Acidic Residues Necessary for Pyrophosphate-energized Pumping and Inhibition of the Vacuolar H\(^+\)-pyrophosphatase by \(N,N'\)-Dicyclohexylcarbodiimide*

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On the basis of a revised topological model of the vacuolar H\(^+\)-pyrophosphatase (V-PPase; EC 3.6.1.1) derived from the analysis of four published sequences using two structure-predicting programs, TopPred II and MEMSAT, eight acidic amino acid residues located near or within transmembrane \(\alpha\)-helices were identified. The codons specifying these amino acids in the cDNA encoding the V-PPase from *Arabidopsis thaliana* were singly mutated to examine their involvement in pyrophosphate (PPi) hydrolysis and PPi-dependent H\(^+\) translocation and the functional significance of the similarities between the sequences encompassing Glu\(^{229}\) (227–245) of the V-PPase and the \(N,N'\)-dicyclohexylcarbodiimide (DCCD)-binding transmembrane \(\alpha\)-helix of the c-subunits of F-ATPases (Nyren, P., Sakai-Nore, Y., and Strid, A. (1993) *Plant Cell Physiol.* 34, 375–378). Three functional classes were identified after helteroslogous expression of mutated enzyme in *Saccharomyces cerevisiae*. Class I (E119Q, E229Q, D573N, E667Q, and E751Q) mutants exhibited PPi hydrolytic and H\(^+\) translocation activities and DCCD sensitivities similar to wild type. The one class II mutant obtained (E427Q) was preferentially impaired for H\(^+\) translocation over PPi hydrolysis but retained sensitivity to DCCD. Class III (E305Q and D504N) mutants exhibited a near complete abolition of PPi hydrolysis and PPi-dependent H\(^+\) translocation and residual activities with decreased DCCD sensitivity. In none of the mutants was diminished insertion of the V-PPase into the membrane or an increase in the background conductance of the membrane to H\(^+\) evident. The decoupled character of E427Q mutants and the enhancement of H\(^+\) pumping in E427D mutants by comparison with wild type, in conjunction with the retention of DCCD inhibitability in both E427Q and E427D mutants, implicate a role for Glu\(^{227}\) in DCCD-insensitive H\(^+\) translocation by the V-PPase. The proportionate diminution of PPi hydrolytic and H\(^+\) translocation activity and conservation of wild type DCCD sensitivity in E229Q mutants refuted the notion that Glu\(^{229}\) is the residue whose covalent modification by DCCD is responsible for the abolishment of PPi-dependent H\(^+\) translocation. Instead, the diminished sensitivities of the residual activities of E305Q and D504N mutants, but not E305D or D504E mutants, to inhibition by DCCD is consistent with the involvement of acidic residues at these positions in inhibitory DCCD binding. The results are discussed with regard to the possible involvement of Glu\(^{227}\) in coupling PPi hydrolysis with transmembrane H\(^+\) translocation and earlier interpretations of the susceptibility of the V-PPase to inhibition by carbodiimides.

The membranes constituting the vacuolysosomal complex of plant cells are unusual in possessing an H\(^+\) translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) (2). The V-PPase bears no systematic resemblance to soluble PPases at the sequence level (3, 4) and is considered to belong to a fourth class of H\(^+\)-phosphohydrolase distinct from the F-, P- and V-ATPases (4). Moreover, unlike the V-ATPase, which is ubiquitous in the membranes bounding the acidic intracellular compartments of all eukaryotic cells, the V-PPase appears to be restricted to plants and a few species of phototrophic bacteria (2, 5). Notwithstanding the intrinsic evolutionary interest of this phenomenon, it poses a problem: the lack of sequence-divergent homologs from phylogenically remote organisms. Because all published V-PPase sequences are from the same group of organisms, vascular plants, and exhibit greater than 85% sequence identity at the amino acid level (6), most attempts to identify conserved amino acid residues of potential mechanistic significance by sequence alignment procedures have been unproductive. Crucial, therefore, has been the development of methods for the expression of functional pump in the yeast, *Saccharomyces cerevisiae* (7, 8). When constructs of the yeast-Escherichia coli shuttle vector pYES2, containing the entire open reading frame of the cDNA (AVP; Ref. 9) encoding the *M. 66,000 substrate-binding subunit* of the V-PPase from *Arabidopsis thaliana* are employed to transform *S. cerevisiae*, endomembrane-associated enzyme active in PPi-dependent H\(^+\) translocation is generated (7). Since the heterologously expressed pump is indistinguishable from the native plant enzyme, thereby establishing the sufficiency of AVP for the elab-

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1 The abbreviations used are: V-PPase, vacuolar H\(^+\)-pyrophosphatase; DCCD, \(N,N'\)-dicyclohexylcarbodiimide; Mes, 4-morpholine-ethanesulfonic acid; NEM, N-ethylmaleimide; EDAC, 1-ethyl-3-(3-diethylaminopropyl)carbodiimide; V-, F-, and P-ATPases, vacuolar-, mitochondrial-, and plasma membrane-type ATPases, respectively.

2 The substrate-binding subunit of the V-PPase migrates at *M* 66,000 (64,500–72,000) on SDS gels, but its probable mass deduced from its amino acid sequence is 79–81 kDa. Thus, when referring to this polypeptide the terms “*M* 66,000 subunit” and “81-kDa subunit” are used interchangeably. Moreover, although it is now known that this subunit is probably the sole polypeptide species constituting the enzyme, it is referred to as the “substrate-binding subunit” because substrate hydrolysis and substrate-protectable covalent modification were the first functions assigned to it.
oration of active V-PPase in *S. cerevisiae*, approaches based on site-directed mutagenesis, epitope tagging, and expression of fusion proteins are now applicable to investigations of the membrane organization and catalytic mechanism of the V-PPase.

By the parallel application of mutational and protein chemical methods, we have demonstrated a specific requirement for a cytosolically oriented Cys residue at position 634 for inhibition of the V-PPase by maleimides and the dispensability of all conserved Cys residues, including Cys634, for catalysis (8, 10).

Our current studies of the V-PPase are directed at elucidating the involvement of acidic (Asp, Glu) residues located near or within hydrophobic spans in substrate turnover and/or H⁺ translocation.

Two factors prompted investigation of these acidic residues. The first was the need to gain insight into the identity and location of acidic residues with the potential for undergoing cycles of protonation and deprotonation within the hydrophobic core of the membrane. On the basis of analyses of other H⁺ pumps and H⁺-coupled transporters, acidic residues associated with transmembrane spans might be expected to directly participate in H⁺ uptake, translocation, and release by the V-PPase. The second factor was the observations of Nyren et al. (1), who noted that the sequences encompassed by positions 227–245 of the V-PPase from Arabidopsis bear a resemblance to the C-terminal regions of the c-subunits of F-ATPases. The C-terminal sequence flanking Glu229 in AVP is 71, 65, and 67% similar (35, 47, and 39% identical) to rhodospirillum rubrum c-subunit (positions 58–74), *P. sativum* chloroplast subunit III (positions 61–77), and *P. sativum* mitochondrial subunit 9 (40). The C-terminal sequences of transmembrane residues 227–245 of AVP are aligned. Identities and conservative substitutions are indicated by white and shaded boxes, respectively. The DCCD-reactive Glu residues of the F-ATPase subunits are shown in bold type. All characterized V-PPases contain the consensus sequence LFE(A/S)ITGYGLGGSSMALF (6).

**MATERIALS AND METHODS**

**Heterologous Expression and Mutagenesis of V-PPase**—The cDNA encoding the V-PPase from *A. thaliana* (AVP; Ref 9) was heterologously expressed in vacuolar protease-deficient *S. cerevisiae* haploid strain BJ5459 (MATa, ural3–52, trp1, lys2–801, leu22, his3–200, pep4: HHS3, prb1Δ16, can1, GAL) (8, 13). Transformation of BJ5459 with yeast-E. coli shuttle vector pYES2 containing the entire open reading frame of AVP inserted between the GAL1 promoter and CYC1 termination sequences (pYES2-AVP; Ref 7), isolation of the Ura^+ transformants, and growth of the cells for the preparation of membranes were performed as described (8). *E. coli* DH5α and CJ236 (dut + ung +) were employed for the amplification of pYES2-AVP and the generation of single-stranded, uracilated template for site-directed mutagenesis, respectively.

Mutagenesis was performed directly on pYES2-AVP vector (8). In all cases the mutagenic oligonucleotides were designed to singly substitute each conserved Asp or Glu codon with an Asn or Gln codon on the basis of the cDNA sequence of AVP (9). The sequences of the eight oligonucleotides (positions of conserved Asp or Glu codons shown in bold type and positions of degeneracy shown in brackets) were: Glu219—Gln, CGGCTCTGT[T]CAGGAGTCTACGAC; Glu229—Gln, TCTTTT[T]CAGGATTCTAGC; Glu298—Gln, GATCGATTCTG[C]AGGATCATCAGG; Glu305—Gln, GCTATTACTGG; Glu308—Gln, GCTATTACTGG; Glu427—Gln, TCTTTT[T]CAGGATTCTAGC; Glu428—Gln, GGCAATT[C]AGGATTCTAG; Asp504—Asn, CCAACCGTAT[AG]TGTGTTTACG; Glu505—Asn, GCTATTACTG[C]AGGATCATCAGG; Asp508—Asp, GATCGATTCTG[C]AGGATCATCAGG; Glu606—Glu, GATCGATTCTG[C]AGGATCATCAGG; Glu672—Glu, GATCGATTCTG[C]AGGATCATCAGG; Asp773—Asn, CCAACCGTAT[AG]TGTGTTTACG; Glu773—Glu, CAGGATCATCAGG; Asp804—Asp, GATCGATTCTG[C]AGGATCATCAGG. 

URACILATED SINGLE-STRANDED TEMPLATE DNA WAS ISOLATED FROM pYES2-AVP TRANSFORMED *E. coli* CJ236, AND SITE-DIRECTED MUTATIONS WERE INTRODUCED BY SECOND STRAND SYNTHESIS FROM THE TEMPLATE USING MUTANT Oligonucleotides (14, 15). IN ALL CASES, MUTAGENESIS WAS CONFIRMED BY SEQUENCING THE TARGET REGION BEFORE YEAST TRANSFORMATION. IN SELECTED CASES, WHEN A PRONOUNCED ALTERATION OF V-PPASE FUNCTION WAS OBSERVED, THE SEQUENCE OF THE TARGET REGION OF THE AVP INSERT OF pYES2-AVP WAS DETERMINED AFTER EXTRACTION OF THE VECTOR FROM THE YEAST TRANSFORMANTS.

**Preparation of Vacuolar Membrane-enriched Vesicles—** Yeast vacuolar membrane-enriched vesicles were prepared as described (8).

**Reaction of V-PPase with N,N′-Dicyclohexylcarbodiimide—** The standard mixture for reaction with DCCD contained 30 mM Tris-Cl, pH 8.0, the indicated concentrations of ligands (MgSO₄, K⁺ or MgCl₂, K⁺, or Tris-Cl, K⁺) and membrane protein (9.7–10.7 mg/ml). Reaction was initiated by the addition of DCCD (0–500 μM dissolved in ethanol), and the samples were incubated at 37 °C for the results of mutagenesis demonstrate that Glu229 is unlikely to play a role in H⁺ translocation or inhibition of the V-PPase by DCCD. Instead, the characteristics of the mutants, combined with the inferred topology of the V-PPase, are better accommodated by a scheme in which membrane-embedded residues Glu305 and Asp504 contribute to DCCD binding, whereas Glu427, which is located at the interface between a transmembrane span and its adjoining cytosolic loop, is required for coupling PPi hydrolysis with H⁺ translocation.

**FIG. 1. Comparison of the deduced amino acid sequence of AVP (9) with the amino acid sequences of the c-peptides of representative F-ATPases.** The F-ATPase c-peptide sequences shown are *R. rubrum* subunit c (38), *P. sativum* chloroplast subunit III (39), and *P. sativum* mitochondrial subunit 9 (40). The C-terminal sequences of the c-subunits of all characterized V-PPases are aligned. Identities and conservative substitutions are indicated by white and shaded boxes, respectively. The DCCD-reactive Glu residues of the F-ATPase subunits are shown in bold type. All characterized V-PPases contain the consensus sequence LFE(A/S)ITGYGLGGSSMALF (6).
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times indicated. After terminating the reaction by the addition of Mg$^{2+}$ (1.3 mM), the samples were cooled on ice before assay aliquots for V-PPase activity. Control samples were treated in an identical manner after the addition of equal volumes of ethanol. All stock DCC solutions were prepared fresh daily.

Mutagenesis of V-PPase Activity and Protein—PP, hydrolytic activity was measured as the rate of liberation of P$_i$ from PP$_i$ at 37 °C in reaction media containing 0.3 mM Tris-PP, 1.3 mM MgSO$_4$, 100 mM KCl, 1 mM NaF, 5 mM gramicidin-D, 1 mM Tris-EGTA, and 30 mM Tris-Mes (pH 8.0) (8). Since yeast-soluble PPase, unlike the V-PPase, is exquisitely sensitive to inhibition by fluoride ($K_{i}^{W}$ (soluble PPase) = 20 µM, $K_{i}^{W}$ (V-PPase) = 3.4 mM) (16), inclusion of 1 mM NaF in the assay media effectively abolishes the contribution of the former to total hydrolysis (8).

PP$_i$- and ATP-dependent H$^+$ translocation was assayed fluorimetrically using acridine orange (2.5 µM) as transmembrane pH difference indicator in assay media containing vacuolar membrane-enriched vesicles (200 µg), 100 mM KCl, 0.4 mM glycerol, 1 mM Tris-EGTA, and 5 mM Tris-HCl (pH 8.0). Reaction was initiated by the addition of Tris-PP$_i$ (1.0 mM) to media containing MgSO$_4$ (1.3 mM) in the case of V-PPase-mediated H$^+$ translocation or by the addition of MgSO$_4$ (3 mM) to media containing Tris-ATP (3 mM) in the case of V-ATPase-mediated H$^+$ translocation. The decrease in fluorescence was measured at excitation and emission wavelengths of 495 and 540 nm, respectively (8). The initial rates of the ATP- and Tris-PP$_i$-translocation and steady state pH gradients were estimated as $\Delta$pH/min (at time zero) and $\Delta$pH/mg (after 5–10 min), where $\Delta$pH% = percentage decrease in fluorescence as described (17). Coupling ratio (the ratio of the rate of H$^+$ pumping to the rate of PP$_i$ hydrolysis) was estimated as (ΔpH%/min) (µmol of PP$_i$ hydrolyzed/min).

Protein was estimated by a modification of the method of Peterson (18). Western Analyses—For Western analyses of the heterologously expressed V-PPase, membrane samples were delipidated by extraction with acetone:ethanol (1:1; -20 °C) (19), dissolved in denaturation buffer, and subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 11% (w/v) slab gels in a Bio-Rad minigel apparatus (7). The electrophoresed samples were electrotransferred to 0.45-µm nitrocellulose filters in standard Towbin buffer (20), containing 10% (v/v) methanol for 30 min at a current density of 2.5 mA/cm² in a Millipore semi-dry blotting apparatus. After reversible staining of the transferred protein bands with Ponceau-S, the filters were processed for reaction with each of five types of topological element: two classes of amino acids according to their propensities for being associated with the cytosolic face of the membrane while preserving the orientation of all of the other spans.

The deduced amino acid sequences of the V-PPases encoded by the cDNAs isolated from A. thaliana (AVP, GenBank™ accession no. M81892) (9), Beta vulgaris (BVP1, L32792; BVP2, L32791) (25), and Hordeum vulgare (HVP, D193472) (12) were processed in parallel using both programs.

RESULTS

Revised Topological Model—A revised topological model of the V-PPase was derived from the deduced sequences of the polypeptides encoded by four cDNAs: AVP from A. thaliana (9), BVP1 and BVP2 from B. vulgaris (25), and HVP from H. vulgare (12). The model shown in Fig. 2 was the only one of the three predicted by the TopPred II and MEMSAT programs of Claros and von Heijne and Jones et al. (24) capable of accommodating a cytosolic orientation for both the C terminus and the hydrophilic loop containing the N-ethylmaleimide (NEM)-reactive cysteine, Cys$^{634}$, inferred from the characteristics of apoaequorin fusions (26) and the results of peptide mapping and Cys mutagenesis, respectively (8, 10).

Examination of the structure of the M$_2$ 66,000 subunit of the V-PPase by TopPred II consisted of three main stages. (i) The first stage was the construction of hydrophobicity profiles using a trapezoid sliding window (27). Depending on the height and width of the hydrophobicity maxima and the preset “upper cutoff” and “lower cutoff” values for the computed hydrophobicity indices, spans were categorized as either “certain” or “putative.” (ii) The second stage was enumeration of the difference in representation of positively charged amino acid residues between the two sides of the membrane and tests of the adherence of any given model to the positive-inside rule, with the bias in favor of Arg and Lys residues in hydrophilic loops with a cytosolic disposition in most polytopic membrane proteins (28). (iii) The third stage was application of the charge-difference rule (29), wherein the net charge difference between the 15 N-terminal and the 15 C-terminal residues flanking the most N-terminal transmembrane span is computed. Transmembrane orientation is correlated with the disposition of charged residues in the immediate vicinity of the first membrane span. The segment C-terminal to the first span is generally positively charged with respect to the N-terminal flanking regions in membrane proteins possessing a luminaly oriented N terminus (29).

Deployment of the MEMSAT program entailed analysis of segments of the sequence of the V-PPase in terms of their likelihood of being located within a particular topological element. Based on statistical analysis of the distribution of amino acids in membrane proteins, the MEMSAT program ranks amino acids according to their propensities for being associated with each of five types of topological element: two classes of hydrophilic loop, designated cytoplasmic (inside) loop (L$_i$) and luminal (outside) loop (L$_o$), and three classes of transmembrane helix domain, designated helix inside (H$_i$), helix middle (H$_m$), and helix outside (H$_o$) (24).

The consensus structure consistent with the predictions from both programs, the disposition of Cys$^{634}$, and the C-terminal apoaequorin fusion data was a 15-span model containing a luminaly localized N terminus and cytoplasmically localized C terminus (Fig. 2). While MEMSAT ranked a 16-span model highest, with the additional span encompassing residues 743–761, two models containing 14 and 15 spans ranked just below this model. In the 14-span model, the two lowest scoring transmembrane spans in the 16-span model (V and VI) were excluded, thus preserving the orientation of the N and C termini and the remaining C-terminal spans. In the 15-span model, the last transmembrane span in the 16-span model (XVI) was excluded, thus transferring the C terminus from the luminal to the cytosolic face of the membrane while preserving the orientation of all of the other spans.

All three models were consistent with a cytosolic disposition for Cys$^{634}$ (8, 10), but only one, the 15-span model, was compatible with the finding that fusion of apoaequorin with the C terminus of AVP generates a vacuolar membrane-associated polypeptide capable of sensing cytosolic Ca$^{2+}$ in transgenic A. thaliana plants (26). Assuming that the fusion of apoaequorin with the C terminus does not itself, change the topology of the V-PPase, these data constrain the C terminus to the cytosolic
face of the membrane and exclude the 16- and 14-span models.

Notable is the basic equivalence between the predictions deriving from TopPred II and MEMSAT. Whereas TopPred II constrains the length of transmembrane spans at a specific value (21 amino acid residues in this study) and is based on the assumption that all spans are perpendicular to the phospholipid bilayer, MEMSAT selects the best fit within a user-defined range of minimum and maximum lengths (17–25 amino acids residues in this study), thereby diminishing bias in favor of any one angle of intersection. Nevertheless, the margins of 11 of the 15 spans predicted by the two programs differed by no more than 4 amino acid residues and the average length of the spans (21.5 by MEMSAT versus a fixed value of 21 for TopPred II) were virtually identical. Of the spans predicted by MEMSAT, only two, spans IX (408–425) and XV (671–687), were shorter than the 20 amino acids required to traverse the entire bilayer, but in both cases the counterpart helices predicted by TopPred II included all 17 of these residues. Accordingly, when the MEMSAT settings were altered to increase the minimum span length from 17 to 19 residues, the overall topology of the V-PPase was unchanged; spans IX and XV were simply lengthened.

The three transmembrane spans (V (230–252), VI (292–316), X (452–472)) not identified in the original 13-span model (9) were neglected because of the proximity of adjacent maxima in the hydrophobicity profiles (span X) and the use of window sizes so broad as to obscure hydrophobic segments adjacent to regions of extreme hydrophobicity (spans V and VI). TopPred II, by contrast, permitted better resolution of the neighboring transmembrane spans by the application of a narrower sliding window (11 amino acid residues).

Two of the three transmembrane spans overlooked in the original 13-span model, helices V and VI, were the least likely in terms of their hydrophobicity and expectation maximization scores. Their MEMSAT scores (339 and 449, respectively) were significantly higher than the default cutoff value of 100 but markedly lower than the average score of 2713 for the other transmembrane spans. However, because the low scores of these helices were largely attributable to Arg246 (in helix V) and Asp298 (in helix VI), each residue of which was predicted to be displaced by seven positions from the cytosolic face of the membrane according to MEMSAT, and therefore appropriately positioned for mutual electrostatic screening, both spans were retained in the model.

The orientation of the N terminus was deduced from the charge-difference rule (29). Examination of the N-terminal residues immediately adjacent to the first transmembrane span (positions 14–34) revealed no positively charged residues and two negatively charged residues (Glu9, Glu13), giving a net charge of −2. The corresponding regions of BVP1, BVP2 (25), and HVP (12) had the same charge, attributable to Arg246 (in helix V) and Asp298 (in helix VI), each residue of which was predicted to be displaced by seven positions from the cytosolic face of the membrane according to MEMSAT, and therefore appropriately positioned for mutual electrostatic screening, both spans were retained in the model.

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A net charge difference of at least 3 (2 for the N-terminal 15 residues before the first span versus 11 for the C-terminal 15 residues after the span for AVP, 4 for BVP1 and BVP2, 8 for HVP) in all four cases was consistent with a luminal orientation for the N terminus.

The cytosolically disposed loops of the 15-span model for AVP contain a significantly greater number of Arg and Lys residues than the luminally oriented loops (83% versus 17%, respectively). Further, the majority of the residues located in hydrophilic loops are cytosolically oriented (79.7%, 45.6% of total), in accord with the inside-positive rule (28) and with the expectation that the overall distribution of hydrophilic loops would be biased toward the side of the membrane responsible for catalysis and ligand binding.

Three Classes of Mutant—According to the 15-span model (Fig. 2), a total of eight conserved acidic amino acid residues (Glu119, Glu229, Glu305, Glu427, Asp504, Asp573, Glu667, and Glu751) were tentatively identified as being near or within putative transmembrane spans. To examine their involvement in PPi hydrolysis, H+ translocation, and DCCD inhibition, these residues were singly substituted. In all cases the corresponding amides; in those cases where acid → amide substitutions had an influence on V-PPase activity, enzyme containing structurally conservative Asp → Glu or Glu → Asp substitutions was also generated.

Three classes of V-PPase mutant were distinguishable on the basis of their hydrolytic and pumping activities after heterologous expression in S. cerevisiae strain BJ5459: (i) those exhibiting rates of PPi hydrolysis and PPi-dependent H+ translocation similar to wild type, (ii) those exhibiting selective impairment of H+ translocation, and (iii) those exhibiting gross impairment of both PPi hydrolysis and PPi-dependent H+ translocation (Table I and Fig. 3).

In all class I mutants except one (D573N), PPi hydrolytic activity and the rate and extent of PPi-dependent H+ translocation were diminished proportionately. Glu → Gln substitutions at positions 119, 229, 667, and 751 generated enzyme with at least 15% of wild type PPi hydrolytic and PPi-dependent H+ pumping activity and coupling ratios, enumerated as ΔF% / μmol PPi hydrolyzed.

**Fig. 3.** PPi-dependent H+ translocation by vacuolar membrane-enriched vesicles prepared from pYES2-AVP-transformed S. cerevisiae BJ5459 cells expressing either wild type or mutated V-PPase. Membrane vesicles (200 μg of membrane protein) were assayed for H+ translocation with the fluorescent ΔpH indicator, acridine orange, in a total reaction volume of 0.9 ml containing 1.3 mM MgSO4, 0.4 M glycerol, 100 mM KCl, 1 mM Tris-EGTA, and 5 mM Tris-HCl (pH 8.0). Intravesicular acidification was initiated at the times indicated by the addition of 1 mM Tris-PPi.

A net charge difference of at least 3 (2 for the N-terminal 15 residues before the first span versus +1 for the C-terminal 15 residues after the span for AVP, 4 for BVP1 and BVP2, 8 for HVP) in all four cases was consistent with a luminal orientation for the N terminus.

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**TABLE I**

| Class | Substitution | H+ translocation | PPi hydrolysis | Coupling ratio |
|-------|--------------|------------------|---------------|---------------|
|       |              | Initial rate      | Steady state  |               |
|       |              | ΔF% / mg/min      | ΔF% / mg     | μmol / mg/min |
| I     | E119Q        | 125              | 185           | 1.3           |
|       |              | 94               |               |               |
|       | E229Q        | 23               | 41            | 0.2           |
|       |              | 99               |               |               |
|       | E229D        | 279              | 254           | 1.5           |
|       |              | 186              |               |               |
|       | D573N        | 253              | 215           | 0.9           |
|       |              | 278              |               |               |
|       | E667Q        | 58               | 129           | 0.5           |
|       |              | 119              |               |               |
|       | E751Q        | 108              | 153           | 1.2           |
|       |              | 94               |               |               |
| II    | E427Q        | 10               | 22            | 0.5           |
|       |              | 19               |               |               |
|       | E427D        | 221              | 235           | 1.0           |
|       |              | 226              |               |               |
| III   | E305Q        | ND               | ND            | 0.1           |
|       |              | NA               |               |               |
|       | E305D        | ND               | ND            | 0.3           |
|       |              | NA               |               |               |
|       | D504N        | ND               | ND            | 0.7           |
|       |              | NA               |               |               |
|       | D504E        | ND               | ND            | 0.0           |
|       |              | NA               |               |               |

* ND, not detectable.

* NA, not applicable.
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**Fig. 4.** ATP-dependent H⁺ translocation by vacuolar membrane-enriched vesicles prepared from pYES2-VPase-transformed S. cerevisiae BJ5459 cells expressing either wild type or mutated V-PPase. A, ATP-dependent intravesicular acidification by membranes containing wild type or E229Q, E305Q, E305D, D504N or D504E mutated V-PPase. PPi was not included in the assay media. B, ATP-dependent intravesicular acidification by membranes containing E229Q-mutated V-PPase measured in the presence or absence of PPi. Membrane vesicles (200 μg of membrane protein) were assayed for H⁺ translocation as described in Fig. 5, except that ATP-dependent pumping was measured in reaction media containing 3 mM Mg⁺² and 3 mM Tris-ATP. Tris-PPi (1 mM) was added when indicated.

**Fig. 5.** SDS-polyacrylamide gel electrophoresis and Western analysis of wild type and mutated V-PPase after heterologous expression in S. cerevisiae BJ5459. Dilipidated vacuolar membrane-enriched vesicles (5 μg of membrane protein) prepared from pYES2-VPase-transformed cells were electrophoresed, electrotransferred to nitrocellulose filters, and probed with V-PPase peptide-specific antibody, PABHK1. All of the bands migrated at M, 66,000. Also shown are the PPi hydrolytic activities of the corresponding membrane samples before delipidation.

Maintenance of Membrane Integrity and Efficiency of V-PPase Insertion—In none of the mutants was there an increase in membrane H⁺ conductance of sufficient magnitude to account for the effects of these substitutions. Vacuolar membrane-enriched vesicles harboring any of the V-PPase mutants achieved similar rates and extents of MgATP-dependent H⁺ translocation by the endogenous, chromosomally coded V-ATPase associated with this fraction as membranes containing wild type V-PPase (Fig. 4A), indicating that the background conductance of the membrane to H⁺ was unaltered by mutagenesis of the V-PPase. Moreover, the possibility of a substrate (Mg⁺²PPi) elicited increase in H⁺ conductance associated with the decoupled mutant, E427Q, was excluded by the finding that the rate of MgATP-dependent intravesicular acidification of membranes containing this form of the enzyme was the same regardless of whether or not PPi was added to the V-ATPase assay medium (Fig. 4B). Similarly, the decreased PPi hydrolytic and/or H⁺ pumping activities of some of the mutants were not explicable in terms of a decrease in the amounts of intact membrane-associated V-PPase through premature maturation, changes in expression level, or expression of polypeptide with decreased stability. Vacuolar membrane-enriched vesicles from cells expressing either wild type or mutated V-PPase contained similar levels of PABHK₁-reactive, M, 66,000 (AVP-specific) polypeptide, irrespective of the type or position of the substitution, and in none of the membrane samples was there an indication of a change in the electrophoretic mobility of the PABHK₁-reactive band (Fig. 5).

Kinetics of Inhibition by DCCD—If DCCD inhibits the V-PPase through its interaction with a carboxyl group located in a transmembrane span, introduction of acid → amide substitutions at these positions in the heterologously expressed enzyme would be predicted to confer decreased sensitivity to this reagent.

As a prelude to optimizing the conditions for reaction with DCCD, the ligand requirements for inhibition were investigated. Of the ligands tested, Mg⁺²⁺ was the only one that influenced the susceptibility of the wild type heterologously expressed enzyme to inhibition by DCCD (Fig. 6). In contrast to the requirements for protection of the V-PPase from inhibition by maleimides (8, 10), free PPi and K⁺ did not influence the inhibitory action of DCCD, and substrate, Mg⁺²⁺ + PPi, did not...
showed an approximately 2-fold decrease, was Mg\textsuperscript{2+} residual activity by more than 3- and 4-fold, respectively (Fig. 5).

The kinetics of inhibition of wild type V-PPase by DCCD were consistent with a scheme in which the modification of two reactive sites on the enzyme is necessary for inactivation and Mg\textsuperscript{2+} confers protection by binding to a high affinity site. The time dependence of inhibition by DCCD was described by the integrated second order rate equation 1/\(A_t\) = 1/\(A_0\) + \(kt\) such that a plot of the reciprocal of V-PPase activity (A) at time t approximated a straight line of slope k and intercept 1/\(A_0\), where \(A_0\) is activity at time zero and k is the rate constant (Fig. 7A). Mg\textsuperscript{2+} decreased the second order rate constant for inactivation by DCCD as a hyperbolic function of Mg\textsuperscript{2+} concentration to yield an apparent affinity constant of 10–15 \(\mu\)M (Fig. 7B).

A screen of all eight acid → amide mutants for Mg\textsuperscript{2+}-protectable inhibition by DCCD revealed that only two (E305Q and D504N) were markedly less sensitive to DCCD (Fig. 8 and Table II). While acid → amide substitutions at positions 119, 229, 427, 573, 667, and 751 had little or no effect on DCCD inhibitability (Fig. 8 and Table II). The data were fitted to the integrated second order rate equation 1/\(A_t\) = 1/\(A_0\) + \(kt\), where \(A_0\) is activity at time zero, \(A_t\) is activity at time t, and k is a second order rate constant. B. plot of second order rate constants, estimated from the data in A, against Mg\textsuperscript{2+} concentration. Concentration of Mg\textsuperscript{2+} required for 50% diminution of k = 10–15 \(\mu\)M.

It was by inspection of the revised topological model of the V-PPase that the eight acidic residues located within or near transmembrane spans were identified as targets for substitution. Although six other acidic residues with a similar disposition were evident from the model (Fig. 2), these were either not conserved in all four of the sequences analyzed (Glu\textsuperscript{123}13, Glu\textsuperscript{298}298, Asp\textsuperscript{324}324) or located in relatively hydrophilic environments (Glu\textsuperscript{225}225, Asp\textsuperscript{335}335, Glu\textsuperscript{445}445) and considered less likely to participate in transmembrane H\textsuperscript{+} translocation and/or DCCD binding. Three main conclusions, discussed below, derive from the results of substituting these acidic residues.

Glu\textsuperscript{225} Is Not Essential for PP, Hydrolysis or H\textsuperscript{+} Translocation—E229Q mutated V-PPase shows some impairment of PP, hydrolysis and H\textsuperscript{+} translocation but, since both processes are diminished in parallel, the diminution of coupling ratio is small. Moreover, in direct opposition to the imputed role of Glu\textsuperscript{225} in inhibition by DCCD, E229Q-substituted V-PPase is no less sensitive to inhibition by DCCD than wild type or E229D mutated enzyme, indicating that an acidic residue at this position is not required for inhibition by this reagent. On this basis, and in contrast to the speculations in Ref. 1, it is unlikely that the alignments between the V-PPase sequences C-terminal to Glu\textsuperscript{225} and the second DCCD-reactive transmembrane span of the c-peptides of F-ATPases (Fig. 1) signifies a functional equivalence. Similarly, Asp\textsuperscript{373}373 and Glu residues 119, 667, and 751 do not appear to be critical for PP, hydrolysis, PP-dependent H\textsuperscript{+} translocation, or inhibition by DCCD. Substitution of these residues by their corresponding amides exerts little or no effect on PP, hydrolytic activity, H\textsuperscript{+} pumping, coupling ratio, or DCCD inhibitability.

Glu\textsuperscript{305} and Asp\textsuperscript{504} Are Critical for Catalysis and Contribute to DCCD Binding—E305Q and D505N mutants exhibit less similar for all four of the sequences analyzed, with only minor differences in the lengths and locations of a few of the transmembrane spans. The results of both were compatible with the available, albeit limited, biochemical data on the localization of Cys\textsuperscript{634}634 and the characteristics of AVP-apoaequorin fusions (26). By contrast, the 12-span model of the V-PPase proposed by Tanaka et al. (12), on the basis of hydropathy analyses of the deduced sequence of HVP alone, places Cys\textsuperscript{634} on the wrong side (on the luminal face) of the membrane and contains a highly improbable transmembrane span (span VI in their model) incorporating 30–40 amino acid residues.

DISCUSSION

Both of the predictive methods applied to the substrate-binding subunit of the V-PPase, TopPred II and MEMSAT, yielded a tentative model containing two transmembrane spans in addition to the 13 proposed previously (9) and an opposed orientation for the N and C termini (luminal N terminal; cytosolic C terminus). Although tests of the validity of the new model will depend eventually on the results of direct, independent, and complementary structural studies, we consider it a significant improvement over those proposed previously. The outcomes from both programs were remarkably
activity in the case of Asp 305 suggests that the steric con-
phate (Mg2PPi), is required for activity: a high affinity binding
those associated with the substrate, dimagnesium pyrophos-
tion of the V-PPase from inhibition by the water-soluble car-
concentration measurements of inhibition by DCCD in the absence or presence of
Mg2
by DCCD in the absence (●) or presence of Mg2
Mg2
1
DCCD in the presence or absence of

Glu305 and Asp504 are the sole residues involved in inactivation of the V-PPase by this carbodiimide. Doubly mutated enzyme containing acid
amine substitutions at both of these posi-

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Mutagenesis of Acidic Residues of V-PPase

than 10% wild type PP, hydrolytic activity, no detectable PP,
dependent H+ translocation, and residual activities markedly
less sensitive to inhibition by DCCD than that of wild type
enzyme. These characteristics, together with the recovery of
DCCD inhibitable activity shown by E305D and D504E mutants, are
consistent with the involvement of acidic residues at these
positions in inhibition by DCCD. It is unlikely, however, that
Glu305 and Asp504 are the sole residues involved in inactivation
of the V-PPase by this carbodiimide. Doubly mutated enzyme
containing acid → amide substitutions at both of these posi-
tions is no less sensitive than either single mutant to DCCD,
implies the participation of residues other than Glu305 and
Asp504. The finding that structurally conservative Asp → Glu
or Glu → Asp substitutions cause a decrease in hydrolytic
activity in the case of Glu504 and only a minor increase in
activity in the case of Asp305 suggests that the steric
constraints for catalysis are more stringent than for DCCD bind-
ing. Evidently, a difference of one methyl group in the carboxyl
side chain is sufficient to severely impair catalytic function
while leaving DCCD inhibitability unaffected.

Mg2
exerts its high affinity effects indirectly
through conformational coupling rather than through direct
screening of the residues that would otherwise be covariantly
modified. By implication, Glu305 and Asp504, although contrib-
ting to DCCD binding, may not themselves bind Mg2

Another explanation compatible with the effects of mutagen-

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that neither Glu\textsuperscript{305} nor Asp\textsuperscript{504} directly participate in DCCD binding or that either Glu\textsuperscript{305} or Asp\textsuperscript{504} does but not the other. For instance, by neutralizing the ϒ- or γ-carboxyl groups on the side chains of these residues, acid → amide substitutions at one or both of these positions may simulate the electrostatic screening action of Mg\textsuperscript{2+} binding and thereby diminish the sensitivity of the V-PPase to inhibition by DCCD in the absence of Mg\textsuperscript{2+}, while at the same time impairing overall catalytic activity.

Glutamate is Required for Efficient Coupling—E427Q mutants, although still active in PP, hydrolysis, mediate H\textsuperscript{+} translocation at less than 6% of the wild type rate to yield an 8–9-fold diminished coupling ratio. While it may be premature to conclude that these results demonstrate a direct role for Glu\textsuperscript{427} in H\textsuperscript{+} transfer, since its substitution by Gln might cause a structural change that indirectly effects enzyme function, the large recovery of wild type H\textsuperscript{+} pumping versus the modest increase in PP, hydrolytic activity by a Glu → Asp substitution nonetheless implies an important role for an acidic residue at this position for H\textsuperscript{+} translocation per se. Whereas the capacity for PP, hydrolysis is increased by only 2-fold when the E427Q substitution is replaced with an E427D substitution, the rate of H\textsuperscript{+} translocation is increased by more than 20-fold.

If Glu\textsuperscript{427} does indeed directly participate in H\textsuperscript{+} transfer, two corollaries follow. First, since E427Q- or E427D-mutated V-PPase is as sensitive to DCCD as wild type enzyme, inhibition by DCCD does not have a direct bearing on H\textsuperscript{+} recovery of wild type H\textsuperscript{+}-dissociable side chains other than those on acidic residues also participate in H\textsuperscript{+} translocation.

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