Localization of a carboxylic residue possibly involved in the inhibition of vacuolar H$^+$-pyrophosphatase by *N,N′*-dicyclohexylcarbodi-imide

Su J. YANG*, Shih S. JIANG†, Soong Y. KUO†, Shu H. HUNG†, Ming F. TAM‡ and Rong L. PAN†[1]

Department of Radiological Technology, Chungtai Institute of Health Science and Technology, Taichung 40605, Taiwan, Republic of China, †Department of Life Science, College of Life Science, National Tsing Hua University, Hsin Chu 30043, Taiwan, Republic of China, and ‡Institute of Molecular Biology, Academica Sinica, Nankang, Taipei 11529, Taiwan, Republic of China

A vacuolar H$^+$-pyrophosphatase (EC 3.6.1.1) that catalyses PP_i hydrolysis and the electrogenic translocation of protons from the cytosol to the vacuole lumen, was purified from etiolated hypocotyls of mung bean seedlings (*Vigna radiata* L.). Group-specific modification was used to identify a carboxylic residue involved in the inhibition of vacuolar H$^+$-pyrophosphatase. Carbodi-imides, such as *N*,*N′*-dicyclohexylcarbodi-imide (DCCD) and 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide, and Woodward's reagent K caused a progressive decline in the enzymic activity of vacuolar H$^+$-pyrophosphatase in a time- and concentration-dependent manner. The stoichiometry of labelling of the vacuolar H$^+$-pyrophosphatase by [^{14}C]DCCD determined that DCCD modifies one carboxylic residue per subunit of the enzyme. Protection studies suggest that the DCCD-reactive carboxylic residue resides at or near the substrate-binding site. Furthermore, peptide mapping analysis reveals that Asp^{283}, located in the putative loop V of a tentative topological model of vacuolar H$^+$-pyrophosphatase on the cytosolic side, was labelled by radioactive [^{14}C]DCCD. Cytosolic loop V contains both DCCD-sensitive Asp^{283} and a conserved motif sequence, rendering it a candidate for the catalytic site of vacuolar H$^+$-pyrophosphatase. A topological picture of the active domain of vacuolar H$^+$-pyrophosphatase is tentatively proposed.

Key words: chemical modification, peptide mapping, vacuole.

INTRODUCTION

Tonoplasts of higher plants contain two proton-pumping enzymes, a vacuolar H$^+$-ATPase (EC 3.6.1.3) and an H$^+$-pyrophosphatase (H$^+$-PPase, EC 3.6.1.1) [1,2]. Both enzymes are essential in the maintenance and regulation of cell turgor and in the storage and secondary transport of ions, sugars and metabolites [3–6]. Vacuolar H$^+$-PPase has been found solely in higher plants and is recognized as a new type of ion translocator that uses exclusively PP_i as energy source [1,2]. Vacuolar H$^+$-PPase is composed of a single polypeptide chain of approx. 64–75 kDa as measured by conventional biochemical methods and approx. 80–81 kDa as predicted from the open reading frame of the DNA sequence [7–10]. It was also demonstrated that vacuolar H$^+$-PPase functions as a homodimer during proton translocation and PP_i hydrolysis as determined by radiation inactivation [11,12]. The properties of the cDNA sequence and the deduced amino acid sequence of vacuolar H$^+$-PPase from *Arabidopsis thaliana*, *Hordeum vulgare*, *Beta vulgaris*, *Vigna radiata*, *Nicotiana tabacum* and *Oryza sativa* L. have been reported [13–18], revealing that vacuolar H$^+$-PPase is a unique proton translocase different from other known proton-pumping enzymes and is distinct in structure and function from other soluble PPases. Hydropathic plots predict that vacuolar H$^+$-PPase is composed of 13–15 transmembrane spans, with its C-terminus on the cytoplasmic side [13,14,19]. Many soluble PPases, such as those from the yeast cytosol, *Escherichia coli*, rat liver cytosol and mitochondrial matrix, have been well characterized and sequenced [20–24]. Many essential amino acid residues involved in the enzymic reaction of soluble PPases have been demonstrated [25–28]. A putative substrate-binding site accommodating a conserved motif D/E(X)$_7$KXE in loop V of vacuolar H$^+$-PPase [29] is suggested from site-directed mutagenesis studies and the analysis of alignments for all the sequenced soluble PPases [20] and those from vacuolar H$^+$-PPases [13–18,29].

The structure–function relationship of vacuolar H$^+$-PPase has been studied extensively by conventional chemical modification and/or site-directed mutagenesis [10,19,30–32]. It has been shown that vacuolar H$^+$-PPase contains an arginine residue and a tyrosine residue crucial to enzymic activity [10,30]. A cytosolically oriented substrate-protectable cysteine residue was further shown to be involved in the inhibition of enzymic activity by *N*-ethylmaleimide, but not to participate in the catalytic reaction [31,32]. Evidence for the necessity of several acidic residues to vacuolar H$^+$-PPase was also demonstrated by site-directed mutagenesis and the employment of a group-specific modifier, *N*,*N′*-dicyclohexylcarbodi-imide (DCCD) [9,19]. However, the exact location of the DCCD-modifiable acidic residue(s) has not been directly identified. In the present study we showed that Asp^{283} of vacuolar H$^+$-PPase, located on loop V, was specifically labelled by [^{14}C]DCCD. Our results indicate that Asp^{283} is involved in the inhibition by DCCD and is probably located at or near the active site of vacuolar H$^+$-PPase. A structural diagram of the active domain of vacuolar H$^+$-PPase is accordingly proposed.

MATERIALS AND METHODS

Plant materials

Seeds of mung bean (*Vigna radiata* L.) were soaked overnight in tapwater and then germinated at room temperature in the dark for 4 days. Hypocotyl tissues of etiolated seedlings were excised, chilled on ice and then used as starting plant materials.

Abbreviations used: AVP, *Arabidopsis* vacuolar H$^+$-PPase; DCCD, *N*,*N′*-dicyclohexylcarbodi-imide; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide; PPase, pyrophosphatase.

[1] To whom correspondence should be addressed (e-mail rlpan@life.nthu.edu.tw).

Preparation of tonoplasts

Preparation of tonoplasts was performed by the method of Maeshima and Yoshida [9], with minor modifications. Hypocotyls from mung bean seedlings were homogenized with a Polytron in homogenization medium [50 mM Mops/KOH (pH 7.6)/0.25 M sorbitol/5 mM Tris/EGTA/1 mM PMSF/1.5 % (w/v) poly(vinyl pyrrolidone)/1 % (w/v) ascorbic acid] with a ratio of tissue fresh weight to medium of 1:1.3 (w/v). The homogenate was filtered and subjected to differential centrifugation at 3600 ***g*** for 10 min and 120000 ***g*** for 20 min. The precipitate was resuspended in suspension medium [10 mM potassium phosphate (pH 7.8)/0.3 M sucrose/1 mM Tris/EGTA/2 mM dithiothreitol (DTT)]. The suspension was overlaid on a medium containing 5 mM Mops/KOH, pH 7.3, 0.25 M sorbitol, 1 mM Tris/EGTA and 2 mM DTT. After centrifugation at 120000 ***g*** for 30 min, the interface portion was collected and diluted with a solution containing 5 mM Mops/KOH, pH 7.3, 0.25 M sorbitol, 1 mM Tris/EGTA and 2 mM DTT. The suspension was centrifuged at 130000 ***g*** for 20 min; the resulting white pellet (vacuolar vesicles) was then resuspended in a medium containing 20 mM Tris/Mes, pH 7.5, 20 % (v/v) glycerol, 1 mM DTT, 1 mM Tris/EGTA and 2 mM $MgCl_2$ at a protein concentration of 3 mg/ml.

Purification of vacuolar H^+-PPase

Vacuolar H^+-PPase was purified from tonoplast membrane by methods modified from those of Maeshima and Yoshida [9] and Britten et al. [7], as detailed below. Tonoplast vesicles were centrifuged at 120000 ***g*** for 1 h and the pellet was resuspended in buffer I [20 mM Tris/acetate (pH 7.5)/20 % (v/v) glycerol/1 mM DTT/1 mM Tris/EGTA/2 mM $MgCl_2$] at a protein concentration of 1 mg/ml. Sodium deoxycholate and KCl were added to final concentrations of 2 mg/mg of protein and 50 mM respectively. After incubation for 10 min at 4 °C, deoxycholate-treated tonoplast vesicles were centrifuged at 150000 ***g*** for 30 min. The membrane pellet was redissolved in buffer I (14 ml) and solubilized by 0.4 % (w/v) L-α-lysophosphatidylcholine for 30 min at room temperature. After centrifugation at 150000 ***g*** for 50 min, the supernatant (5–10 mg of protein) was concentrated to 200 μl and then applied to a FPLC column of Superose 12 HR 10/30 pre-equilibrated with the running buffer [20 mM Tris/HCl (pH 7.5)/20 % (v/v) glycerol/0.1 % (w/v) Triton X-100/1 mM Tris/EGTA/2 mM $MgCl_2$]. The flow rate was 0.4 ml/min; chromatography was conducted at room temperature. Fractions (2.5 ml) with PPase activity were collected and then applied to a Mono Q HR 5/5 FPLC column pre-equilibrated with running buffer. The column was eluted at a flow rate of 0.5 ml/min with a salt gradient (25 ml) of 0–0.5 M NaCl. Fractions (1 ml) with highest activity were collected and stored immediately at −70 °C for later use.

Enzyme assay and protein determination

PPase activities were determined by measuring the release of P_i from PP_i. Aliquots of purified H^+-PPase (2–5 μg) were incubated in a 1.0 ml reaction medium at 33 °C for 15 min. The reaction solution included 25 mM Mops/KOH, pH 7.2, 1 mM $MgSO_4$, 50 mM KCl, 1 mM potassium pyrophosphate, 80 μg/ml phosphatidylcholine and 0.02 % (w/v) Triton X-100. After incubation, the reaction was terminated by adding the stop solution [1.7 % (w/v) ammonium molybdate/2 % (w/v) SDS/0.02 % 1-amino-2-naphthol-4-sulphonic acid]. The released P_i was determined spectrophotometrically (SLM-Aminco U2000) as described elsewhere [33,34].

Protein concentration was measured by a modified Lowry method [35] with BSA as the standard.

Chemical modification and labelling stoichiometry

Purified H^+-PPase (10 μg) was incubated with carboxy-group modifiers in the medium containing 50 mM Mops/KOH, pH 7.2, 20 % (v/v) glycerol, 1 mM EGTA and 1 mM DTT at 33 °C for the durations indicated. $MgSO_4$ and protection agents were added (when present) as described in the figure legends. The concentration of ethanol carried over into the incubation mixture was kept below 5 % (v/v), at which concentration it had no effect on enzymic activity. At the end of the incubation, the enzyme activity was assayed immediately. In contrast, the reaction mixture of [^{14}C]DCCD-labelled H^+-PPase was stopped by dilution 1:30 with 6 % (w/v) trichloroacetic acid. After incubation on ice for 30 min, the solution was centrifuged at 10000 ***g*** for 5 min. The pellet was collected for further stoichiometry studies or as the starting material for peptide mapping analysis. Radioactivity was measured with a Packard liquid-scintillation analyser, model 1600CA. The stoichiometry of modification was determined on the basis of an assumed molecular mass of PPase of 145 kDa [10].

Proteolysis and peptide separation by HPLC

After incubation with [^{14}C]DCCD, the PPase (1 mg) was precipitated with trichloroacetic acid or passed through a Sephadex G-25 column to remove the free modifier. The solution was then concentrated and equilibrated in 0.1 M ammonium bicarbonate buffer (pH 7.8) to bring it directly to the conditions for tryptic digestion. The digestion was performed for 12–16 h at 37 °C by trypsin treated with tosylphenylalanylchloromethane ('TPCK') at a trypsin-to-PPase ratio of 1:100 (w/w). After digestion, the mixture was concentrated to a volume of approx. 70 μl with a Speed-Vac, filtered through a 0.45 μm pore-size membrane filter and finally analysed on a reverse-phase HPLC column (Vydac C_{18} analytical column, 2.1 mm × 25 cm) with a Shimadzu HPLC system. A flow rate of 0.2 ml/min was used to deliver a linear gradient from 0 % to 100 % (v/v) solvent B over 100 min at room temperature. For all HPLC separations, solvent A was 0.08 % (v/v) trifluoroacetic acid in water and solvent B was 0.05 % (v/v) trifluoroacetic acid in acetonitrile. A second HPLC run was performed with a linear 20–50 % (v/v) acetonitrile gradient over 150 min. Eluted peptides were monitored by A_{220} with a Shimadzu UV–visible spectrophotometer. Peptide fractions containing radioactivity were collected, then concentrated with a Speed-Vac. Amino acid analysis was performed with an amino acid analyser (Applied Biosystems Model 473A).

Chemicals

DCCD was purchased from Sigma; [^{14}C]DCCD (56 mCi/mmol) was from Amersham. All other chemicals were of analytical grade and were used without further purification.

RESULTS

Inactivation of H^+-PPase by carbodi-imides

Vacuolar H^+-PPase was purified from the tonoplasts of etiolated mung bean seedlings by a two-step detergent solubilization and a subsequent series of chromatographies, as described previously [10–12]. A highly purified vacuolar H^+-PPase, with only a single polypeptide of 73 kDa visible on SDS/PAGE, was obtained (results not shown; see [10–12]). Incubation of purified H^+-

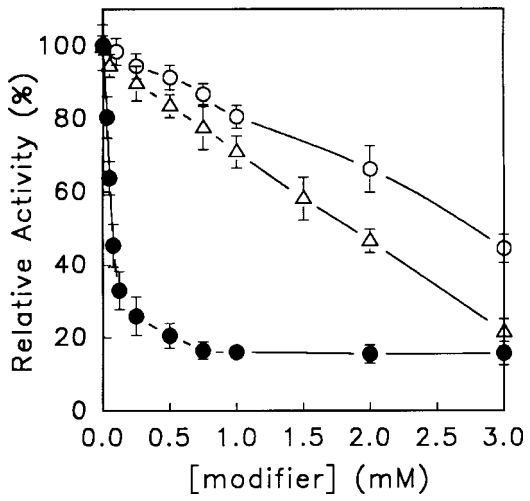

Figure 1 Inactivation of vacuolar H+-PPase by carboxy-group modifiers

Purified vacuolar H+-PPase (5 μg) was incubated at 33 °C for 10 min in Mops/KOH buffer, pH 7.2, with various concentrations of carboxy-group modifiers. At the end of the incubation, aliquots were removed and assayed for enzymic activity as described in the Materials and methods section. The control activity was approx. 180.3 μmol of PP_i consumed/h per mg of protein. Symbols: ●, DCCD; ○, EDC; △, Woodward's reagent K. Results are means ± S.D. for at least three independent experiments.

PPases with carboxy-group modifiers resulted in a marked loss of enzyme activity, in a concentration-dependent manner (Figure 1). Of the carboxy-group modifiers, hydrophobic carbodi-imide DCCD caused a striking abolition of the enzymic activity of vacuolar H+-PPase at concentrations higher than 1 mM. Further investigation indicated that the inhibition of vacuolar H+-PPase by DCCD was also time-dependent (results not shown). Prolonged incubation (more than 4 min) of vacuolar H+-PPase with 1.0 mM DCCD inhibited its enzymic activity completely. In contrast, hydrophilic analogues of carbodi-imide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide (EDC) and Woodward's reagent K, exerted a smaller inhibitory effect on purified vacuolar H+-PPase. We therefore employed DCCD as the modifier of carboxylic residues of vacuolar H+-PPase in subsequent studies.

Protection against inactivation

Vacuolar H+-PPase could be protected from inhibition by DCCD by magnesium pyrophosphate, indicating that the modifier competed with the physiological substrate for the target residue. Table 1 summarizes the protective effects of substrate analogues (such as *p*-nitrophenyl phosphate and glucose 6-phosphate) and the product (P_i) of vacuolar H+-PPase. Substrate analogues also provided protection against inhibition by DCCD, although less than that afforded by PP_i. The presence of Mg^{2+} was crucial for the enzyme's protection by substrate and substrate analogues against inhibition by DCCD. However, the product (P_i) exerted a negligible protective effect towards vacuolar H+-PPase. Protection by ATP, ADP and AMP, all containing a phosphate moiety similar to PP_i at one end of the molecule, was examined. Their protective effect followed the decreasing order AMP > ADP > ATP, probably owing to steric hindrance at the active cleft of the enzyme. From the foregoing, we believe that the DCCD-reactive carboxylic residue might be located at or near the active site of the enzyme.

Table 1 Protection of PPase activity against inactivation by DCCD

Purified PPase (0.6 mg/ml) was incubated with 0.1 mM DCCD for 10 min at 33 °C in a medium containing 5 mM protectors in the absence or presence of 5 mM $MgSO_4$. Conditions for further PPase activity assay were as described in the Materials and methods section. The percentage protection was calculated from the equation:

Protection (%) = $100(SA_{protected} - SA_{unprotected})/(SA_{control} - SA_{unprotected})$

where SA denotes specific activity. Results are means ± S.D. for at least three independent experiments.

Ligand	Activity as substrate ($\mu m \cdot h^{-1} \cdot mg$ of protein)	Activity as substrate (% of control)	Protection (%) + $MgSO_4$	Protection (%) − $MgSO_4$
Potassium pyrophosphate	180.0	100	80.2 ± 7.3	3.9 ± 10.5
p-Nitrophenyl phosphate	8.25	4.58	48.1 ± 6.5	16.6 ± 5.2
Glucose 6-phosphate	9.72	5.40	61.4 ± 8.4	< 1
P_i	–	–	< 1	< 1
ATP	10.59	5.88	27.9 ± 4.6	4.3 ± 3.8
ADP	16.37	9.10	34.9 ± 7.1	15.4 ± 5.2
AMP	< 1	–	61.9 ± 8.5	10.0 ± 5.9

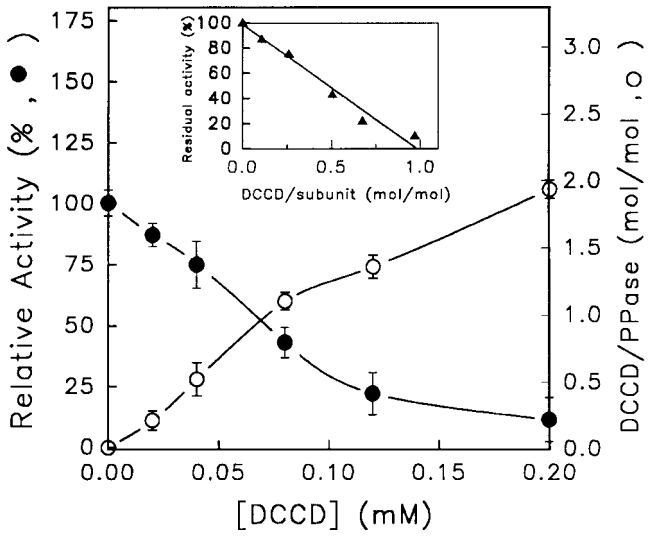

Figure 2 Stoichiometry of labelling of vacuolar H+-PPase with [14C]DCCD

Purified vacuolar H+-PPase (20 μg) was incubated with various concentrations of [14C]DCCD in 50 mM Mops/KOH buffer, pH 7.2, at 33 °C for 10 min as described in the Materials and methods section. The enzyme concentration was measured by the modified Lowry method, taking 145 kDa as the molecular mass. Relative activities (●) and the numbers of moles of DCCD incorporated per mole of PPase (○) are plotted against the concentration of DCCD. The control activity was approx. 182.5 μmol of PP_i consumed/h per mg of protein. Results are means ± S.D. for at least three independent experiments. The insert is a plot of the residual activity against [14C]DCCD labelling of the enzyme.

Stoichiometry of labelling

The identification and location of the modified residue were further determined by peptide mapping analysis. PPase activity was inhibited to different degrees by various concentrations of [14C]DCCD; the extent of radioactive labelling was determined (Figure 2). The degree of incorporation of radioactivity was correlated with a progressive loss of vacuolar H+-PPase activity. The enzymic activity of H+-PPase was completely inhibited by the incorporation of 1.78 mol of DCCD/mol of PPase or 0.89 mol of DCCD/mol of PPase subunit, taking the molecular

Table 2 Amino acid sequence of the [^{14}C]DCCD-labelled peptide

Peptide fractions with ^{14}C radioactivity from HPLC were collected for amino acid sequencing. The amino acid sequence of the DCCD-labelled fragment and its similarity to those of the vacuolar H^+-PPases from other species are shown.

DCCD-labelled fragment		NPAVI	F	DN	K	V	XX	NVG	
	Trypsin cut ↓								
Vigna radiata	^{272}R	NPAVI	A	DN		V	GD	NVG	286
Beta vulgaris	^{270}R	NPAVI	A	DN		V	GD	NVG	284
Oryza sativa	^{277}R	NPAVI	A	DN		V	GD	NVG	291
Arabidopsis thaliana	^{276}R	NPAVI	A	DN		V	GD	NVG	290
Nicotiana tabacum	^{271}R	NPAVI	A	DN		V	GD	NVG	285
Hordeum vulgare	^{267}R	NPAVI	A	DN		V	GD	NVG	281

mass of purified vacuolar H^+-PPase as 145 kDa. This result indicates that the incorporation of approx. 1 mol of DCCD/mol of subunit was sufficient to abolish the activity of vacuolar H^+-PPase completely.

Identification of the DCCD-modified peptide

[^{14}C]DCCD-modified enzyme was digested with tosylphenylalanylchloromethane-treated trypsin for 12–16 h in 0.1 M ammonium bicarbonate buffer, pH 7.8. The resulting tryptic digest was subjected to C_{18} reverse-phase column chromatography for peptide separation. Only a single peak with radioactivity was obtained, at 40 % (v/v) acetonitrile, confirming the stoichiometry of the DCCD-binding ratio as approx. 1 mol of DCCD/mol of PPase subunit (results not shown). After a second HPLC run, the amino acid sequence of the DCCD-labelled peptide was determined with an amino acid analyser; the sequence is shown in Table 2. The radioactively labelled residue is assigned as Asp^{283}. The sequence of this radioactively labelled fragment closely matches that of residues 273–286 of vacuolar H^+-PPase from mung bean (*Vigna radiata*), with an N-terminal sequence compatible with cleavage on the carboxy side of Arg^{272}. In addition, this DCCD-labelled fragment is highly similar to those of the PPase family from other species, as shown in Table 2. Figure 3 further shows that residues 273–286 are located cytosolically at putative loop V of a tentative topological model of vacuolar H^+-PPase adapted from the prediction made by Zhen et al. [19]. Taking these results together, we conclude that Asp^{283} is probably the target residue responsible for the inhibition and chemical labelling of H^+-PPase by DCCD.

DISCUSSION

The carboxylic residue modifier DCCD causes a progressive inhibition in the enzymic activity of vacuolar H^+-PPase in a time- and concentration-dependent manner. Labelling stoichiometry indicates that the incorporation of 1 mol of DCCD/mol of subunit suffices to abolish the enzymic activity of vacuolar H^+-

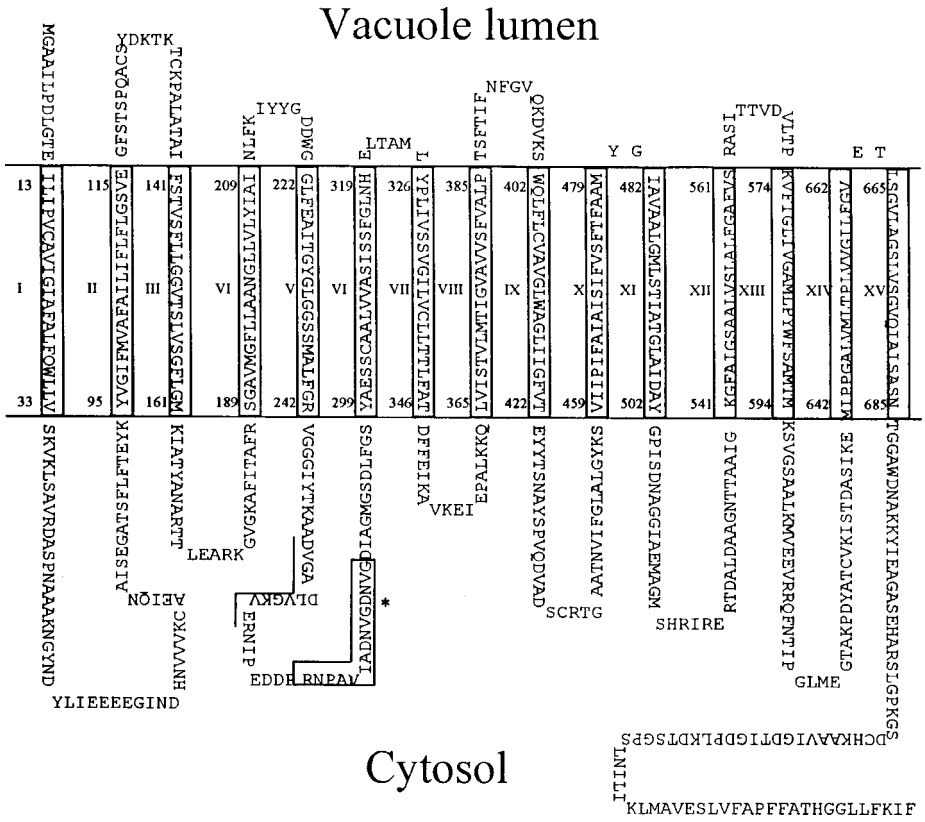

Figure 3 Tentative location of the DCCD-labelled peptide fragment of vacuolar H^+-PPase

This tentative topological model of mung bean vacuolar H^+-PPase is adapted from the prediction made by Zhen et al. for the enzyme from *Arabidopsis* [19]. The DCCD-labelled fragment in putative cytosolic loop V is shown in a box, with an asterisk at the DCCD-reactive Asp^{283}. The conserved D(X)$_7$KXE motif in loop V is underlined.

PPase. Protection studies suggest that the DCCD-reactive carboxylic residue might reside at or near the substrate-binding site of vacuolar H^+-PPase. Furthermore, peptide mapping analysis reveals that Asp^{283} of vacuolar H^+-PPase from mung bean, located cytosolically in the putative loop V of a currently accepted model, was labelled by radioactive [^{14}C]DCCD. Putative loop V on the cytosolic side is therefore tentatively assigned as the substrate-binding domain of vacuolar H^+-PPase.

Analysis of the amino acid sequence of putative loop V as follows renders it a plausible candidate for the catalytic site of vacuolar H^+-PPase; structural features of the possible active domain are accordingly described.

(1) The putative hydrophilic loop V encompassing Asp^{283} faces the cytosolic side of vacuolar H^+-PPase [19]. This orientation assignment is confirmed by the inhibition of vacuolar H^+-PPase by water-soluble, membrane-impermeable carbodi-imide analogues. EDC and Woodward's reagent K inhibit the enzymic activities of both membrane-bound and purified H^+-PPases in a substrate-protectable and competitive manner for a common binding site (results not shown).

(2) Putative loop V contains a conserved motif sequence, $D/E(X)_7KXE$, found in most PP_i hydrolases [29]. X-ray crystallographic and site-directed mutagenesis analyses have revealed the striking result that soluble PPases from many species contain 24 conserved residues; 17 of these residues are polar and possibly involved in enzyme catalysis, as suggested by Rea et al. [29]. These potentially important residues are accommodated in two configurations, $E(X)_7KXE$ and $D(X)_4DXK(X)_4D$, beginning at positions 48 and 146 of the enzyme from *Saccharomyces* [29]. Variants of both motifs are found at loops V and II, beginning at positions 243 and 116 respectively in the deduced amino acid sequence of *Arabidopsis* vacuolar H^+-PPase (AVP) [13]. Although the vacuolar H^+-PPase and soluble PPases seem to be evolutionarily remote, they might exhibit similar motifs related to the need for both types of PPase to interact with the same substrate and cofactors. However, comparison studies on deduced amino acid sequences of the polypeptide encoded by AVP and those from other species, such as *H. vulgare*, *B. vulgaris*, *V. radiata*, *N. tabacum* and *O. sativa* L. [14–18], demonstrate that the $D/E(X)_4DXK(X)_4D$ motif of loop II in the sequence of AVP is not conserved. Nevertheless, the $D(X)_7KXE$ motif of loop V is found in vacuolar H^+-PPases of all species. Furthermore, loop II of AVP is on the luminal side of the vacuole, even though it has been demonstrated by site-directed mutagenesis that this loop might participate directly in catalysis, as proposed by Kim et al. [31]. Therefore the $D/E(X)_7KXE$ motif-containing loop V is the most reasonable candidate for containing the catalytic site. The DCCD-binding site, Asp^{283} (in the sequence of the *V. radiata* enzyme), is encompassed in putative loop V and is close to the $D/E(X)_7KXE$ motif. Therefore the Asp^{283} residue might be essential to the function of vacuolar H^+-PPase.

(3) The putative hydrophilic loop V contains 41.8% of the spacing and alternating acidic and basic amino acid residues. This region probably folds into a suitable active pocket for interaction with substrate, inhibitor and activators of vacuolar H^+-PPase. The DCCD-reactive Asp^{283} residue itself might provide a negatively charged side chain to stabilize the Mg^{2+}–PP_i complex in the active site for the enzymic reaction.

(4) The Asp^{283}-containing loop V is a large hydrophilic loop on the cytosolic side. It seems reasonable to assume that this bulky fragment, and therefore the Asp^{283} residue, has a crucial role in the structure and function of vacuolar H^+-PPase.

(5) Several carboxylic residues located near or within transmembrane spans were examined by site-directed mutagenesis for a possible involvement in PP_i-dependent proton pumping and the inhibition of AVP by DCCD [19]. The results predict that Glu^{305} and Asp^{504} of the V-PPase were probably responsible for the inhibitory DCCD binding. However, our own results indicate directly that Asp^{283} (Asp^{287} in AVP) is the sole target residue for [^{14}C]DCCD labelling. Nevertheless, Glu^{305} and Asp^{287} (Glu^{301} and Asp^{283} in *V. radiata* respectively) are encompassed within the same trypsin-digested fragment. We could not exclude the possibility of the involvement of Glu^{301} in the inhibition of enzymic activity by DCCD. We suggest that Asp^{283} of *V. radiata* is the preferential target for DCCD and/or that its modification might exert a remote effect on nearby residues, including Glu^{301}, within this loop.

(6) An analysis of the helix wheel shows that transmembrane fragments of loops III, V and XIV contain mainly hydrophilic amino acid residues, which are clustered asymmetrically on one side of the helix (results not shown). We speculate that these transmembrane helices might form the proton channel coupling to loop V as the active domain. This requires verification.

Taking these results together, we conclude that Asp^{283} is probably the target residue responsible for the inhibition and chemical labelling of H^+-PPase by DCCD and that the Asp^{283}-containing loop V might fold into an active cage for catalytic reactions.

This work was supported by a grant from the National Science Council, Republic of China (NSC 87-2311-B-007-009) to R.L.P.

REFERENCES

1 Rea, P. A. and Poole, R. J. (1993) Annu. Rev. Plant Physiol. Mol. Biol. **44**, 157–180
2 Barkla, B. J. and Pantoja, O. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**, 159–184
3 Rea, P. A. and Sanders, D. (1987) Physiol. Plant. **71**, 131–141
4 Meis, L. D. (1989) Biochim. Biophys. Acta **973**, 333–349
5 Matsuura-Endo, E. C., Maeshima, M. and Yoshida, S. (1990) Eur. J. Biochem. **187**, 745–751
6 Sze, H., Ward, J. M. and Lai, S. P. (1992) J. Bioenerg. Biomemb. **24**, 371–381
7 Britten, C. J., Turner, J. C. and Rea, R. A. (1989) FEBS Lett. **256**, 200–206
8 Sarafian, V. and Poole, R. J. (1989) Plant Physiol. **91**, 34–38
9 Maeshima, M. and Yoshida, S. (1989) J. Biol. Chem. **264**, 20068–20073
10 Yang, S. J., Jiang, S. S., Tzeng, C. M., Kuo, S. Y., Hung, S. H. and Pan, R. L. (1996) Biochim. Biophys. Acta **1294**, 89–97
11 Tzeng, C. M., Yang, C. Y., Yang, S. J., Jiang, S. S., Kuo, S. Y., Hung, S., Ma, J. T. and Pan, R. L. (1996) Biochem. J. **316**, 143–147
12 Yang, S. J., Ko, S. J., Tsai, Y. R., Jiang, S. S., Kuo, S. Y., Hung, S. and Pan, R. L. (1998) Biochem. J. **331**, 395–402
13 Sarafian, V., Kim, Y., Poole, R. J. and Rea, P. A. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1775–1779
14 Tanaka, Y., Chiba, K., Maeda, M. and Maeshima, M. (1993) Biochem. Biophys. Res. Commun. **181**, 962–967
15 Kim, Y., Kim, E. J. and Rea, P. A. (1994) Plant Physiol. **106**, 375–382
16 Hung, S. H., Chiu, S. J., Lin, L. Y. and Pan, R. L. (1995) Plant Physiol. **109**, 1125–1127
17 Lerchl, J., Konig, S., Zrenner, R. and Sonnewald, U. (1995) Plant Mol. Biol. **29**, 833–840
18 Sakakibara, Y., Kobayashi, H. and Kasamo, K. (1996) Plant Mol. Biol. **31**, 1029–1038
19 Zhen, R.-G., Kim, E. J. and Rea, P. A. (1997) J. Biol. Chem. **272**, 22340–22348
20 Cooperman, B. S., Baykov, A. A. and Lahti, R. (1992) Trends Biochem. Sci. **17**, 262–266
21 Heikinheimo, P., Lehtonen, J., Baykov, A. A., Lahti, R., Cooperman, B. S. and Goldman, A. (1996) Structure **4**, 1491–1508
22 Baykov, A. A., Hyytia, T., Volk, S. E., Kasho, V. N., Vener, A. V., Goldman, A., Lahti, R. and Cooperman, B. S. (1996) Biochemistry **35**, 4655–4661
23 Lahti, R., Kolakowski, Jr., L. F., Heinonen, J., Vihinen, K. P. and Cooperman, B. S. (1990) Biochim. Biophys. Acta **1038**, 338–345
24 Kankare, J., Salminen, T., Lahti, R., Cooperman, B. S., Baykov, A. A. and Goldman, A. (1996) Biochemistry **35**, 4670–4677
25 Baltscheffsky, M., Shakhov, Y. A. and Nyren, P. (1982) FEBS Lett. **146**, 177–180
26 Samejima, T., Tamagawa, Y., Kondo, Y., Hachimori, A., Kaji, H., Akeda, A. and Shiroya, Y. (1988) J. Biochem. (Tokyo) **103**, 766–772
27 Romero, I. and Celis, H. (1992) J. Bioenerg. Biomem. **24**, 617–624

28 Volk, S. E., Dudarenkov, V. Y., Kapyla, J., Kasho, V. N., Voloshina, O. A., Salminen, T., Goldman, A., Lahti, R., Baykov, A. A. and Cooperman, B. S. (1996) Biochemistry **35**, 4662–4669
29 Rea, P. A., Kim, Y., Sarafian, V., Pool, R. J., Davies, J. M. and Sanders, D. (1992) Trends Biochem. Sci. **17**, 348–353
30 Kuo, S. Y. and Pan, R. L. (1990) Plant Physiol. **93**, 1128–1133
31 Kim, E. J., Zhen, R. G. and Rea, P. A. (1995) J. Biol. Chem. **270**, 2630–2635
32 Zhen, R. G., Kim, E. J. and Rea, P. A. (1994) J. Biol. Chem. **269**, 23342–23350
33 Fiske, C. H. and Subbarow, Y. (1925) J. Biol. Chem. **66**, 378–400
34 Wang, M. Y., Lin, Y. H., Chow, W. M., Chung, T. P. and Pan, R. L. (1989) Plant Physiol. **90**, 475–481
35 Larson, E., Howlett, B. and Jagendorf, A. T. (1986) Anal. Biochem. **155**, 243–248

Received 4 January 1999/11 June 1999; accepted 5 July 1999