Plant Physiol. (1987) 83, 569–572
0032-0889/87/83/0569/04/$01.00/0

H^+-ATPase Activity from Storage Tissue of *Beta vulgaris*[1]

IV. *N,N'*-DICYCLOHEXYLCARBODIIMIDE BINDING AND INHIBITION OF THE PLASMA MEMBRANE H^+-ATPase

Received for publication August 1, 1986 and in revised form October 16, 1986

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ABSTRACT

The molecular weight and isoelectric point of the plasma membrane H^+-ATPase from red beet storage tissue were determined using *N,N'*-dicyclohexylcarbodiimide (DCCD) and a H^+-ATPase antibody. When plasma membrane vesicles were incubated with 20 micromolar [^{14}C]-DCCD at 0°C, a single 97,000 dalton protein was visualized on a fluorograph of a sodium dodecyl sulfate polyacrylamide gel. A close correlation between [^{14}C]DCCD labeling of the 97,000 dalton protein and the extent of ATPase inhibition over a range of DCCD concentration suggests that this 97,000 dalton protein is a component of the plasma membrane H^+-ATPase. An antibody raised against the plasma membrane H^+-ATPase of *Neurospora crassa* cross-reacted with the 97,000 dalton DCCD-binding protein, further supporting the identity of this protein. Immunoblots of two-dimensional gels of red beet plasma membrane vesicles indicated the isoelectric point of the H^+-ATPase to be 6.5.

The role of the plant cell plasma membrane ATPase in catalyzing H^+-transport is now well established (16, 23). The importance of the H^+-ATPase in driving secondary transport of a number of solutes, in intracellular pH regulation, and possibly in auxin action has prompted a large number of biochemical studies of this enzyme. Several methods have been used to identify the mol wt of the plasma membrane H^+-ATPase, including localization of the phosphorylated intermediate of the enzyme on SDS-polyacrylamide gels following labeling with [γ-^{32}P]ATP (5, 7, 25). Results of these labeling studies have identified mol wt for the H^+-ATPase of 100,000 D for corn roots (5) and red beets (7), and 105,000 D for oat roots (25). In addition, Serrano (19), and Anthon and Spanswick (2) have used a combination of selective solubilization and density gradient centrifugation to partially purify the H^+-ATPase, and have reported mol wt of 100,000 D in oat roots (19) and 90,000 D in tomato roots (2).

Here we report the use of two additional tools, [^{14}C]DCCD[3] and a H^+-ATPase antibody, to identify the mol wt and isoelectric point of the H^+-ATPase from red beet plasma membrane preparations. DCCD is a general inhibitor of ATPases (21), and has been demonstrated to inhibit the H^+-ATPases of *Neurospora crassa* plasma membrane (22) and vacuolar membrane (4) as well as the plasma membrane and tonoplast H^+-ATPase from red beets (3, 6). In addition, DCCD has been shown to bind to a low mol wt protein presumed to be a subunit of the red beet and oat root tonoplast H^+-ATPase (14, 17) and to a 19,500 D subunit of the yeast vacuolar H^+-ATPase (24).

MATERIALS AND METHODS

Plant Material. Red beets (*Beta vulgaris* L.) were obtained from local fresh market vegetable sources and stored at 4°C for up to 10 d before using the hypocotyl tissue for plasma membrane preparation.

Membrane Preparation. Red beet plasma membrane vesicles were prepared as previously described (3). *Neurospora crassa* plasma membrane vesicles were a gift from Drs. Karl Hager and Carolyn Slayman (Yale University).

Protein Determination. Protein was determined by the method of Schaffner and Weismann (18).

SDS-Gel Electrophoresis. One-dimensional SDS-PAGE was performed according to the method of Laemmli (12). All gels were 10% polyacrylamide. Membrane samples were prepared for electrophoresis by adding 1 volume of membranes (approximately 5 mg protein/ml) to 1 volume of sample buffer consisting of 4.0 ml 50% glycerol, 0.5 ml 0.04% bromophenol blue, 1.0 ml 2-mercaptoethanol, 2.0 ml 20% SDS and 2.5 ml 0.5 M Tris HCl (pH 6.8). Samples were heated at 30°C for 30 min prior to electrophoresis. Low mol wt protein standards were obtained from Bio-Rad Laboratories (Richmond, CA).

Isoelectric Focusing. A modification of the method of O'Farrell (15) for the IEF dimension was used. To prepare 10 tube gels, 5.5 g urea was dissolved in 4.3 ml H_2O, 0.5 ml LKB (pH 3.5–10.0) ampholyte, 1.0 ml acrylamide (38% acrylamide and 2% BIS[*N,N'*-methylene-bis-acrylamide]) and 200 μl 100% (w/v) CHAPS detergent (Bio-Rad Laboratories). The gels were polymerized by the addition of 10 μl 10% ammonium persulfate (w/v) and 7 μl *N,N,N',N'* tetramethylethylenediamine. Red beet plasma membrane samples were prepared for IEF by adding one volume of 2% (w/v) SDS, 1 mM DTT, and 25 mM Tris/Mes (pH 7.0) and centrifuging in a Beckman type 50 rotor at 140,000*g* for 30 min. The supernatant was then concentrated approximately 5-fold in a Centricon microconcentrator (Amicon, Danvers, MA). To the concentrated samples was added 1 volume of glycerol, 1 mg urea per 1 μl of sample, and 0.25 μl of sample concentrate (200 μl 100% [w/v] CHAPS, 50 μl 2-mercaptoethanol), and 100 μl LKB ampholytes (pH 3.5–10.0) per 1 μl of sample. The prepared sample was kept on ice until ready for use. The upper (basic) buffer was 50 mM NaOH and the lower (acidic) buffer was 25 mM H_3PO_4. The IEF gels were prefocused at 200 V for 15 min, 400 V for 30 min, and 800 V for 30 min. Samples were run for 12 h at 800 V. At the end of the run, each IEF tube was equilibrated in 62.5 mM Tris-Cl (pH 6.8), 2% (w/v) SDS, and 2-mercaptoethanol for 30 min. Second dimension (SDS) gel electrophoresis was performed as described above.

[1] Supported by National Science Foundation grant DMB 84-04990.

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[3] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; IEF, isoelectric focusing.

Protein Blotting. Proteins were transferred from gels to nitrocellulose paper at 1.0 A for 1 h in a transfer cell (Hoefer TE 42) with a buffer consisting of 20 mM Tris, 150 mM glycine, 20% (v/v) methanol, and 0.01% (w/v) SDS (8).

H^+-ATPase Antibody. A rabbit polyclonal antibody raised against the plasma membrane H^+-ATPase from *Neurospora crassa* (11) was obtained from K. M. Hager and C. W. Slayman (Yale University).

Immunodetection of the H^+-ATPase on Protein Blots. The nitrocellulose blot was placed in 50 ml of 3% (w/v) BSA in PBS for 1 h, and then into *Neurospora crassa* H^+-ATPase antiserum (500 μl of antiserum diluted in 40 ml of 3% [w/v] BSA in PBS) for 1 h. The blot was washed twice in 100 ml of PBS with 0.025% (v/v) Tween 20 (Bio Rad Laboratories) for 15 min each time, and then briefly rinsed in 100 ml PBS. The blot was then placed into 40 ml of 1% BSA (w/v) PBS with 25 μl of horseradish peroxidase conjugated goat anti-rabbit IgG antibody (Bio Rad Laboratories) for 1 h, and then washed twice (15 min each time) in 100 ml of PBS with 0.025% (v/v) Tween 20. The H^+-ATPase antibody was detected by developing the blot in 100 ml of PBS containing 0.015% (v/v) H_2O_2 and 0.5 mg/ml 4-chloro-1-naphthol. Biotinylated mol wt standards (9) present on the protein blot were used to estimate the mol wt of the H^+-ATPase.

DCCD Labeling and Inhibition of the Plasma Membrane H^+-ATPase. For electrophoretic identification of the DCCD-labeled protein, 500 or 65 μg of membrane protein was diluted in resuspension buffer (250 mM sucrose, 1 mM DTT, and 5 mM Trish/Mes [pH 7.0]) to a final volume of 0.5 ml. [^{14}C]DCCD (0.05 μCi/μl) in ethanol was added to a final concentration of 20 μM (or as indicated). Membranes incubated with [^{14}C]DCCD were placed on a stir plate at 0 or 28°C for 105 min and then pelleted at 140,000 *g* in a Beckman type 50 rotor for 15 min. The membrane pellet was resuspended in resuspension buffer to a final protein concentration of approximately 3.5 mg/ml. This sample was then prepared for SDS-PAGE. After electrophoresis, the gels were impregnated with Enhance (Amersham, Arlington Heights, IL), dried, and exposed to Kodak XAR-5 autoradiograph film at −80°C (13) for 4 to 7 d. Radioactivity in polyacrylamide gel bands was determined by scintillation counting in Liquiscint (National Diagnostics).

Inhibition of the plasma membrane H^+-ATPase by DCCD was as follows. Membrane protein (65 μg) was diluted to 0.5 ml in resuspension buffer. Five μl DCCD in ethanol at the appropriate concentrations was added to the membranes while stirring at 28°C to give the indicated final concentration of DCCD. The membranes in DCCD were incubated for 105 min and the reaction was stopped by adding 5 ml of ice-cold resuspension buffer and pelleting the membranes at 140,000 *g* for 15 min. Membrane pellets were resuspended to approximately 0.75 μg protein/μl. ATPase activity was assayed as previously described (3) and liberated Pi determined by the method of Ames (1).

RESULTS

The effect of DCCD on ATPase activity is shown in Figure 1. As the concentration of DCCD is increased from 0 to 300 μM, the activity of the enzyme in the absence of vanadate decreases rapidly, while ATPase activity in the presence of vanadate remains essentially unchanged over the entire range of DCCD concentrations. This result indicates that it is the vanadate sensitive H^+-ATPase that is inhibited by DCCD (and not some other contaminating ATPase activity such as that of the mitochondria). The I_{50} (concentration of inhibitor resulting in 50% inhibition of enzymic activity) for DCCD inhibition of vanadate-sensitive ATPase activity was approximately 20 μM as calculated by a Dixon-Webb plot (10) shown in the inset of Figure 1. Because ATPase activity was measured after separating membranes from DCCD by centrifugation, the inhibition shown here represents

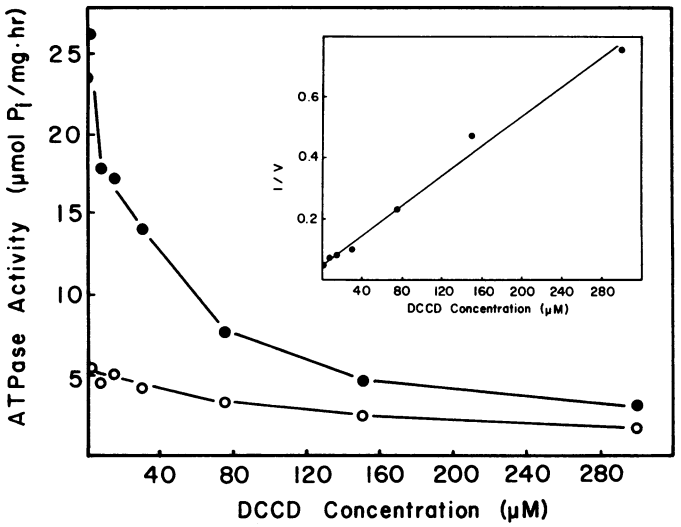

FIG. 1. Concentration dependence of DCCD inhibition of ATPase activity from plasma membrane vesicles of red beet. Membranes (65 μg protein) were incubated in a volume of 500 μl at 28°C for 105 min in the presence of the indicated concentrations of DCCD, pelleted by centrifugation and assayed for ATPase activity in the absence (●) or presence (○) of 50 μM vanadate. The inset shows a Dixon-Webb plot to determine the I_{50} for DCCD inhibition of vanadate sensitive ATPase activity.

irreversible inhibition of the H^+-ATPase, presumably by covalent DCCD binding to the enzyme. This irreversible inhibition would be consistent with the mechanism of DCCD inhibition in other systems (21).

The specificity of DCCD binding is shown in Figure 2. Incubation of plasma membrane vesicles with 20 μM [^{14}C]DCCD at 0°C clearly labels a single protein of approximately 97,000 D. To examine the relationship between DCCD inhibition of the H^+-ATPase and DCCD binding to the 97,000 D protein membrane vesicles were incubated over a range of [^{14}C]DCCD concentrations at 28°C for 105 min. After incubation, the membranes were subjected to SDS-PAGE, Coomassie stained, and fluorographed. When incubated at 28°C DCCD binding is not as specific for the 97,000 D protein band as observed for DCCD binding carried out at 0°C (Fig. 2). At 28°C many polypeptides were labeled with [^{14}C]DCCD, especially at concentration above 7.5 μM; however, the same 97,000 D band specifically labeled at 0°C remained the predominantly labeled band at all DCCD concentration at 28°C. The intensity of the 97,000 D protein band on the fluorograph increased as the concentration of [^{14}C]DCCD was increased (data not shown). The band on the Coomassie gel corresponding to the 97,000 D band on the fluorograph at each [^{14}C]DCCD concentration was cut out of the gel and counted for radioactivity. The cpm in the 97,000 D protein band increased as the [^{14}C]DCCD concentration increased (Fig. 3). This increase in cpm paralleled the increase in inhibition of ATPase activity as the DCCD concentration was increased (Fig. 3).

The identity of the 97,000 D DCCD binding protein as the plasma membrane H^+-ATPase is further supported by cross-reactivity of an antibody raised against the *Neurospora crassa* plasma membrane H^+-ATPase with the 97,000 D protein band. This antibody cross-reactivity was demonstrated by immunoblot analysis of proteins resolved by SDS-PAGE (Fig. 4). The *N. crassa* H^+-ATPase antibody reacts strongly with a 104,000 D protein from *N. crassa* plasma membrane vesicles (lane 5, Fig. 4) and also reacts with a 97,000 D protein from red beet plasma membrane vesicles (lane 4, Fig. 4). A close comparison of [^{14}C]-DCCD labeled protein transferred to nitrocellulose and subjected to both fluorography and immunological detection indicated

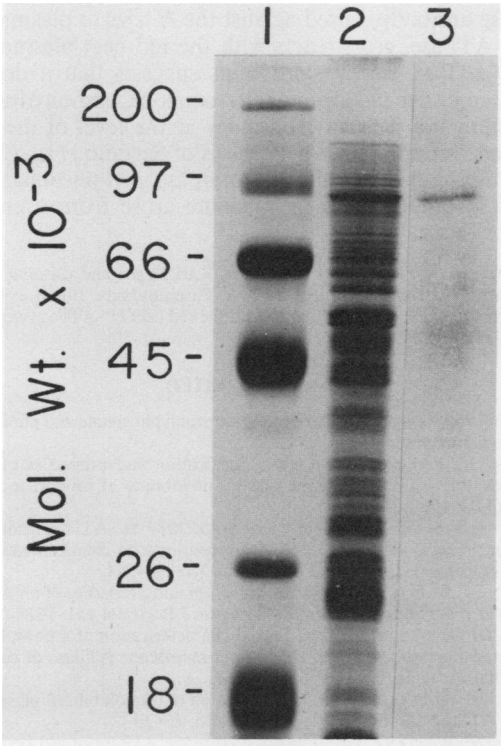

FIG. 2. SDS-PAGE of red beet plasma membrane: Lane 1, mol wt standards; lane 2, Coomassie stained plasma membrane vesicles (approximately 50 μg protein); lane 3, fluorograph of [^{14}C]DCCD labeled plasma membrane vesicles (approximately 200 μg protein). [^{14}C]DCCD labeling was carried out in a 500 μl volume with 500 μg membrane protein and 20 μM DCCD (20 nmol DCCD/mg protein) at 0°C for 105 min. Film was exposed for 4 d at −80°C.

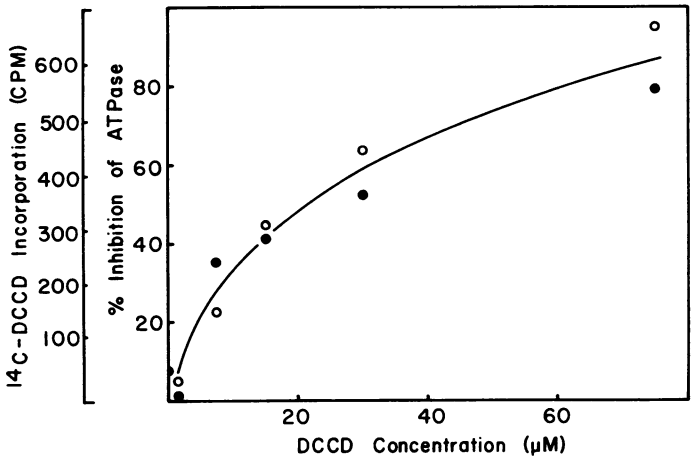

FIG. 3. [^{14}C]DCCD binding to a 97,000 D plasma membrane protein (○), and DCCD inhibition of vanadate sensitive ATPase activity (●), both plotted as a function of DCCD concentration. Membranes (65 μg protein) were incubated for 105 min at 28°C in [^{14}C]DCCD, subjected to SDS-PAGE, Coomassie stained, and fluorographed. The band on the Coomassie stained gel corresponding to the 97,000 D protein band on the fluorograph was cut out and radioactivity was determined by scintillation counting. The data for ATPase inhibition is replotted from Figure 1.

that an identical protein bands bind [^{14}C]DCCD and *N. Crassa* H+-ATPase antibody. The immunoblot analysis shows that the red beet plasma membrane H+-ATPase has a lower mol wt than the *N. crassa* H+-ATPase. In both cases, the antibody-reactive protein band corresponds to a prominent Coomassie-stained

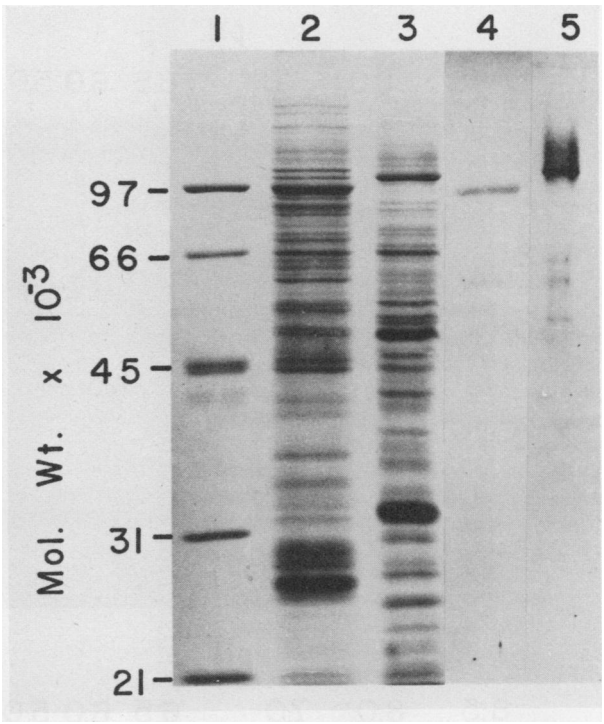

FIG. 4. SDS-PAGE of *N. crassa* and red beet plasma membrane vesicles. Lane 1, mol wt standards. Lanes 2 and 3, Coomassie stained plasma membrane protein of red beet and *N. crassa*, respectively. Lanes 4 and 5, immunodetected protein blot of red beet and *N. crassa*, respectively, using the *N. crassa* H+-ATPase antiserum.

protein band of the same mol wt (lanes 2 and 3, Fig. 4). This suggests that the H+-ATPase is a significant component of the plasma membrane of both organisms.

The *N. crassa* H+-ATPase antibody was also used to identify the isoelectric point (pI) of the red beet plasma membrane H+-ATPase by protein blotting and immunodetection of a two-dimensional gel of these membranes (Fig. 5B). The results indicate that the pI is approximately 6.5. Comparison of the immunoblot with the two-dimensional Coomassie stained gel suggests that the protein spot indicated by the arrow in Figure 5A represents the H+-ATPase. Attempts to confirm the isoelectric point by two-dimensional gel electrophoresis of the [^{14}C]DCCD labeled H+-ATPase were unsuccessful for reasons that remain unclear. We suspect that the presence of urea and/or ampholytes may destabilize the *N*-acylurea complex formed by DCCD binding to the H+-ATPase during IEF.

DISCUSSION

Through the use of [^{14}C]DCCD and a polyclonal antibody raised against the *Neurospora crassa* plasma membrane H+-ATPase we have determined a mol wt of 97,000 D for the red beet storage tissue plasma membrane H+-ATPase. This value is similar to the values reported by [γ-^{32}P]ATP labeling studies and by some purification schemes (2, 5, 7, 19, 25). We can attribute the slight differences to either the variation inherent in mol wt standards and the method of SDS gel electrophoresis for estimating mol wt, or to possible proteolytic damage to the enzyme during preparation for electrophoresis. In one experiment, red beet plasma membrane vesicles were prepared in the presence of 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml chymostatin. The use of these protease inhibitors did not serve to increase the mol wt of the H+-ATPase as detected by immunoblotting.

The H+-ATPase isoelectric point of approximately 6.5 identified by immunodetection of a two-dimensional Western blot is

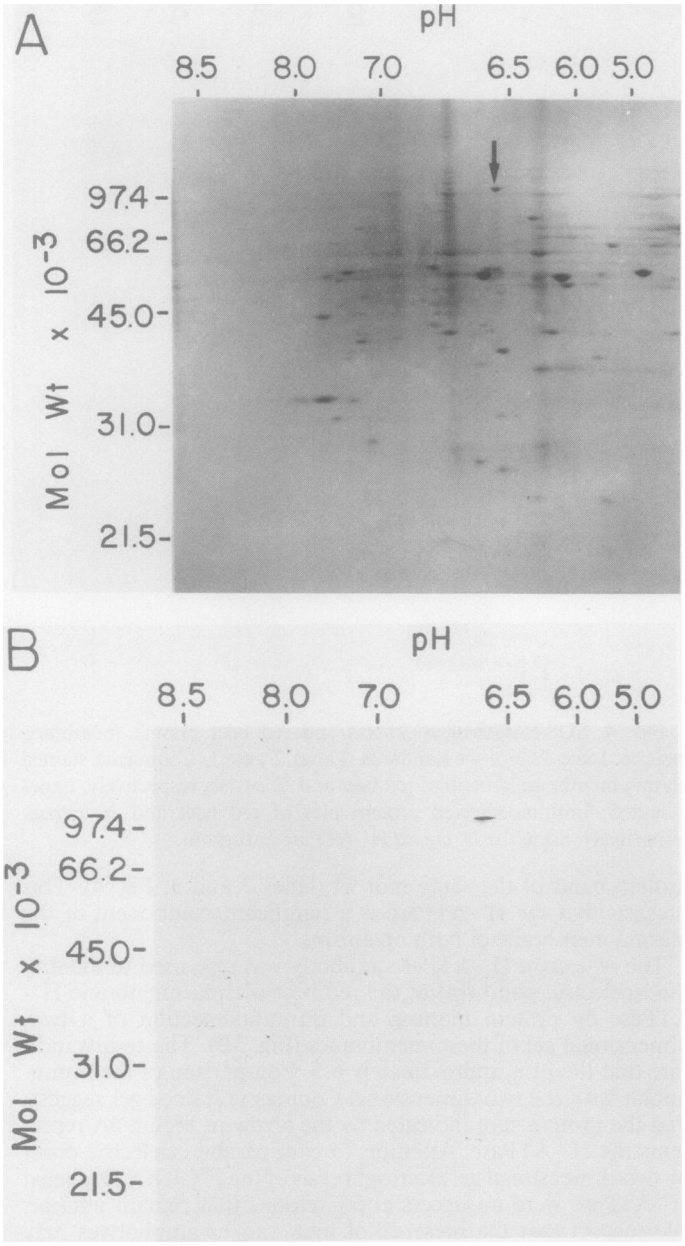

FIG. 5. Two-dimensional gel electrophoresis of red beet plasma membrane vesicles. A, Coomassie stained gel (approximately 100 μg protein). The H^+-ATPase is indicated by the arrow. B, Immunodetected protein blot using the *N. crassa* H^+-ATPase antiserum.

interesting since the pH optimum for this enzyme is also 6.5 (3). To our knowledge, this is the first report identifying a plant plasma membrane protein on a two-dimensional gel.

In other experiments, the H^+-ATPase antibody was used in an attempt to purify the red beet plasma membrane H^+-ATPase. The antibody was covalently bound to CNBr-activated Sepharose. The H^+-ATPase (solubilized in 0.1% [w/v] Zwittergent 3-14 [Behring Diagnostics, San Diego, CA] and 25% [v/v] ethylene glycol) was passed over the column but was not retained. In addition, attempts to immunoprecipitate the solublized H^+-ATPase with the antibody proved unsuccessful. We suspect that since the antibody was raised against the SDS denatured *N. crassa* H^+-ATPase, it simply does not recognize the red beet plasma membrane H^+-ATPase in its native form. However, the fact that the H^+-ATPase antibody, raised against the *N. crassa* plasma membrane H^+-ATPase, cross-reacts with the red beet plasma membrane H^+-ATPase on Western blots suggests that a degree of homology in either the amino acid sequence, carbohydrate residues, or both, must exist. Homology at the level of the amino acid sequence supports the hypothesis of Serrano *et al.* (20) that cation pumping ATPases that form an aspartyl phosphate intermediate and are sensitive to vanadate arose from a common ancestral ATPase.

Acknowledgment—The authors thank Drs. Karl Hager and Carolyn Slayman for their generous gift of the *N. crassa* H^+-ATPase antibody. Initial experiments indicating cross-reaction of this antibody with the red beet H^+-ATPase were carried out by Dr. K. Hager.

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