low levels of NO_3^- (1–2 mM), as the relative inhibition by NO_3^- of Cl^--stimulated ATPase activity is less than that of Mg, ATPase.

Inhibition by DIDS. When we showed that DIDS inhibited ionophore-stimulated ATPase activity (35), membrane potential (8), and pH gradient generation (11), we were uncertain about the specificity of DIDS. Here we show that DIDS specifically inhibited the vanadate-resistant ATPase with a K_i of about 10 μM, but had little effect on the vanadate-sensitive ATPase activity (Fig. 9). These results agree with our previous report that low concentrations of DIDS preferentially inhibited the Cl^--stimulated but not the K^+-stimulated ATPase activity (9).

DIDS inhibited ATP-dependent methylamine accumulation (Fig. 10) and ATP-dependent SCN^- uptake (Fig. 11) in the same concentration range that inhibited ATPase activities (Fig. 12). The presence of Cl^- was not necessary for DIDS action. DIDS inhibited Mg,ATPase activity (Fig. 12a) as well as Cl^--stimulated ATPase and gramicidin-stimulated ATPase activities (Fig. 12c). Though we have not tested whether DIDS competed for Cl^- activation site(s) on the ATPase, the presence of Cl^- did confer some protection from DIDS inhibition. At 2 to 10 μM DIDS, Cl^--stimulated ATPase activity (with or without gramicidin) was relatively less inhibited than Mg,ATPase activity (Fig. 12d).

DISCUSSION

Cl^--Stimulated ATPase Activity and Dissipated Membrane Potential. The vanadate-insensitive ATPase activity is stimulated by anions, especially Cl^-, but is largely unaffected by cations (Table I and II) as previously reported (2, 8, 11, 14, 16, 25, 33). Chloride stimulation of an ATPase could be caused by one or more of the following reasons: (i) Cl^- dissipates an electrical potential by moving into the vesicles via a lipid pathway or an anion channel separate from the H^+-ATPase; (ii) Cl^- directly

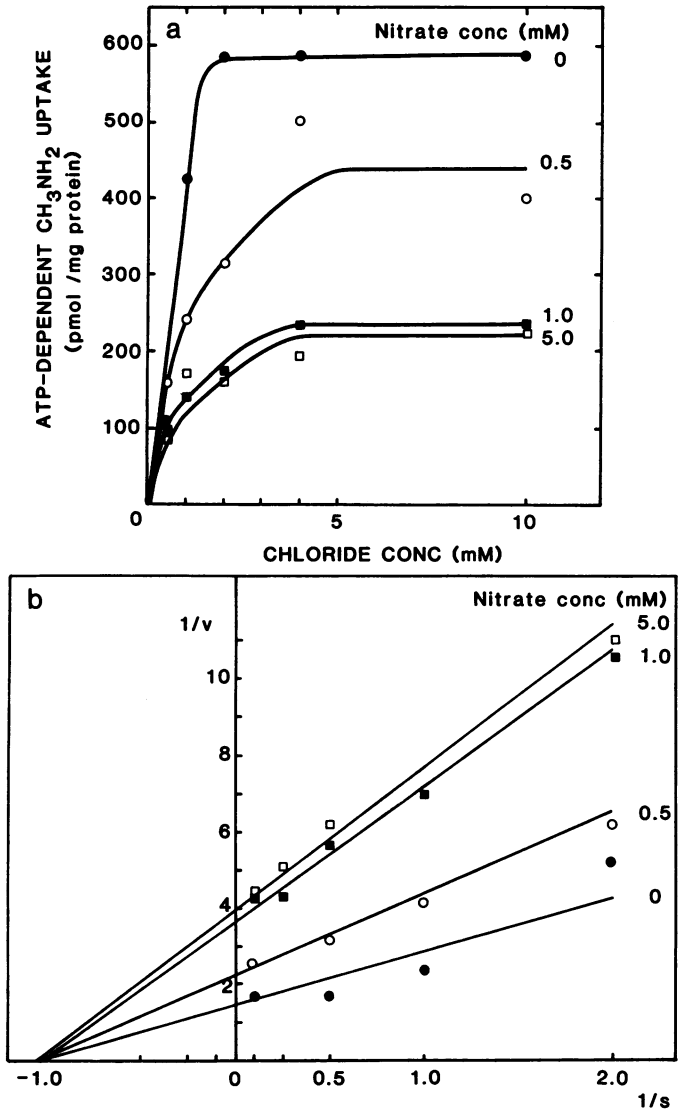

FIG. 8. (a) Effect of NO_3^- concentration on ATP-dependent methylamine uptake into low-density microsomal vesicles from oat roots in the presence of various Cl^- concentrations. Assay conditions were as described in legend to Figure 1a. BTP-NO_3 (0.5–5 mM) and BTP-Cl (0.5–10 mM) were added as indicated. Triplicate samples were filtered after 3 min incubation. Result shown is from one experiment. (b) Lineweaver-Burk analysis of the data shown in (a).

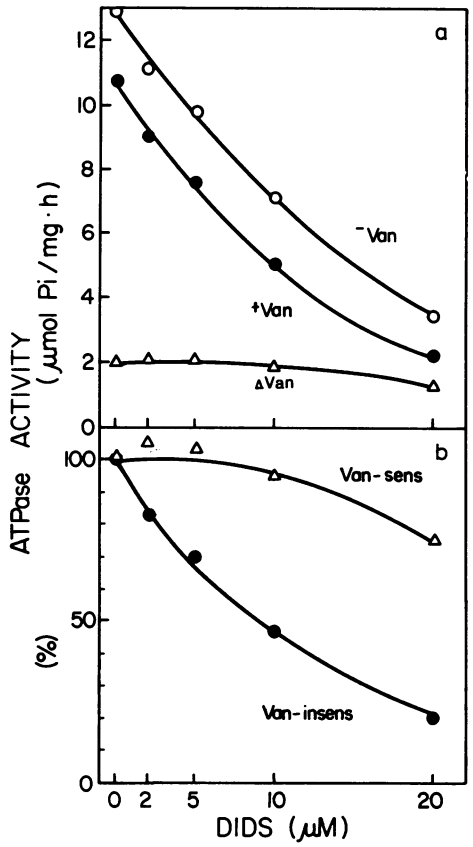

FIG. 9. (a) Effect of DIDS concentration on Mg,ATPase activity of sealed microsomal vesicles from oat roots in the presence (●) or absence (○) of Na orthovanadate (100 μM). Reaction conditions were as described in Figure 6a. (b) DIDS effect on the relative activity of the vanadate-sensitive (△) and vanadate-insensitive (●) ATPases.

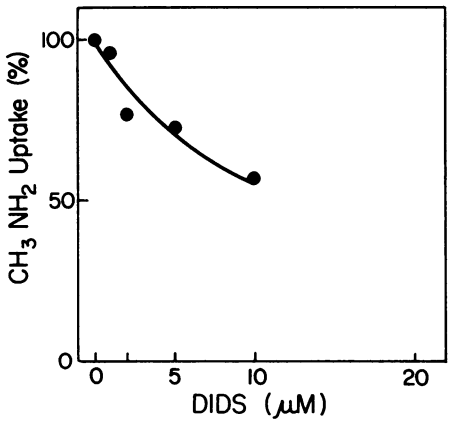

FIG. 10. Effect of DIDS concentration on ATP-dependent methylamine uptake into low-density vesicles from oat roots. Assay conditions were as described in Figure 1a with 10 mM BTP-Cl. In the absence of DIDS, 729 pmol methylamine per mg protein was taken up (100%). Results are the mean of two experiments.

stimulates the H^+-pumping ATPase activity without being transported by the ATPase; and (iii) Cl^- is transported by an anion channel associated with the H^+-ATPase as proposed by Bennett and Spanswick (2). We have made a preliminary attempt to distinguish between these possibilities.

Several observations suggested that collapse of a membrane potential by Cl^- (Fig. 1) could not account completely for the stimulation of ATPase activity (Fig. 2) and ΔpH formation (Fig. 1). (a) Using microsomal vesicles from tobacco callus, ionophores (CCCP or gramicidin) that collapse membrane potential (38) had little or no effect on Mg,ATPase activity when measured in the absence of Cl^- (34) as shown in this study with oat root vesicles (Fig. 3) and other tissues (2, 33). Similarly, a membrane potential built up by a H^+-pumping ATPase in plasma membrane vesicles of *Neurospora* could be completely collapsed by Cl^- or NO_3^-, but these ions had essentially no stimulatory effect on ATP hydrolysis (29). (b) SCN^- at 10 mM collapses the membrane potential but barely stimulated Mg,ATPase activity in the absence of gramicidin (Table I). (c) Collapse of a pH gradient by NH_4 salts caused some stimulation of ATPase activity but additional stimulation is observed in the presence of 10 mM Cl^- (Table I). (d) In the presence of gramicidin which dissipates $\Delta\psi$ and ΔpH, Cl^- stimulated ATPase activity (Fig. 3). (e) Detergent-solubilized and partially purified ATPase from oat roots is stimulated by Cl^- (31). These results and the similar dependence on Cl^- concentration (K_m = 1.5–2.0 mM) of pH gradient generation (Fig. 1) and gramicidin-stimulated ATPase (Fig. 3) would

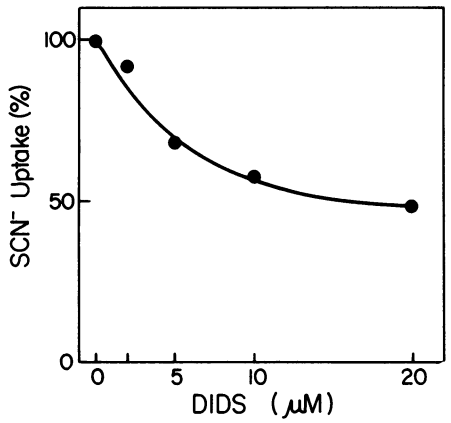

FIG. 11. Effect of DIDS concentration on ATP-dependent SCN^- uptake into low-density vesicles from oat roots. Assay conditions were as described in Figure 1b except BTP-Cl was absent. Results are the mean of two experiments.

support the idea that the Cl^--stimulated pH gradient generation was mostly due to direct Cl^- stimulation of the H^+-pumping ATPase. Taking together the ATPase results (Fig. 3; Table II) and what we know about $\Delta\psi$ and ΔpH under the various conditions, we estimated by two independent ways that Cl^- stimulation of the H^+-pumping ATPase associated with sealed vesicles is 75% or more attributed to a direct effect on the enzyme activity (possibility ii) and 25% or less due to dissipation of the $\Delta\psi$ (possibility i).

Direct Inhibition of the Vanadate-Insensitive ATPase by NO_3^- and DIDS. The results from our previous (8–11) and present studies (Figs. 6, 7, 9) clearly indicate that NO_3^- and DIDS preferentially inhibit the vanadate-insensitive, Cl^--stimulated H^+-ATPase. The vanadate-sensitive or K^+-stimulated ATPase is not inhibited (Figs. 7 and 9). Since all assays contained 0.2 mM azide, residual mitochondrial ATPase activity was eliminated. Nitrate has been shown by numerous investigators to inhibit an anion-sensitive ATPase (6, 18, 27, 32) and a tonoplast ATPase from red beets (1, 30, 41).

Nitrate inhibition of a Cl^--stimulated ATPase and generation of a pH gradient would suggest that NO_3^--sensitive site(s) are required for the catalytic as well as the vectorial activity of the enzyme. Although the presence of Cl^- conferred slight protection to the ATPase activity (Fig. 6d), the inhibition by NO_3^- was noncompetitive with Cl^- (Fig. 8b). Nitrate inhibited Mg,ATPase activity even in the absence of Cl^- (Fig. 6a; Ref. 10). Since a few millimolar NO_3^- was effective in decreasing Cl^--dependent and Cl^--independent ATP hydrolysis as well as Cl^--dependent H^+ pumping, NO_3^- probably acts by directly altering enzyme activity rather than by a chaotropic effect (17). The physiological significance of NO_3^- regulation of a H^+-pumping ATPase is not clear.

Our studies (Figs. 11 and 12) suggest that DIDS specifically and directly inhibited the anion-sensitive, electrogenic H^+-ATPase activity. We have been interested in the effect of DIDS (8–11, 35) because it is a specific anion channel blocker in red blood cells (5), corn protoplasts (21), and *Chara* (20). Bennett and Spanswick (2) have suggested that DIDS inhibited H^+ pumping into vesicles by blocking Cl^- transport via an anion channel because DIDS inhibited the saturable component of the Cl^--dependent H^+ pumping. A similar model was proposed by Pazoles *et al.* (28) for an anion-activated Mg,ATPase activity associated with chromaffin granules. However, we show that DIDS inhibited Mg,ATPase activity as well as membrane potential generation which were measured in the absence of Cl^- (Figs. 9 and 11). Furthermore, Cl^--dependent ATPase activity and Cl^--stimulated ΔpH generation were inhibited by similar concentrations of DIDS as those needed to inhibit Mg,ATPase activity

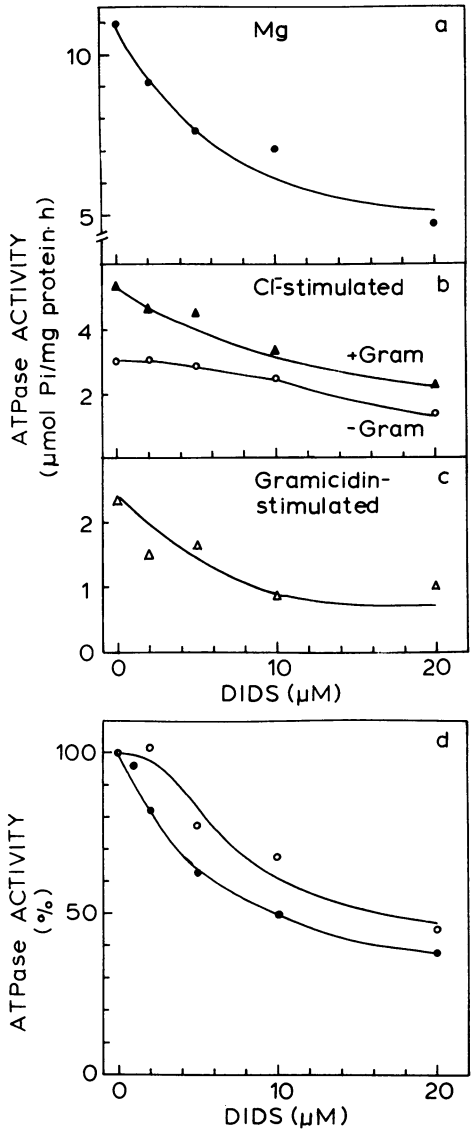

FIG. 12. Effect of DIDS concentration on ATPase activity in low-density microsomal vesicles from oat roots. Reaction mixtures were as described in Figure 6 in the presence of 0 to 20 μM DIDS. Results are the mean of four experiments. (a) Mg,ATPase activity. (b) Cl^--stimulated ATPase activity in the presence (▲) or absence (○) of 2.5 μM gramicidin. (c) Gramicidin-stimulated Cl^--Mg,ATPase calculated from (b) as the difference in activity in the presence and absence of gramicidin. (d) Relative Mg,ATPase (●) and Cl^--stimulated ATPase (○) activities plotted from data of (a) and (b).

indicating that the same enzyme was inactivated either in the presence or absence of Cl^- (Figs. 10 and 12c). If DIDS inhibited Cl^- transport alone into vesicles, one would expect DIDS to decrease membrane potential collapse by Cl^-. However, this was not observed; instead DIDS decreased membrane potential formation by Mg,ATP. A direct inhibition by DIDS of the anion-sensitive H^+-pumping ATPase would also result in inhibition of Cl^--dependent ΔpH formation (Fig. 10; Refs. 2 and 11) and ATP-dependent, potential-driven $^{36}Cl^-$ uptake into vesicles (24).

We interpret the present data as follows: DIDS directly interacted with the H^+-ATPase complex. This interaction results in inhibition of the catalytic and vectorial activity without necessarily invoking inhibition by DIDS of anion transport. This conclusion is supported by our finding that octylglucoside-solubilized ATPase is also DIDS-sensitive (31). Chloride uptake is

probably driven by the electrical gradient (positive inside) generated by the electrogenic H^+-ATPase. Chloride could move in via a lipid pathway or a protein channel that may or may not be sensitive to DIDS. It is interesting that a direct action of DIDS on the H^+-pumping ATPase in addition to an anion (Cl^- or Br^-) channel was also proposed in the chromaffin granules (28). However, in clathrin-coated vesicles, DIDS inhibited an anion channel separate from the H^+-ATPase (42).

Anion-Binding Site(s) Regulate Catalytic and Vectorial Activity. Taken together, our results show that the vanadate-insensitive ATPase is anion-sensitive in at least three ways: (a) Cl^- directly stimulated ATPase activity; (b) NO_3^- specifically inhibited ATPase activity; and (c) DIDS inhibited directly the Mg,ATPase activity. These three properties are characteristic of the vanadate-insensitive ATPase but not the vanadate-sensitive H^+-pumping ATPase. All three compounds potentially associate with anion-binding sites, though our results do not permit us to distinguish whether Cl^-, NO_3^-, or DIDS acts at the same or separate site(s) of the enzyme complex. DIDS will react with sulfhydryl as well as amino groups (5), but it is less likely that the preferential inhibition of the anion-sensitive ATPase is caused by interaction with sulfhydryl groups (42) as assays were conducted in the presence of DTT.

How the interaction of anion-sensitive sites with Cl^-, NO_3^-, or DIDS regulate the catalytic and vectorial H^+ pumping activity of the ATPase is not clear. We propose a simple model. The vanadate-insensitive ATPase complex has anion-binding sites which are directly involved in catalysis and are important for maintaining the conformation of the enzyme. In the presence of SO_4^{2-} or IDA, the enzyme is moderately active in ATP hydrolysis and H^+ pumping. When Cl^- is associated with the anion-binding site(s), the enzyme is in an active configuration so H^+ pumping is increased. However, binding of NO_3^- or DIDS either prevents catalysis or modifies the enzyme configuration resulting in less activity. So far there is no evidence that the anion-sensitive site(s) of the H^+-ATPase are associated with channels that conduct anions, though this possibility is considered. Until we understand the molecular and transport properties of the anion-sensitive H^+-ATPase, the physiological significance of the anion-sensitive site(s) will remain speculative.

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