Transmembrane Carboxyl Residues Are Essential for Cation-dependent Function in the Gastric H,K-ATPase*

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The K+-dependent ATPase activity of the H,K-ATPase was irreversibly inhibited by the carboxyl activating reagent, dicyclohexylcarbodiimide (DCCD). The inhibition was first order and displayed a concentration dependence with the $K_{0.5}$ (DCCD) = 0.65 ± 0.04 molar KCl protected 70% of the ATPase activity from DCCD-dependent inhibition in a concentration-dependent manner with a $K_{0.5}$ (K+) = 0.58 ± 0.1 molar KCl. DCCD modification selectively inhibited the K+-dependent rather than ATP-dependent partial reactions including eosin fluorescence responses and ligand-stabilized initial tryptic cleavage patterns of the membrane-associated enzyme. DCCD modification also inhibited the binding of $^{86}$Rb+ and the fluorescent responses of the K+-competitive, fluorescent inhibitor 1-(2-methylphenyl)-4-methylamino-6-methyl-2,3-dihydropyrrolo[3,2-c]quinoline. [14C]DCCD was incorporated into the H,K-ATPase in a time course identical to that describing the inactivation of the K+-dependent ATPase activity of the H,K-ATPase. A component of the [14C]DCCD incorporated into the H,K-ATPase was K+-sensitive where K+ reduced the [14C]DCCD incorporated into the enzyme by 1.6 nmol of [14C]DCCD/mg of protein. Membrane-associated tryptic peptides resolved from the [14C]DCCD-modified H,K-ATPase exhibited various K+-sensitivities with peptides at 23, 9.6, 8.2, 7.1, and 6.1 kDa containing 10–78%, 23–52%, 24–38%, 2%, and 3–4% K+-sensitivity, respectively.

The N-terminal sequence of the K+-sensitive, ~23- and 9.6-kDa peptides was LVNE$^{857}$, a C-terminal fragment of the ATPase α-subunit. The mass of the smaller peptide limited the residue assignment to the transmembrane M7/M8 domains and an intervening extracytoplasmic loop. An N-terminal sequence, SD$^{40}$IM, was obtained from a 3.3-kDa, [14C]DCCD-labeled peptide resolved from a V8 digest of the partially purified α-subunit. This mass was sufficient to include LVNE but would exclude M8 and the intervening loop between M7 and M8. Glu$^{857}$ is a unique residue present in each of the proteolytic preparations of the H,K-ATPase modified by [14C]DCCD. These data provide functional evidence of the selective inactivation of the K+-dependent partial reactions of the H,K-ATPase and show that Glu$^{857}$ located at the M7 boundary in the C terminus of the pump molecule is a significant site of DCCD modification. These data are interpreted to indicate that this carboxyl residue is important for cation binding function.

The H,K-ATPase of gastric fundic mucosa is an ion pump responsible for H+ movement across the secretory membrane of the parietal cell. The full-length sequence obtained from cDNA clones of rat, rabbit, and hog identify the catalytic α-subunit of the ATPase as a P type ion pump with a mass of 114 kDa (1–3). The minimal organizational unit of the pump is an αβ heterodimer composed of the catalytic α-subunit and a smaller, glycosylated β-subunit (4–6).

Various lines of evidence suggest that the ion transport cycle proceeds through a cyclical series of enzyme conformations generally represented as $E_1$ and $E_2$ (7). These conformations are defined by the fluorescent responses of intrinsic and extrinsic fluorescent probes (8–11), proteolytic peptide maps (12, 13), antibody binding (14–16), and measurements of transient pump currents (17).

Residues responsible for nucleotide binding and catalysis are located on the cytoplasmic enzyme face of the H,K-ATPase, where both chemical labeling studies in the H,K-ATPase (18, 19) and site-specific mutations of the genes of other P type ion pumps have provided insight into the identity of residues important for ATP binding (20–22) and the coupling of conformational change to ion transport (23).

In contrast to nucleotide binding, cation binding also occurs on the extracytosolic face of the pump, where the transported ion is exchanged for H+ during the active transport cycle. Chemical labeling studies have identified at least two regions at this pump face that contain residues essential for overall catalytic activity. Modification of Cys$^{813}$ and/or Cys$^{822}$ and Cys$^{856}$ by omeprazole and a residue within the M1/M2 extracytoplasmic domain by the K+-competitive, photoactive derivative, $[^3]$HMeDAZIP$^1$ fully inhibits the H,K-ATPase (13, 24, 25). Because of the K+-competitive inhibition, it has been suggested that the MeDAZIP binding domain is involved in cation binding.

To provide additional information about the chemical residues important for cation binding functions we have modified the H,K-ATPase with the hydrophobic carboxyl activating reagent, DCCD. DCCD readily inactivates the H,K-ATPase and its partial reactions associated with cation binding. We show that [14C]DCCD is incorporated into the H,K-ATPase and is enriched in an ~23-kDa peptide purified from a tryptic membrane preparation containing a competent cation binding domain. Sequence analysis of the [14C]-labeled peptides obtained from various proteolytic digests suggests that a carboxyl residue(s) within the C-terminal portion of the molecule at the M7 boundary is essential for cation binding-dependent activities.

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MATERIALS AND METHODS
Preparation of Membrane-bound H,K-ATPase

Purified, microsomal H,K-ATPase was prepared by previously described methods (26). Aliquots of the preparation were suspended in 1 mM EDTA and 10 mM Pipes/Tris, pH 7.4, at 4°C. Digitonin (detergent/protein (29), 10 mM stock in Me2SO, was added to a percentage of the untreated control. MDPQ was prepared daily from a 0.2 mM stock in Me2SO.

Fluorescence Measurements

All measurements were performed on an SLM-Aminco SPF 500™ spectrophotofluorimeter, fitted with a magnetic device for stirring the cuvette's contents and water temperature control.

Eosin Fluorescence—Eosin fluorescence was measured in a 2.3-ml aliquot of assay buffer containing 100 mM histidine, pH 7.4, 2 mM MgCl2, 2 mM Chlchlo, 2 μg of nigericin, 1 μM eosin Y and 300 μg of enzyme. The enzyme was incubated for 2 min in assay buffer prior to the addition of either KCl or ATP to stabilize the base-line fluorescence. Fluorescence intensity was measured at excitation/emission at 470 nm and 620 nm, slit widths. Steady-state values for each response were determined 90 s after the addition of each ligand. The fluorescence response, corrected for dilution/ionic strength artifacts, was reported as a percentage of the untreated control. MDPQ was prepared daily from a 10 mM stock in Me2SO.

MDPQ Fluorescence—Steady-state fluorescence was measured in a 2.3-mI assay buffer containing 50 mM Pipes/Tris, pH 7.4, 2 mM MgCl2, 52 μg of enzyme, 2.0 μg of nigericin, 0.3 mM MDPQ, and either 10 mM KCl or 10 mM choline chloride at excitation/emission at 470 nm and 620 nm, slit widths. Steady-state values for each response were determined 90 s after the addition of each ligand. The fluorescence response, corrected for dilution/ionic strength artifacts, was reported as a percentage of the untreated control. MDPQ was prepared daily from a 10 mM stock in Me2SO.

Measurement of 86Rb+ Occlusion

86Rb+ occlusion was measured as described previously (27). The H,K-ATPase was incubated in buffer containing 100 mM choline chloride, 2 mM CDTA, 0.3 mM 86RbCl (1.5 × 106 cpm/60 μg of protein), and 40 mM Pipes/Tris, pH 6.7 for 5 min at room temperature and 30 min at 4°C. Digitonin (detergent/protein = 2.0 (w/v)) was added with gentle vortexing 5 min prior to protein dilution (1:10) into an ice-cold stop solution containing 100 mM choline chloride, 2.0 mM MgCl2, 0.2 mM NaN3, and 40 mM Pipes/Tris, pH 6.7. The quenched suspension was vacuum-filtered through Dowex 50X—200 cation exchange columns constructed as described by Shani et al. (28) with vacuum pressure applied to obtain a flow rate of 0.2 ml s⁻¹. Each column was washed with 1.5 ml of ice-cold sucrose buffer containing 300 mM sucrose and 40 mM Pipes/Tris, pH 6.7.

Tryptic Preparations of the H,K-ATPase

Initial Cleavage Peptides—The H,K-ATPase was suspended at 37°C in medium composed of 100 mM imidazole, pH 7.0, 2 mM EDTA, 1 mg/ml protein, and either 30 mM KCl or 3 mM ATP. Trypsin was added at a protein/trypsin ratio of 75 or 50 (w/v) to medium containing KCl or ATP, respectively. Aliquots of the tryptic digests were removed at intervals, stopped by the addition of trypsin inhibitor protein (TIP) (TIP/trypsin = 10 (w/v)), and placed on ice. The membrane-bound peptides were collected by centrifugation for 30 min at 2,000 g, in a Beckman Airfuge, resuspended, and resolved on 10% Laemmli minigels.

Extensive Trypsin Proteolysis—The H,K-ATPase (1.0 mg/ml) was suspended in medium composed of 40 mM Tris-CI, pH 7.4, 1 mM EDTA, and 25 mM KCl or 40 mM Tris-CI, pH 8.0, 1 mM EDTA, and 0.25 mM KCl. Trypsin was added at a protein/trypsin ratio of 4:10 (w/v), and the suspension was digested for 30 min at 37°C. Proteolysis was stopped by the addition of trypsin inhibitor protein (TIP/trypsin = 6 (w/v)). The membrane-bound peptides were diluted 101, collected by centrifugation, labeled with 0.2 mM F-5-M and resolved on a 16.5% discontinuous Tricine gel using the method of Schagger and von Jagow (29).

14C DCCD Incorporation

Time-dependent Incorporation of 14C DCCD—The H,K-ATPase was suspended at 1.5 mg/ml protein in buffer composed of 20 mM Pipes/Tris, pH 7.4, with or without 30 mM KCl. 14C DCCD was added in a 10% volume (0.2–0.3 mM final concentration and ~110–130 cpm/ml) to the suspension equilibrated at 37°C and incubated for time periods up to 1 h. At intervals, aliquots (55 μg of protein) were quenched by a 1.0-ml addition of ice-cold solution containing 10% Cl,CCO0H and 0.15 mg/ml serum albumin. Membrane lipids were removed as described by Gorga (30), and the protein was resuspended in 2% SDS. The quantity of covalently incorporated 14C DCCD was calculated from the specific activity of the 14C DCCD recovered within the lipid extracted protein. Protein was measured with the Pierce assay system using γ-globulin as a standard.

H,K-ATPase Inactivation versus 14C DCCD Incorporation—The H,K-ATPase was suspended in 25 mM Tris-CI, pH 7.4, 1 mM EDTA, and either 30 mM KCl or 30 mM choline chloride. 14C DCCD (0.2–1.5 mM) (specific activity ~110–114 cpm/pmol) was added to the enzyme and equilibrated for 10 min at 37°C. The reaction was stopped as described above, and aliquots were taken for analysis of ATPase activity and covalently incorporated 14C DCCD.

Measurement of K⁺–sensitive 14C DCCD Incorporation—The H,K-ATPase was suspended in 25 mM Pipes/Tris, pH 7.4, 1 mM EDTA, and either 25 mM KCl or 25 mM choline chloride. 0.2 mM 14C DCCD (final concentration) was added in a one-tenth volume and incubated for 20 min at 37°C. The reaction was stopped by 14-fold dilution into ice-cold buffer composed as above. The membrane fraction was recovered by centrifugation and washed once in a wash buffer composed of 50 mM Tris-CI, pH 8.2 and 250 mM sucrose. The washed enzyme was digested with TPCK trypsin at a protein/trypsin ratio of 4 (w/v) for 30 min at 37°C. The proteolysis was stopped by the addition of TIP (TIP/trypsin = 6), and the membrane-associated peptides were collected by centrifugation for 45 min at 105,000 g. In one experiment, the pelletized peptides were resuspended in Tricine sample buffer containing 0.2 mM F-5-M and resolved on the 16% gel system. In a second experiment, increasing amounts of the labeled peptides obtained under K⁺- and K⁺-free labeling conditions were applied to each lane. In each experiment, the peptides were transferred to PVDF and washed three times in distilled water for 10-min periods prior to drying and exposure on a BAS-IIIs Fuji Imaging plate for 48–72 h. The exposed imaging plates were read by the Fuji BAS1000 imager and quantified by resident BAS software. The Mᵣ of the smaller peptides was calculated from a log curve fit to the 2.3–14.1-kDa standards from the 14C-labeled low molecular weight standards obtained from American Corp. The integrals of the density profiles of each lane were corrected for a linear background measured at the base of the 6.1-kDa peptide and extending to a 34-kDa 14C-labeled peptide.

Preparation of the 14C DCCD-modified ATPase for V8 Proteolysis

2.5 mg of the H,K-ATPase was treated with 14C DCCD in medium composed of 1 mg/ml enzyme, 1 mM EDTA, 25 mM choline chloride, 25 mM Pipes/Tris, pH 7.4, and 14C DCCD (0.3 mM final concentration at 130 cpm/pmol). The suspension was incubated for 20 min at 37°C and stopped by a 1:1 dilution into ice-cold buffer without DCCD. The protein was separated from the reaction buffer by centrifugation for 45 min at 105,000 g, and the pellet fraction was resuspended in nonreducing Laemmli sample buffer containing 0.2 mM F-5-M. The labeled H,K-ATPase was partially purified by preparative electrophoresis using the 2.8-cm column of the Bio-Rad Prep Cell constructed with a 1-cm stacking gel composed of 3.5% acrylamide and a 4.5-cm running gel composed of 7% acrylamide using the continuous elution method described by Treuheit et al. (31). The labeled preparation was electrophoresed at 6 watts constant current and collected within continuous fractions displaying an overlapping 280-nm elution profile and radioactive peak. The selected fractions were pooled, reduced to 50 μl by ultrafiltration using the Centricon 30™ microconcentrators, and diluted 10-fold into a storage buffer containing 10 mM Tris-CI, pH 7.4, and 0.025% SDS. The protein concentration was determined by the Pierce protein assay using γ-globulin as a standard, and aliquots were stored at ~80°C until V8 protease digestion.

V8 Proteolysis of Partially Purified H,K-ATPase

An aliquot containing 100 μg of the 14C DCCD-labeled, partially purified H,K-ATPase was thawed and incubated with 5 μg of V8 protease for 4 h at 37°C. The reaction was stopped by the addition of a 5× Tricine sample buffer, and the sample was equilibrated for 20 min at ambient temperature prior to the application of 20-μg aliquots of the H,K-ATPase peptides to a 14.5-cm × 1.5-nm Tricine gel equilibrated with a 10-well comb. The peptides were resolved during the electrophoresis at 30 mA (constant current) for 19.5 h. The resolved peptides were then transferred to PVDF by electrophoresis for 20 h at 150 mA in a glycine transfer buffer as described previously (27). The fluorescent, transferred peptides were identified by visualization on a UV light source (312 nm) and photographed using the Ultra Violet Products 7500.

32318
gel documentation system. The fluorescence image was stored as a Tiff file for comparison to the phosphor image profile of the $^{14}$C-DCCD-labeled peptides obtained by the Fuji BAS1000 imager. The $^{14}$C-DCCD-labeled peptides were identified on the PVDF transfer from a 1:1 size overlay of the density profile of the $^{14}$C-DCCD phosphor image. The labeled peptides were selected, removed from the PVDF, and submitted for sequence analysis.

**Sequence Analysis**

Sequence analysis was obtained using methods described previously (27). In brief, after the identification of the labeled peptides on PVDF, the peptides from three identical bands were cut from the PVDF paper, pooled, and analyzed with an Applied Biosystems model 475A sequencing system composed of a model 470A protein sequencer, a model 120A phenylthiohydantoin analyzer, and a model 900A data module. The peptide sequence was identified by comparing the amino acid sequence elevated above background in each sequence cycle. Some bands contained a minor sequence as well as the major sequence.

**RESULTS**

**Inactivation of the H,K-ATPase**—The concentration and time dependence of DCCD inactivation of the H,K-ATPase is shown in Fig. 1. At each DCCD concentration, the time course of inhibition was described by a single inactivation constant, $k'$, and a residual factor inversely proportional to the DCCD concentration. This residual activity has been noted in studies of the Na,K-ATPase and attributed in part to the lability of DCCD in aqueous solution (32). This explanation likely accounts for the residual activity in the present experiments, since the residual ATPase activity following DCCD treatment is inhibited by a second addition of DCCD and prior dilution of the inhibitor into aqueous medium reduces the inhibitor-dependent inactivation of the ATPase (data not shown). The inset provides a plot of the log $k'$ obtained from the experiment of Fig. 1 versus the log of the DCCD concentration. From a consideration of the inhibited enzyme as a stable product arising from the rearrangement of the initial inhibitory complex, Pedemonte and Kaplan (32), utilizing the original model of Levy (33), provided a model of the inactivation where $\log k' = \log k/K_c + n \log [\text{DCCD}]$. The data of Fig. 1 show that $k/K_c = 48 \text{ min}^{-1} \text{ mol}^{-1}$ and $n = 0.72 \text{ mol of DCCD bound per mol of inhibited enzyme}$.

**KCl Protection**—K$^+$-dependent protection of the H,K-ATPase is shown in Fig. 2. The data in Fig. 2A indicate that K$^+$ protects the H,K-ATPase over a broad range of DCCD concentrations. In the absence of KCl, DCCD inhibited the H,K-ATPase with a $K_{0.5}$ (DCCD) = 0.65 ± 0.04 mM. DCCD displaced the concentration dependence for the DCCD inhibition approximately 4-fold where full inactivation of the H,K-ATPase was predicted to occur with a $K_{0.5}$ (DCCD) = 2.3 ± 0.2 mM. Fig. 2B provides a time course of the DCCD inactivation. In the absence of KCl, DCCD more rapidly inactivated the H,K-ATPase with a single time constant of $k' = 0.36 \pm 0.04 \text{ min}^{-1}$, which accounted for approximately 90% of the measured activity. As shown in the upper trace of Fig. 2B, DCCD decreased the rate of inhibition to $k/K_c = 0.026 \pm 0.001 \text{ min}^{-1}$. Similar experiments were performed at pH 6.1 to test the effect of pH on the K$^+$-dependent protection. The concentration dependence for DCCD inactivation at the lower pH was decreased to $K_{0.5}$ (DCCD) = 0.18 ± 0.02 mM. KCl partially protected the enzyme by increasing the inhibitory concentration to $K_{0.5}$ (DCCD) = 0.49 ± 0.05 mM. The decrease in the rate of the inactivation process was less pronounced where $k'$ was reduced from $0.63 \pm 0.04$ to $0.37 \pm 0.06 \text{ min}^{-1}$. Since the ratio of the rate of DCCD inactivation of the unprotected to the KCl-protected enzyme, $k'/k_{KCl}$, was greater at pH 7.4, experiments were routinely performed at this pH to optimize the conditions for K$^+$ protection. The concentration dependence of KCl protection at pH 7.4 is shown in Fig. 3. The protective effect of KCl is saturable with a $K_{0.5}$ of 0.58 ± 0.1 mM and, at saturation, protects 70% of the total ATPase activity. A plausible mechanism for K$^+$ protection has been suggested by Pedemonte and Kaplan (32), where Scheme 1 provides the basis for the derivation of a linear plot to obtain approximations for values related to K$^+$ protection including the K$^+$ association constant $K_c$, and $n$, the number of K$^+$ ions bound to the ATPase.

![Scheme 1](image)

The data plotted in the inset of Fig. 3 are reasonably linear, as predicted by this simple mechanism and provide estimates of $K_c = 1.2 \pm 1 \text{ mM}$ KCl and $n = 3.5 \pm 1.1$. Imprecise numbers are derived for $K_c$ and $n$, but $K_c$ is close to that for K$^+$ activation of the H,K-ATPase (34, 35), and $n$, the number of K$^+$ ions bound to the ATPase, is also close to that reported for the $^{86}$Rb$^+$ binding capacity of the vanadate-inhibited enzyme (27).

**DCCD Inactivation of Functional Domains within the H,K-ATPase**—Since inactivation of the ATPase could result variously from covalent modifications within the catalytic domain, residues important for the relay of conformational changes between domains, or residues within the cation binding domain, it was appropriate to evaluate DCCD-inhibited functions within both the nucleotide and cation binding domains. Several partial reactions provide a more specific measure of the DCCD-dependent inhibition of a functional domain.

**Conformational Transitions within the Cytoplasmic Pump Face**—Eosin has been utilized to report conformation change within the cytoplasmic nucleotide domain of the H,K-ATPase (9). Its fluorescence is quenched by ATP via competitive displacement from the nucleotide binding site or indirectly by K$^+$-induced conformational changes at the nucleotide site. The eosin fluorescence responses of the DCCD-modified enzyme are shown in Fig. 4. The kinetic data in Fig. 4A depict a marked difference in the time course of DCCD-dependent inhibition of the eosin fluorescence responses produced by ATP or K$^+$. The
DCCD Inhibition of the H,K-ATPase

The enzyme was incubated with 1.5 mM DCCD for 10 min at 37 °C, quenched by a 50-fold dilution into ice-cold ATPase assay buffer, and immediately assayed for residual ATPase activity. Lines are drawn to the best fit to $y = 1 - (V_{\text{max}} - X/K_{n} + X)$ where $K_{n} = 0.58 \pm 0.11$ mM KCl and $V_{\text{max}} = 0.70 \pm 0.02$. The inset is a transform of the same data set limited to 5 mM KCl. The line is a linear fit to logarithm of activity, $\log c_{\text{t}}/c_{0}$, residual activity of DCCD-inhibited ATPase in KCl medium, $c_{i}$ is residual activity in KCl-free medium, $n = 3.5$, and $Kc = 1.2$ mM.

K$^+$-dependent fluorescence response was rapidly lost with $k = 0.45 \pm 0.01$ min$^{-1}$, while the ATP-dependent response was inhibited over a longer time course estimated at $k = 0.048 \pm 0.02$ min$^{-1}$. From comparisons with the above data, the time course of inhibition of the ATPase catalytic activity is closely related to that describing the loss of the K$^+$-dependent fluorescent response and suggests that enzyme inhibition occurs with the modification of a residue(s) important for cation binding-dependent functions rather than nucleotide binding functions.

**Conformational Transitions of a K$^+$-competitive, Extracytoplasmic Inhibitor—MDPQ, a fluorescent, K$^+$ competitive inhibitor of the H,K-ATPase, is closely associated with the cation binding domain on the extracytoplasmic side of the pump molecule and displays distinctive K$^+$-sensitive fluorescence responses for the phosphoenzyme and dephosphoenzyme species (27). This fluorescent inhibitor was utilized as an indirect probe of the binding domain in the DCCD-modified enzyme. The upper trace in Fig. 4B is a measure of the ATP-enhanced fluorescence response of the phosphoenzyme, and the lower trace is the K$^+$-dependent quench of the dephosphoenzyme.

**Fig. 2. K$^+$ protection against DCCD inactivation of the H,K-ATPase.** A, DCCD concentration dependence. 0.03–1.5 mM DCCD (final) was added to the H,K-ATPase (1 mg/ml) suspended in 20 mM Pipes/Tris, pH 7.4, 2 μg of nigericin, and 150 mM KCl (●) or 150 mM choline chloride (○). The suspension was incubated for 10 min at 37 °C, quenched by a 50-fold dilution into ice-cold ATPase assay buffer, and immediately assayed for residual ATPase activity. Lines are drawn to the best fit to $y = 1 - (V_{\text{max}} - X/K_{n} + X)$ where $K_{n} = 0.65 \pm 0.04$ mM DCCD with $V_{\text{max}} = 1$. ○, $K_{n} = 2.33 \pm 0.2$ mM DCCD with $V_{\text{max}} = 1$. B, DCCD time course. The H,K-ATPase was prepared as in A and incubated with 1.5 mM DCCD (final). At the indicated times, an aliquot (22.5 μg of protein) was removed, and the reaction was quenched by dilution into ice-cold ATPase buffer. At the completion of the time course, all aliquots were assayed for ATPase activity. ○, ATPase activity of the K$^+$-protected enzyme. ▲, ATPase activity of the unprotected enzyme. Lines are drawn to the best fit to $y = A \times \exp^{-kX} + \text{residual}$. ▲, $k = 0.38 \pm 0.04$ min$^{-1}$, $A = 1$, and residual $= 0.08 \pm 0.03$. ○, $k = 0.026 \pm 0.001$ min$^{-1}$, $A = 1$, and residual $= 0$.

**Fig. 3. Concentration dependence of K$^+$ protection.** The H,K-ATPase was suspended at 37 °C in 20 mM Pipes/Tris, pH 7.4, nigericin, and 0.5–30 mM KCl. The enzyme was incubated with 1.5 mM DCCD (final) for 7 min at 37 °C, and the reaction was quenched by a 50-fold dilution into ATPase buffer. The line is drawn to the best fit of the data to $y = V_{\text{max}} \times X/K_{c} + X$ where $K_{c} = 0.58 \pm 0.1$ mM KCl and $V_{\text{max}} = 0.70 \pm 0.02$. The inset is a transform of the same data set limited to 5 mM KCl. The line is a linear fit to $\log c_{\text{t}}/c_{0}$, residual activity of DCCD-inhibited ATPase in KCl medium, $c_{i}$ is residual activity in KCl-free medium, $n = 3.5$, and $Kc = 1.2$ mM.

The fluorescent responses to both ligands were inhibited in the DCCD-modified enzyme over similar time courses where $k = 0.38 \pm 0.05$ and 0.32 ± 1 min$^{-1}$, respectively. The rate of loss of the ATP-dependent MDPQ response was also correlated with the rate of inhibition of the ATP-dependent phosphoenzyme (data not shown).

**Inactivation of $^{86}$Rb$^+$ Occlusion—$^{86}$Rb$^+$ occlusion within the H,K-ATPase provides a direct measure of the cation binding capacity of the enzyme. In the untreated ATPase, 1091 ± 119 pmol of $^{86}$Rb$^+$ are occluded per mg of protein following equilibration in 0.3 mM Rb$^+$. The experiment of Fig. 5 shows that DCCD inhibited 70% of the $^{86}$Rb$^+$ bound to the H,K-ATPase over a monoeXponential time course, where $k = 0.84 \pm 0.2$ min$^{-1}$. 10 mM KCl protected the $^{86}$Rb$^+$ binding site over the time period sufficient to inactivate the enzyme in the absence of the cation.

**Pattern of Proteolytic Cleavage Influenced by DCCD—Ligand binding stabilizes conformational states that are defined by the initial cleavage patterns of the ATPase. A peptide map of the initial cleavage pattern of the DCCD-inhibited protein was obtained to identify the ligand-dependent conformational sensitive to inhibitor modification. The experiments in Fig. 6 show the tryp tic cleavage peptides obtained from the K$^+$- and ATP-stabilized H,K-ATPase. The peptide cleavage pattern of the ATP-stabilized control enzyme is shown in panel A. The $A_{2}$ peptide at 68 kDa is the most prominent peptide obtained under these conditions, and it, along with the $A_{4}$ peptide at 35 kDa, compose the characteristic Coomassie-stained map of the initial cleavage products of the unmodified pump. A faintly stained $A_{3}$ peptide at 41 kDa was also obtained in the latter time points. The ATP-dependent cleavage pattern of the DCCD-inhibited enzyme is shown in panel B. The characteristic peptide map is similar to the unmodified enzyme, where the $A_{3D}$ and $A_{4D}$ (D specifies DCCD-inhibited) peptides are observed at 68 and 35 kDa along with a faintly stained $A_{3D}$ peptide at 41 kDa. Closer inspection reveals a minor variant where an additional, faintly stained $A_{1D}$ peptide at 74 kDa is present in the DCCD-modified enzyme. The peptide map of the KCl-stabilized control enzyme is shown in panel C. An early cleavage event yields the K$^+$ peptide fragment at 75 kDa. Further proteolysis produces a characteristic pattern containing the K$^+$ and K$^+$ peptides at 54 and 41 kDa, respectively. The K$^+$ peptide at 39 kDa begins to appear as a doublet to the K$^+$ peptide in the incubation periods beyond 10 min. In the final time periods, the K$^+$-dependent peptide map is dominated by...
DCCD Inhibition of the H,K-ATPase

Fluorescence. The H,K-ATPase (52 mU) was modified ATPase. The modified preparation also lacks the K4/K5 kDa accumulation in this preparation in contrast to the unmodified... shown). The difference in DCCD incorporated in the presence of KCl (circles) or 1 mM ATP (■). Each line represents the best fit to the data drawn to y = A × exp(kx). For KCl, k = 0.45 ± 0.01 min⁻¹ and A = 0.90 ± 0.01. For ATP, k = 0.048 ± 0.02 min⁻¹ and A = 1.01 ± 0.05. B, MDPQ fluorescence. The H,K-ATPase (52 mU) was equilibrated with 1.5 mM DCCD in K⁻-free buffer. At the indicated times, the enzyme was added directly to the fluorescence assay buffer, and fluorescence responses were measured after additions of 0.1 mM ATP (final) (○) or 10 mM KCl (final) (△).

The coordinated appearance of the K5 and K4/K5 peptides. The peptide map of the K⁻-stabilized, DCCD-modified enzyme is shown in panel D. The K1D peptide at 75 kDa is present in the early cleavage preparation, but the peptide map changes significantly in the latter stages of proteolysis. A K2D peptide at 68 kDa accumulates in this preparation in contrast to the unmodified ATPase. The modified preparation also lacks the K3/K4 peptides that are present in the unmodified protein. In summary, the characteristic peptides of the DCCD-modified preparation are the K2D and K3D peptides at 68 and 55 kDa, while the characteristic peptides of the control preparation are the K3 and K4/K5 peptides at 54 and 41/39 kDa.

Stoichiometry of [14C]DCCD Incorporation—Fig. 7A shows that approximately 1.6 nmol of the 5.2 nmol of [14C]DCCD incorporated into the H,K-ATPase were K⁻-sensitive. Fig. 7B shows the concentration dependence between the K⁻-sensitive [14C]DCCD incorporated into the H,K-ATPase and the inhibition of ATPase activity. The relationship between activity and the amount of label incorporated is reasonably linear and can be extrapolated to full inhibition where approximately 1.1 nmol of [14C]DCCD are incorporated into the H,K-ATPase. This stoichiometry is close to the phosphoenzyme stoichiometry of 1154 pmol of phosphoenzyme/mg of protein (data not shown).

K⁻-sensitive Incorporation of [14C]DCCD—One explanation for the observed K⁻ protection against DCCD-dependent inactivation of the ATPase is that K⁻ binding prevents the activation of a carboxyl residue important for activity. To evaluate the role of K⁻ in the protection of residues from attack by DCCD, the H,K-ATPase was modified in the presence or absence of K⁻. Membrane-associated tryptic peptides were prepared from the modified ATPase, resolved by SDS-PAGE, and transferred to PVDF. The distribution of [14C]DCCD within the resolved peptides was quantitated by analysis of the exposure profile recorded on a phosphor image plate. The phosphor image profile of the tryptic peptides obtained with proteolysis in K⁻-free medium is shown in Fig. 8. The majority of [14C]DCCD was asymmetrically distributed within a small peptide cluster between 9.6 and 6.1 kDa. A smaller doublet band was also present in an ~23-kDa gel region previously shown to contain the C terminus of the H,K-ATPase beginning with the sequence LVNEG™ (27). The recovery of ~23-kDa peptide in the tryptic preparation varied in several experiments utilizing different tryptic batches and H,K-ATPase preparations ranging from 7 to 50% of the total [14C]DCCD distributed into the peptides.

The data of Table I show that when the ~23-kDa peptide is a significant portion of the tryptic preparation (percentage of total K⁻-sensitive), up to 78% of the [14C]DCCD incorporated into this band is K⁻-sensitive. Table I also identifies the distribution of [14C]DCCD into the peptides within the smaller peptide cluster. These peptides, along with the ~23-kDa peptide, account for more than 90% of the [14C]DCCD present in the purified peptides. Within the small peptide cluster, the majority of the total K⁻-sensitive [14C]DCCD was present in the larger 9.6- and 8.2-kDa peptides, while less than 5% of the total K⁻-sensitive [14C]DCCD was present in the 7.1- and 6.1-kDa peptides. These results show that [14C]DCCD is almost exclusively distributed into peptides of ~23, 9.6, and 8.2 kDa and that this label is sensitive to the presence of K⁻ in the labeling buffer.
Identification of Tryptic Peptides Containing \([^{14}C]\)DCCD—

The initial strategy to identify the hydrophobic pump regions selectively modified by \([^{14}C]\)DCCD was to resolve tryptic peptides obtained from the membrane-associated ATPase, identify the peptides incorporating \([^{14}C]\)DCCD, and obtain N-terminal sequence from the modified peptide transferred to a PVDF membrane. To accomplish this, the membrane-associated peptides of the \([^{14}C]\)DCCD-modified H,K-ATPase were collected by centrifugation following tryptic digestion and labeled with the fluorescent thiol reagent, F-5-M, to aid in the visualization of cysteine residues located within transmembrane regions. This permitted the identification of peptides from their fluorescence profile, the phosphor image profile of \([^{14}C]\)DCCD present in the peptides, or a radiometric profile obtained from serial slices of the PVDF membrane taken through the gel regions of interest. A typical fluorescence pattern of the modified peptides is shown in the inset of Fig. 9. The pattern displays a broad, low level fluorescence band at \(23\) kDa and a cluster of several smaller bands ranging from \(9.6\) to \(4.9\) kDa. The \([^{14}C]\)DCCD distribution within the smaller fluorescent peptides was measured by scintillation counting of either the intact, excised band visualized by its fluorescence or by the distribution of \([^{14}C]\)DCCD present in \(1\)-mm slices taken throughout the smaller peptide region. The areas marked with a \(\square\) below the data points in Fig. 9 indicate that the distribution of \([^{14}C]\)DCCD in the presence or absence of \(25\) mM KCl was resolved on a mini format, \(16.5\%\) Tricine gel. The \([^{14}C]\)DCCD incorporated into the peptides was determined by analysis of an exposed phosphor imager cassette. The upper trace provides a densitometric profile of the phosphor image obtained from the peptides labeled in the presence of KCl. The lower image provides the original phosphor image of the peptides modified in the presence or absence of KCl.
TABLE I  
Distribution of K*-sensitive [14C]DCCD into tryptic peptides

| Experiment 8–3 | 34 kDa | 23 kDa | 9.6 kDa | 8.2 kDa | 7.1 kDa | 6.1 kDa |
|----------------|--------|--------|---------|---------|---------|---------|
| pkl (2.4 μg)  | -      | +      | +       | -       | -       | +       |
| pkl (4.4 μg)  | 33     | 34     | 132     | 122     | 226     | 128     |
| % K*-sensitive| 0      | 10     | 7       | 44      | 41      | 2       |
| % total K*-sensitive| 0 | 7 | 7 | 52 | 36 | 2 |
| Experiment 9–20| 34 kDa | 23 kDa | 9.6 kDa | 8.2 kDa | 6.1 kDa |
| pkl (13 μg)   | -      | +      | +       | -       | -       | +       |
| % K*-sensitive| 23     | 78     | 58      | 67      | 40      |
| % total K*-sensitive| 3 | 46 | 23 | 24 | 4 |

% K*-sensitive refers to the fraction of K*-sensitive pkl present in each discrete peptide band. % total K*-sensitive refers to the fractional contribution of each peptide to the total population of K*-sensitive peptides.

was enriched in the region between 9.6 and 8.2 kDa.

Using an alternative strategy, a tryptic membrane preparation was prepared from the H,K-ATPase equilibrated with KCl. This tryptic preparation has been previously shown to contain a functional Rb occlusion site as well as the MDPQ inhibitor site and is typically characterized by the presence of a prominent ~23 kDa, C-terminal peptide. The K*-dependent tryptic preparation was modified with [14C]DCCD, labeled with F-5-M, and resolved by SDS-PAGE. The [14C]DCCD-labeled peptides were visualized by autoradiography. Fig. 9C shows the fluorescent pattern of F-5-M-labeled peptides. This fluorescent peptide distribution differs from preparations obtained with tryptic digestion in the absence of KCl (see Fig. 8), where the K*-dependent preparation displays a more prominent ~23 kDa band and a simplified smaller peptide cluster. The densitometric scan in panel A of the autoradiograph in panel B indicates that the ~23 kDa band is highly enriched in the [14C]DCCD-labeled peptides, while only a minor component of [14C]DCCD was present within the smaller peptide cluster.

Sequence of Residues Containing [14C]DCCD—N-terminal sequence was obtained from both the 9.6-kDa peptide band of

was modified with [14C]DCCD in KCl. Experiment 9–20 reports the mean of data obtained from n = 3 lanes of ATPase modified under each condition. % K*-sensitive refers to the fraction of K*-sensitive pkl present in each discrete peptide band. % total K*-sensitive refers to the fractional contribution of each peptide to the total population of K*-sensitive peptides.

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Distribution of K*-sensitive [14C]DCCD into tryptic peptides

| Experiment 8–3 | 34 kDa | 23 kDa | 9.6 kDa | 8.2 kDa | 7.1 kDa | 6.1 kDa |
|----------------|--------|--------|---------|---------|---------|---------|
| pkl (2.4 μg)  | -      | +      | +       | -       | -       | +       |
| pkl (4.4 μg)  | 33     | 34     | 132     | 122     | 226     | 128     |
| % K*-sensitive| 0      | 10     | 7       | 44      | 41      | 2       |
| % total K*-sensitive| 0 | 7 | 7 | 52 | 36 | 2 |
| Experiment 9–20| 34 kDa | 23 kDa | 9.6 kDa | 8.2 kDa | 6.1 kDa |
| pkl (13 μg)   | -      | +      | +       | -       | -       | +       |
| % K*-sensitive| 23     | 78     | 58      | 67      | 40      |
| % total K*-sensitive| 3 | 46 | 23 | 24 | 4 |

% K*-sensitive refers to the fraction of K*-sensitive pkl present in each discrete peptide band. % total K*-sensitive refers to the fractional contribution of each peptide to the total population of K*-sensitive peptides.

Distribution of K*-sensitive [14C]DCCD into tryptic peptides

| Experiment 8–3 | 34 kDa | 23 kDa | 9.6 kDa | 8.2 kDa | 7.1 kDa | 6.1 kDa |
|----------------|--------|--------|---------|---------|---------|---------|
| pkl (2.4 μg)  | -      | +      | +       | -       | -       | +       |
| pkl (4.4 μg)  | 33     | 34     | 132     | 122     | 226     | 128     |
| % K*-sensitive| 0      | 10     | 7       | 44      | 41      | 2       |
| % total K*-sensitive| 0 | 7 | 7 | 52 | 36 | 2 |
| Experiment 9–20| 34 kDa | 23 kDa | 9.6 kDa | 8.2 kDa | 6.1 kDa |
| pkl (13 μg)   | -      | +      | +       | -       | -       | +       |
| % K*-sensitive| 23     | 78     | 58      | 67      | 40      |
| % total K*-sensitive| 3 | 46 | 23 | 24 | 4 |
DCCD Inhibition of the H,K-ATPase

Table II

| 14C/DCCD-labeled peptides resolved from trypsin and V8 protease digests |
|-------------------------------|--------------------|
|                              | [14C]DCCD-Enriched N-Terminal Fragment |
| A. 23 kDa                    | L (1/3)            |
| B. 9.6 kDa                   | X                  |
| C. 7.1 kDa                   | N (2.5/3)          |
| D. 3.3 kDa                   | X                  |
| E. 3.3 kDa                   | X                  |

Fig. 11. V8 peptide map of the partially purified α-subunit. The native H,K-ATPase was modified with [14C]DCCD, and the α-subunit was partially purified by preparative electrophoresis as described under “Materials and Methods.” The α-subunit was digested with V8 protease and labeled with F-5-M. Aliquots containing 20 μg of protein were resolved by SDS-PAGE using a standard format 16.5% Tricine gel and transferred to PVDF for radiometric analysis and sequence. The left lane provides a phosphor image of V8 peptides labeled with [14C]DCCD. The right lane provides a fluorescent image of the F-5-M-labeled peptides.

The V8 peptide digest are shown in Fig. 11. The phosphor image shown in the left lane identifies a [14C]DCCD-labeled peptide at 3.3 kDa. Inspection of the fluorescent profile in the right lane shows that the [14C]DCCD-labeled peptide does not incorporate the fluorescent label. The sequence obtained from two experiments is shown in Table II. Both sequences identify the N terminus of the peptide as SDIM, a cytoplasmic portion of the molecule preceding the putative M7 transmembrane domain.

DISCUSSION

These data provide biochemical evidence of an essential carboxyl residue within a functionally defined cation binding domain of the H,K-ATPase. The functional criteria are severalfold including 1) K⁺ protection from DCCD inhibition of H,K-ATPase catalysis; 2) selective loss of K⁺-stabilized conformational structure within the cytoplasmic domains; 3) DCCD inhibition of 86Rb⁺ binding; and 4) K⁺ protection from inhibition of 86Rb⁺ binding.

The cation binding site that protects the ATPase from DCCD attack is arguably that responsible for the activation of the ATPase, since its saturation demonstrates the same concentration dependence as K⁺-activated hydrolysis. It is also likely that this site resides near or within the transmembrane domains of the ATPase, since both 86Rb⁺ binding and the MDPQ fluorescence response is present within the tryptic membrane preparation devoid of the M4/M5 cytoplasmic loop containing elements of the nucleotide and catalytic sites of the ATPase (10, 27, 36). Finally, the data indicate that the inactivation of the ATPase is functionally selective, because ATP-dependent responses such as the stabilization of conformationally defined tryptic maps and eosin fluorescence responses are spared, while cation-dependent responses are inhibited.

The specific loss of function within the partial reactions involved in cation binding is similar to results obtained in the Na,K-ATPase and Ca-ATPase following inactivation with carboxyl activating agents including DCCD (37–39), NCD-4 (40, 41), and 4-(diazomethyl)-7-(diethylamino)-coumarin (42). Together these data suggest that the carboxyl amino acids participate in cation-dependent functions within the P type ion pumps, although their mechanism of involvement remains controversial.

Alternatively suggested mechanisms of inhibition include cross-linking via nucelophilic attack on a nearby residue and hydrolysis with production of the native enzyme as well as the incorporation of a stable N-aclysiofore (32, 38, 43, 44). Inspection of the gels of the initial tryptic digests with regard to the presence of mobility shift indicative of cross-linked species are inconclusive in these experiments, although Gorga (30) has reported that cross-linking of the Na,K-ATPase into species of high molecular weight proceeds significantly slower than inactivation of the Na,K-ATPase.

While the functional criteria are compatible with a modification within the cation binding domain, it is important to point out that these functional data do not exclude the possibility that the DCCD-modified residue(s) interfere indirectly with ATPase function, for example by preventing the propagation of conformational changes between specific domains of the enzyme following cation binding. Ultimately, the assignment of a specific structural role of any residue must include high resolution structural detail in addition to biochemical evidence. The biochemical data do, however, identify a residue important for cation binding or linkage of cation binding to conformational changes.

The data in Fig. 7 show that [14C]DCCD is covalently incorporated into the H,K-ATPase over a time course similar to that of ATPase inhibition. A portion of the DCCD, approximately 1–1.6 nmol of [14C]DCCD/mg of protein is reasonably linearly correlated with the loss of K⁺-sensitive activity. While the accuracy of this determination is limited by the incomplete cation protection from DCCD incorporation, the stoichiometry is consistent with the involvement of relatively few molecules in the ATPase. Quantitative analysis of the modified peptides indicated that [14C]DCCD was specifically incorporated into three membrane-associated tryptic peptides resolved at ~23, 9.6, and 8.2 kDa. The modification of these peptides was sensitive to K⁺, which reduced the amount of [14C]DCCD incorporation within the peptides from 44 to 78%.

In one experimental data set, [14C]DCCD was heavily distributed into the ~23-kDa peptide with the N terminus LVNE located at the predicted cytoplasmic boundary of M7. A [14C] DCCD-enriched N-terminal fragment of this peptide was also identified within a 9.6-kDa band, as was a second sequence with the N terminus SIAY originating from the predicted transmembrane sequence, M5. It is more likely that the [14C] DCCD present in the 9.6-kDa band resulted from the modifi-
cation of a residue within the peptide containing the M7/M8 domain rather than the M5/M6 domain, since a second M5 sequence present in the 7.1-kDa band contained less than 5% of the [14C]DCCD distributed into the trypic peptides.

These data are summarized in the hydropathy model of Fig. 12, which suggests that a prominent site of labeling is the M7/M8 region of the pump. The identification of the carboxyl residue within this domain was aided by the purification and analysis of a 3.3-kDa peptide obtained from V8 digests of the partially purified α-subunit. This peptide with the N-terminal sequence SDIM would overlap the LVNE sequence identified within the trypic peptides and provides a single common carboxyl residue at Glu

Comparison of sequence homology among the sarcoplasmic reticulum Ca-ATPase, the Na,K-ATPase, and the H,K-ATPase within the continuous cytoplasmic/M7 domain indicates that while this domain is conserved with 22 and 53% homology within the Ca

While this site is conserved within the Na,K-ATPase, it does not appear to be the major site of DCCD modification, despite structural similarities revealed by trypic cleavage patterns (39, 45). In each pump, trypic cleavage in the presence of K

In summary, the present data provide functional evidence of selective inactivation of K

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DCCD Inhibition of the H,K-ATPase

sensitive DCCD-dependent labeling within an 8.2-kDa peptide band. Additional work is necessary to identify the N terminus and DCCD-modified residue within this band, since additional residues may be implicated as several residues necessary for cation binding in the Na,K-ATPase and the sarcoplasmic reticulum Ca-ATPase are conserved in the H,K-ATPase (48–51). It is also speculated that residues in the M1/M2 domain that bind the K

It should be noted that the present data also show K

DCCD Inhibition of the H,K-ATPase

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