Distance Variations between Active Sites of H+-Pyrophosphatase Determined by Fluorescence Resonance Energy Transfer*

Received for publication, April 20, 2010, and in revised form, May 28, 2010. Published, JBC Papers in Press, May 28, 2010, DOI 10.1074/jbc.M110.134916

Yun-Tzu Huang¹, Tseng-Huang Liu¹, Yen-Wei Chen¹, Chien-Hsien Lee³, Hsueh-Hua Chen⁸, Tsu-Wei Huang⁹, Shen-Hsing Hsu¹, Shih-Ming Lin⁵, Yih-Juian Pan⁵, Ching-Hung Lee⁶, Ian C. Hsu¹, Fan-Gang Tseng⁵, Chien-Chung Fu¹, and Rong-Long Pan¹,²

From the Department of Biomedical Engineering and Environmental Sciences, the Department of Engineering and System Science, and the Institute of Nano Engineering and Microsystems, National Tsing Hua University, Hsin Chu 30013, Taiwan

Homodimeric H+-pyrophosphatase (H+-PPase; EC 3.6.1.1) is a unique enzyme playing a pivotal physiological role in pH homeostasis of organisms. This novel H+-PPase supplies energy at the expense of hydrolyzing metabolic byproduct, pyrophosphate (PPi), for H+ translocation across membrane. The functional unit for the translocation is considered to be a homodimer. Its putative active site on each subunit consists of PPi binding motif, Acidic I and II motifs, and several essential residues. In this investigation structural mapping of these vital regions was primarily determined utilizing single molecule fluorescence resonance energy transfer. Distances between two C termini and also two N termini on homodimeric subunits of H+-PPase are 49.3 ± 4.0 and 67.2 ± 5.7 Å, respectively. Furthermore, putative PPi binding motifs on individual subunits are found to be relatively far away from each other (70.8 ± 4.8 Å), whereas binding of potassium and substrate analogue led them to closer proximity. Moreover, substrate analogue but not potassium elicits significant distance variations between two Acidic I motifs and two His-622 residues on homodimeric subunits. Taken together, this study provides the first quantitative measurements of distances between various essential motifs, residues, and putative active sites on homodimeric subunits of H+-PPase. A working model is accordingly proposed elucidating the distance variations of dimeric H+-PPase upon substrate binding.

H+-PPase³ is widely present in higher plants, as well as in some alga, protozoa, archaebacterial, and bacteria (1, 2). This unique enzyme hydrolyzes PPi to generate H+-motive force across vacuolar membrane in plants and plasmic membrane in prokaryotes (3, 4). In addition, H+-PPase requires Mg²⁺ ion with PPi as the actual substrate (Mg₂PPi) for the hydrolyzing reaction (5, 6). Furthermore, K⁺ is essential for stimulating type I H+-PPase; however, Ca²⁺, Na⁺, and F⁻ inhibit its reaction (7). H+-PPase consists of a single polypeptide with a molecular mass ranging from 60 to 81 kDa for various species (3). Radiation inactivation analysis revealed a functional size of a monomer suffices for enzymatic reaction of H+-PPase, whereas that for H+ translocation is homodimeric (8, 9). Further investigation under high hydrostatic pressure demonstrated that proper interaction between the homodimeric subunits of H+-PPase is crucial for PPi-supported H+ translocation (10). Besides, the dimeric structure of H+-PPase from Thermotoga maritima was shown by electron microscopy (11). Furthermore, the homodimeric mung bean H+-PPase in the lipid bilayer had been visualized by atomic force microscopy to be larger in size at cytoplasmic protrusions but relatively smaller at luminal protrusions (12). After thermoinactivation or trypsin digestion, H+-PPase displayed conformational changes upon substrate binding (13, 14). Mutagenic analysis demonstrated fundamental roles of Lys-261 and Glu-263 in the PP binding motif of mung bean H+-PPase for the substrate binding and the energy conversion (14). All aspartate residues in Acidic motifs and putative PPi binding motif are involved in substrate interaction and in sustaining the physiological functions (14). Notwithstanding, detailed information on the structure/function relationships still requires further efforts.

Fluorescence resonance energy transfer (FRET) is a powerful biophysical technique to determine the distances between a donor and an acceptor for elucidating the conformation and dynamics upon enzyme activation (15–18). To dissect the structure of H+-PPase, FRET was then employed at single molecule level to measure the distances between essential regions within homodimeric H+-PPase of Clostridium tetani E88 (CtH+-PPase) in the present study. The distances between two C termini and two N termini as well as two active sites within CtH+-PPase dimer were measured. Both C termini are close to each other and near the interface of subunits, whereas both N termini at a relatively remote position of the dimeric H+-PPase. Moreover, distance measurements indicated that the K⁺ and substrate analogue could presumably induce conformational changes of active site surrounding several
smFRET Analysis of CtH+-PPase

essential motifs and residues. The primary distance determination in this investigation provides new insights into structural features relevant to the active site and ligand interactions for further understanding the mechanism of enzymatic reaction in H+-PPase.

EXPERIMENTAL PROCEDURES

CtH+-PPase DNA Construction and Mutagenesis—The CtH+-PPase gene (GenBank™ accession number AA035020) was amplified from C. tetani E88 genomic DNA with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) and then cloned into pET23d vector (Novagen, Nottingham, UK) with a C-terminal His6 tag. Mutant derivatives were generated from wild type CtH+-PPase by the QuikChange site-directed mutagenesis method (19), and the sequences were verified by DNA sequencing. Cysteine-less CtH+-PPase mutants were prepared by subsequently replacing the two endogenous cysteines at positions 80 and 542 with serines and/or alanine. A set of single cysteine residues was then introduced into the cysteine-less CtH+-PPase mutants at active sites, C and N termini, respectively (Fig. 1).

Microsome Isolation and Protein Purification—E. coli microsomes were isolated by a procedure described previously with minor modifications (20, 21). DNA constructs of wild type and mutant CtH+-PPases were transformed into Escherichia coli strain C43(DE3) (Lucigen, Middleton, WI), and transformants were grown at 37 °C in Luria-Bertani medium supplemented with 50 µg/ml ampicillin. After a 3-h incubation at 37 °C until A600 = 0.8, 400 µM isopropyl β-D-thiogalactoside was added. Cultures were further grown for 4 h at 37 °C, and cells were finally harvested by centrifugation at 4000 × g for 15 min. The cells were washed with a solution (50 mM Tris/Mes (pH 8.0), 1 mM MgCl2, 75 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, and 20% (w/v) glycerol) and subsequently resuspended in 50 ml of the same medium supplemented with DNase I (20 µg/ml). After lysozyme digestion (200 µg/ml) with gentle shaking at 4 °C for 10 min, the cells were disrupted by sonic disruption. The homogenate was then centrifuged differentially at 4,000 × g for 15 min and 75,000 × g for 35 min at 4 °C. The membrane vesicles were resuspended in 5 mM Tris/Mes (pH 7.5) and 20% (w/v) glycerol and stored at −80 °C for further use.

The CtH+-PPase was solubilized from microsomal membrane (1 mg/ml) in an extraction buffer (25 mM MOPS/KOH (pH 7.2), 400 mM KCl, 4 mM MgCl2, 20% (w/v) glycerol, 1 mM phenylmethanesulfonyl fluoride, and 0.25% (w/v) n-dodecyl-β-D-maltopyranoside). The suspension was gently stirred at 4 °C for 30 min followed by centrifugation at 75,000 × g for 55 min. The supernatant was immediately incubated with Ni2+-nitrilotriacetic acid beads (Qiagen, Valencia, CA) under gentle shaking at 4 °C for 1 h. The supernatant and matrix were injected into a 15-ml column and washed with the elution buffer (25 mM MOPS/KOH (pH 7.2), 400 mM KCl, 4 mM MgCl2, 20% (w/v) glycerol, 1 mM phenylmethanesulfonyl fluoride, and 0.05% (w/v) n-dodecyl-β-D-maltopyranoside) with a step gradient of 10 and 50 mM imidazole. After washing, CtH+-PPase proteins were eluted with 250 mM imidazole in elution buffer.

CtH+-PPase Activity Assays—PPi hydrolytic activity of H+-PPase was assayed according to the previous protocol with minor modifications (20). The reaction mixture consisting of 10 mM Tris/Mes (pH 8.0), 1 mM MgCl2, 100 mM KCl, 250 µM NaF, 250 µM Na3PPi, 1.5 µg/ml gramicidin D, and 20 µM of microsomes were incubated at 37 °C for 10 min. The reaction was then terminated by a stop solution (0.7% (w/v) ammonium molybdate, 2.0% (w/v) sodium dodecyl sulfate, 0.02% (w/v) 1-amino-2-naphthol-4-sulfonic acid, and 1.16 N HCl). Pi released by enzymatic reaction was determined spectrophotometrically (20). H+ translocating activity coupling ratio and ion effects was determined according to previous methods (20, 22). The background concentrations of K+, Na+, and Ca2+ in the assay media were measured as below 0.5 µM determined by inductively coupled plasma mass spectrometry.

Fluorescence Spectroscopy—Fluorophore-labeled CtH+-PPases (5 nM) were immobilized on the coverslips according to the previous protocol with minor modifications (12, 18). Coverslips were cleaned and freshly coated with L-polylysine. Total internal reflection fluorescence microscopy was routinely performed with Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). The sample was placed above an oil immersion Olympus 60×/1.45NA internal reflection fluorescence microscope objective and illuminated with an argon laser (488 nm, 10 milliwatts) (Olympus, Tokyo, Japan) and a diode laser (638 nm, 35 milliwatt) (Omicron Laserage, Rodgau, Germany). The emitted fluorescence from Alexa Fluor 488 and 647 was then collected through the same objective. The fluorescence emissions from Alexa Fluor 488, Alexa Fluor 647, or FRET were separated by an emission splitter (U-SIP, Olympus, Tokyo, Japan) with a 600-nm dichroic mirror (Omega Optical, VT) and band pass filters (Omega Optical) appropriate to the donor and acceptor fluorophores (520-DH-40 for donor and 670-DH-40 for acceptor). The image was then recorded by a Sensicam electron-multiplying charge coupled device camera (Cooke, MI).

Single Molecule Imaging Assays—CtH+-PPase dimer was labeled with cysteine reactive fluorophores (maleimide-reactive probe; Molecular Probes, Invitrogen) after standard labeling protocols provided by manufacturer. For single molecule imaging, purified CtH+-PPase in 10 mM MOPS/KOH (pH 7.2) and 0.05% (w/v) n-dodecyl-β-D-maltopyranoside was reacted with a 5-fold molar excess of both fluorophore Alexa Fluor 488 and 647, simultaneously. After 2 h of incubation at 4 °C, unreacted fluorophore was removed by buffer exchange on an Amicon® Ultra-4 centrifugal filter device with a cutoff of 30 kDa (Millipore, Bedford, MA). All buffer solutions were degassed and passed through 0.22-µm filter before use.

Images for donor and acceptor emissions were analyzed at least 100 frames using Image-Pro Plus software (Media Cybernetics, MD). Background-subtracted fluorescence intensities were determined, and the corrected FRET efficiencies (E) are defined by Equation 1 (23),

$$ E = \frac{I_d}{I_d + \gamma I_a} $$

(Eq. 1)

where $I_d$ and $I_a$ are the background-subtracted fluorescence intensities from donor and acceptor channels, respectively,
when FRET occurs (23). The correction factor $\gamma$ is given by Equation 2 (24),

$$\gamma = \frac{\eta_A}{\eta_D}$$

where $\eta_A$ and $\eta_D$ are the detection efficiencies of donor and acceptor signals, yielding $\eta_A/\eta_D$ of $\sim 1.0$ (23, 24). The $\psi_A$ and the $\psi_D$ are the quantum yields of fluorescence donor and acceptor, respectively. The efficiencies ($E$) measured from each molecule are converted into histogram. A Gaussian fit to the histogram with the efficiency value ($E^*$) is obtained using SigmaPlot software (SPSS, Chicago, IL).

Distance calculation is based on the Förster theory with the energy transfer efficiency depending on the inverse sixth power of the distance between fluorescence donor and acceptor. The distance ($R$) is given by Equation 2 (15),

$$R = R_0 \left(1 - \frac{E^*}{E^*} \right)^{1/6} (\text{Å})$$

where the Förster distance ($R_0$) is the interfluorophore distance at which the energy transfer efficiency is 50% (15). For calculating $R_0$, the orientation factor $\kappa^2$ was conventionally taken as the isotropic-averaged value of $2/3$ (25). A Förster distance of 56 Å between Alexa Fluor 488 and 647 was, therefore, obtained (25).

**RESULTS AND DISCUSSIONS**

Construction and Characterization of CtH$^+$-PPase Mutants—The DNA sequence and topological analyses suggest that the single polypeptide of CtH$^+$-PPases is composed of 673 amino acids with its secondary structure containing 16 transmembrane domains (TMs), cytoplasmic and periplasmic loops, and N and C termini (Fig. 1) (21). In addition, several highly conserved regions in the loops 5 and 15, including PPi binding motif (DVGADLVGKVE$^{190}$), and two Acidic motifs (Acidic I motif, DNVGDNVGD$^{215}$; Acidic II motif, DTVGDPFKD$^{637}$) are predicted to form a part of the active site for implementing PPi hydrolysis function. The native H$^+$-PPase gene from *C. tetani* E88 was cloned into pET23d vector and heterologously expressed in *E. coli* strain C43(DE3) under control of the T7/lac promoter. Microsomal CtH$^+$-PPase, successfully prepared as described under “Experimental Procedures,” were observed on SDS-PAGE and immunoblot analysis (Fig. 2A). The molecular mass of the CtH$^+$-PPase subunit was then calculated to be 65 kDa, relatively smaller than those from higher plants (7, 20). Microsomal CtH$^+$-PPase displayed PP, hydrolysis and PP$_i$-associated H$^+$ translocation activities, whereas that transformed by vector alone showed only negligi-
smFRET Analysis of CtH⁺-PPase

**FIGURE 2. Heterologous expression, characterization and purification of CtH⁺-PPase.** Wild type CtH⁺-PPase with the His₆ tag was inserted into vector pET23d and heterologously expressed in *E. coli* strain C43(DE3). A, SDS-PAGE and Western blotting are shown. Aliquots of 20 μg of microsomes and 5 μl of purified CtH⁺-PPase proteins from each sample were subjected to SDS-PAGE, and then Western analysis were carried out using monoclonal anti-His antibody. Lower panel, SDS-PAGE; upper panel, immunoblot analysis using anti-His antibody. Lane 1, microsomal fractions of *E. coli* with vector alone; lane 2, microsomal fractions of *E. coli* containing wild type CtH⁺-PPase; lane 3, purified CtH⁺-PPase. B, PPi hydrolyzing activities are shown. C, shown is PPi-supported H⁺ translocation. V, microsomal fraction prepared from the construct with wild type CtH⁺-PPase. The concentrations of ions, if present: 1 mM Mg²⁺, 100 mM K⁺, 100 mM Na⁺, 0.1 mM Ca²⁺, and 10 mM F⁻. Proton pumping was initiated by adding 1.0 mM PPi. At the end of each reaction, 3 μg/ml gramicidin D was added to stop the fluorescence quenching of acridine orange. GD, gramicidin D. Values are the means ± S.D. from at least three independent experiments. D, Three-dimensional atomic force microscopy image of purified CtH⁺-PPase adsorbed onto mica. The scale bar is 100 nm.

PPi hydrolysis activities are stimulated by ~11-fold under 100 mM K⁺, evidently demonstrating that CtH⁺-PPase belongs to type I (K⁺-dependent) H⁺-PPase (1). Both H⁺ translocation and PPi-hydrolyzing activities of CtH⁺-PPase on microsome were not significantly affected by 100 mM Na⁺ but decreased considerably in the presence of 10 mM F⁻ and 0.1 mM Ca²⁺, respectively (Fig. 1, B and C). Furthermore, CtH⁺-PPase was successfully purified from microsomes to its homogeneity, as also verified by SDS-PAGE and immunoblot analysis (Fig. 2A). Subsequently, purified CtH⁺-PPases were adsorbed randomly on the mica surface for atomic force microscopy imaging and subjected to analysis as described previously (12). Fig. 2D exhibits a prototypical globular structure of CtH⁺-PPase (13). The volume of CtH⁺-PPase calculated is ~328.5 ± 37.2 nm³ (V; n = 50), similar to mung bean H⁺-PPase in a dimeric and symmetric structure (12).

To map the structure of homodimeric CtH⁺-PPase, a series of mutants with a single cysteine on each subunit was generated for further anchoring of fluorescent dyes. The primary sequence of CtH⁺-PPase contains two cysteines, with Cys-80 located on the end of TM3 toward the periplasm, and Cys-542 in the loop 13-facing cytoplasm (Fig. 1). Cys-542 is highly conserved among various organisms, albeit other members of the H⁺-PPase, especially those from plants, commonly possess a serine in the position of CtH⁺-PPase at Cys-80 (26). To construct functional cysteine-less mutants of CtH⁺-PPase, site-directed mutagenesis was used to displace these two cysteines to alanine or serine. As shown in Fig. 3A, all CtH⁺-PPase mutants were stably expressed at the wild type level on the inner membrane. The C542A mutant showed 87 and 68% of PPi hydrolysis and H⁺ translocation activities of wild type, whereas the C80A mutant even brought a significant increase in PPi hydrolysis activity (130% of wild type). The characterization of C80A and C542A mutations explicitly indicates that none of native cysteines is essential for the enzyme functions. Furthermore, the resulting cysteine-less mutants (C80A/C542A, C80A/C542S, C80S/C542A, and C80S/C542S) also displayed similar functional properties to the wild type. C80S/C542S showing relatively high enzymatic activity subsequently provided a valuable tool for the following cysteine labeling in FRET analysis.

A single cysteine was then planted by replacement of residues at the loop 5 and 15, N and C termini on cysteine-less CtH⁺-PPase (C80S/C542S), respectively. Fig. 1 exhibits the locations of 14 sites at which single cysteine residues were introduced by site-directed mutagenesis. Glu-2 and an additional cysteine (C-1) upstream of the first amino acid, Met-1, are located at the N terminus, whereas Leu-668 is at the C terminus. Ala-191, Arg-199, Val-203, Ala-205, Val-208, Val-212, Val-215, and Ala-216, within or close to highly conserved motifs in loop 5, are individually replaced by cysteine residue. Furthermore, His-622, Val-627, and Val-631 are positioned in loop 5, are individually replaced by cysteine residue. Furthermore, the resulting cysteine-less mutants (C80A/C542A, C80A/C542S, C80S/C542A, and C80S/C542S) also displayed similar functional properties to the wild type. C80S/C542S showing relatively high enzymatic activity subsequently provided a valuable tool for the following cysteine labeling in FRET analysis.
of E2C and R199C mutants were almost completely inhibited (<30% of wild type and cysteine-less mutant), implicating that Glu-2 and Arg-199 are deeply involved in the function of Cth⁺-PPase. Moreover, C-1, V212C, and L668C mutants revealed ~40 and 50–70% less in PPi hydrolysis and H⁺ translocation, respectively. Coupling efficiency of A191C mutant was decreased to 59% of wild type, suggesting its possible association with H⁺ translocation (Fig. 3, B–D).

**Determination of Intersubunit Distance for Both Termini and Two Active Sites**—The single molecule FRET (smFRET) technique was further used to determine a set of intersubunit distances within homodimeric Cth⁺-PPase. Purified Cth⁺-PPase mutant with a single cysteine on each subunit was randomly labeled with the donor fluorophore Alexa Fluor 488 and the acceptor fluorophore Alexa Fluor 647. The following species with donor and acceptor fluorophores presumably linked to two cysteine residues on homodimeric Cth⁺-PPase were generated: donor-donor, acceptor-acceptor, and donor-acceptor. For the donor-donor moieties on the C80A mutant (containing sole cysteine at position 542), excitation by laser at 488 nm produced a strong fluorescence signal corresponding to its emission (at 520 nm) in the donor channel. Nevertheless, no fluorescence signal occurred in acceptor channel (at 670 nm), indicating that there was no fluorescence cross-talk. Besides, the excitation at 638 nm also did not generate any corresponding fluorescence signal (at 670 nm) as expected (Fig. 4A, top). Similarly, as for the acceptor-acceptor species, a brilliant fluorescence signal was detected after excitation at 638 nm. Likewise, no corresponding fluorescence was visible upon the excitation at 488 nm (Fig. 4A, middle). As both donor and acceptor were labeled on C80A mutant (donor-acceptor species), illumination by the 488-nm laser beam generated a donor fluorescence signal in the donor channel as anticipated. Concomitantly, the emission corresponding to the acceptor was observed in the acceptor channel as well, implicating explicitly energy transfer from donor to acceptor. This phenomenon never occurred in both donor-donor and acceptor-acceptor species. Afterward, this donor-acceptor species was further excited by the 638-nm laser beam, and acceptor fluorescence was also visualized in acceptor channel, illustrating the presence of acceptor located at the appropriate position (Fig. 4A, bottom). Taken together, these results verify that both donor and acceptor fluorophores coexisted in the same single Cth⁺-PPase molecule, and energy transfer from donor to acceptor occurred accordingly as the 488-nm laser was excited, demonstrating feasibility of this system for further smFRET measurements (17). Moreover, the FRET efficiency histogram of the C80A mutant (542–542 pair) was then determined. The peak in gaussian distribution from the FRET efficiency histogram of C80A mutant is 0.513 ± 0.088 (n = 93), corresponding to a distance of 55.5 ± 3.4 Å between the 542–542 pair (Fig. 4B, Table 1) that possibly locates at the entrance of both active sites (26, 27).

To further ensure the practicability of this technique for Cth⁺-PPase, a technique combining monomaleimide gold nanoparticle (GNP) labeling with transmission electron microscopy (TEM) was employed to validate the distance measured from smFRET (12). GNPs (1.4 ± 0.2 nm) were anchored to Cys-542 of the C80A Cth⁺-PPase mutant. A TEM image field for C80A mutant exhibited the bound GNPs as solid spheres (Fig. 5A). GNPs bound to homodimeric C80A mutant were found in pairs (Fig. 5B), concurring with the notion of the dimeric structure for this novel enzyme. The histogram shows a gaussian distribution of distances between the centers of the gold particles observed from the TEM images (Fig. 5C). The distance between the 542–542 GNPs pair was accordingly estimated to be 55.6 ± 7.3 Å, a value in a good agreement with that obtained by smFRET, demonstrating that the current system is workable for further distance measurements by FRET below (12, 17).
smFRET Analysis of CtH⁺-PPase

Distance mapping of two C termini, two N termini, and two active sites on homodimeric subunits were further examined using the smFRET technique to elucidate the structure of essential regions in CtH⁺-PPase. Dual labeling at residue 668 in C terminus on each subunit displayed a relatively high energy transfer efficiency (0.682 ± 0.098, n = 215) (Fig. 4B), resulting in a distance of 49.3 ± 4.0 Å between the 668–668 pair within homodimeric CtH⁺-PPase (Table 1). Sequence alignment analysis indicates that C terminus of H⁺-PPase is relatively conserved among various species (28). Moreover, C terminus was believed to be involved in sustaining PPi hydrolysis and H⁺ translocation reactions as well as for indirect regulation of K⁺ binding as demonstrated by deletion analysis (28). Recently, the observation by atomic force microscopy or TEM using antibodies and GNPs anchored at the His6 tags of two subunits suggested C termini within dimeric mung bean H⁺-PPase is in close proximity, presumably near the interface of subunits (12). The present measurement by smFRET concurs again with the notion that two C termini form a portion of interface between homodimeric subunits of H⁺-PPase.

The signal peptide of H⁺-PPase is predicted to locate close to the N terminus using the SignalP 3.0 Server, showing a possible role of N terminus in H⁺-PPase in protein targeting (data not shown). The present study using smFRET explored the relative position of two N termini within homodimeric CtH⁺-PPase.

The FRET histogram for insertion mutant (C-1)-upstream N termini revealed a lower degree of energy transfer efficiency as 0.252 ± 0.099 (n = 73) (Fig. 4B), corresponding to a distance of 67.2 ± 5.7 Å between the (−1)-(−1) pair (Table 1). This

![Diagram](image)

**TABLE 1**

FRET efficiencies (E*) of mutant CtH⁺-PPases and calculated distances (R) between dye pairs in the absence and presence of ligands

| Cysteine pairs | Ligand-free | K⁺ | IDP | K⁺/IDP |
|---------------|-------------|----|-----|--------|
|                | E*          | R  | E*  | R     | E*  | R  | E*  | R     | E*  | R  |
| (−1)-(−1)     | 25.2 ± 9.9 (73) | 67.2 ± 5.7 | 24.7 ± 9.9 (135) | 67.5 ± 6.9 | 26.8 ± 10.1 (73) | 66.2 ± 7.3 | 24.1 ± 9.7 (74) | 67.8 ± 7.0 |
| 80-80         | 42.7 ± 9.6 (149) | 58.8 ± 4.2 | 41.9 ± 9.1 (89) | 59.1 ± 3.8 | 38.8 ± 10.5 (109) | 60.4 ± 4.9 | 44.8 ± 10.7 (116) | 58.0 ± 4.3 |
| 191-191       | 19.6 ± 6.1 (90) | 70.8 ± 4.8 | 29.7 ± 8.5 (115) | 64.6 ± 4.2 | 37.5 ± 10.0 (83) | 61.0 ± 4.7 | 48.5 ± 10.6 (107) | 56.6 ± 4.1 |
| 205-205       | 49.1 ± 9.4 (103) | 56.3 ± 3.7 | 50.4 ± 9.5 (90) | 55.9 ± 3.7 | 28.6 ± 8.9 (73) | 65.2 ± 4.6 | 25.7 ± 10.0 (95) | 66.8 ± 5.2 |
| 542-542       | 51.3 ± 8.8 (93) | 55.5 ± 3.4 | 52.2 ± 9.0 (109) | 55.2 ± 3.4 | 48.5 ± 7.7 (86) | 56.6 ± 3.0 | 50.1 ± 9.7 (135) | 56.0 ± 3.7 |
| 622-622       | 54.7 ± 10.1 (135) | 54.3 ± 3.8 | 48.1 ± 7.5 (108) | 56.7 ± 2.9 | 37.4 ± 9.4 (75) | 61.0 ± 4.3 | 37.4 ± 8.7 (123) | 61.0 ± 3.9 |
| 631-631       | 58.7 ± 13.4 (103) | 52.8 ± 5.2 | 51.8 ± 11.8 (76) | 55.3 ± 4.6 | 46.2 ± 11.0 (98) | 57.4 ± 4.5 | 53.2 ± 8.7 (106) | 54.8 ± 3.3 |
| 668-668       | 68.2 ± 9.8 (215) | 49.3 ± 4.0 | 67.5 ± 7.9 (135) | 49.6 ± 3.0 | 71.9 ± 11.2 (121) | 47.9 ± 4.5 | 72.7 ± 8.2 (97) | 47.6 ± 3.5 |

The value of FRET efficiency (E*) represents gaussian fits to the data ± S.D. Numbers of molecules analyzed are shown in parentheses. The distances (R) were estimated directly from E* using Equation 3. Concentrations of ligands: 100 mM K⁺, 5 mM IDP.
smFRET Analysis of CtH⁺-PPase

FIGURE 5. TEM analysis of GNP-labeled homodimeric CtH⁺-PPase. A, TEM for GNP-labeled CtH⁺-PPase is shown. Purified C80A CtH⁺-PPase was labeled with monomaleimide GNPs according to the protocol described under "Experimental Procedures." The scale bar is 5 nm. B, zoomed images are shown for the GNP pairs labeled at cysteine pairs of homodimeric C80A mutant. The scale bar is 2 nm. C, a histogram shown the distances between GNP pairs.

smFRET measurement implicated that two N termini on homodimeric subunits are relatively distant from each other compared to a theoretical diameter of 77.8 Å (12, 29). In contrast to C termini, N termini are possibly remote from the interface of homodimeric CtH⁺-PPase. In addition, a topological prediction indicates the location of Cys-80 at the end of TM3 to face the periplasmic side (Fig. 1). The C542A mutant containing the sole cysteine at position 80 exhibited a FRET efficiency of 0.427 ± 0.096 (n = 149), suggesting the distance of 58.8 ± 4.2 Å between two TM3s near the periplasmic side (Fig. 4B).

Furthermore, the distance between two Acidic I motifs of the subunits was measured by smFRET using Ala-205 as the target that resides one residue upstream from these motifs (Fig. 1). As shown in Fig. 4B, the FRET efficiency for the A205C mutant was 0.491 ± 0.094 (n = 103), presumably resulting in a distance of 56.3 ± 3.7 Å between two Acidic I motifs. Moreover, the peaks in the FRET efficiency histogram of H622C and V631C mutants were at 0.547 ± 0.101 (n = 135) and 0.587 ± 0.134 (n = 103), corresponding to distances of 54.3 ± 3.8 and 52.8 ± 5.2 Å between two His-622 and two Acidic II motifs (Table 1), which were suggested as an important residue and an essential motif for sustaining an enzymatic reaction, respectively (14, 20). Taken together, the mean distances between two Acidic I motifs, two Acidic II motifs, two His-622, and two Cys-542 residues on homodimeric CtH⁺-PPase subunits are about 55 Å. As for the distance between two PPi binding motifs, Ala-191 flanked by the motif was selected for smFRET measurement. Energy transfer efficiency between the two Ala-191 residues was 0.196 ± 0.061 (n = 90) accordingly (Fig. 4B). The corresponding distance between 191–191 pair was 70.8 ± 4.8 Å (Table 1), indicating that two PPi binding motifs on homodimeric subunits of CtH⁺-PPase are relatively far away from each other.

Ligand Effects on FRET Efficiency between Two Termini and Two Active Sites—Structural dynamics of CtH⁺-PPase was further investigated using smFRET determination upon treatments of essential factor, K⁺, and substrate analogue, imidodiphosphate (IDP) separately or together. Thermoinactivation analysis suggested that substrate interaction might induce a conformational change of vacuolar H⁺-PPase (13). Furthermore, various biochemical studies validated that K⁺ is required for type I H⁺-PPase activities (Fig. 2B) (6, 7, 30). Circular dichroism analysis revealed that K⁺ could elicit the secondary structure variations of H⁺-PPase (31). In the present work the FRET efficiencies for the C80A mutant (542–542 pair) in the presence of 100 mM KCl and 5 mM IDP were 0.522 ± 0.090 (n = 109) and 0.485 ± 0.077 (n = 86) (Fig. 6, A and B), resulting in distances of 55.2 ± 3.4 and 56.6 ± 3.0 Å, respectively (Table 1). Both KCl and IDP treatment of C80A mutant also produced negligible effects on the proximity between the 542–542 pair (E*= 0.501 ± 0.097, n = 135; R = 56.0 ± 3.7 Å) (Fig. 6C and Table 1). Neither K⁺ nor the substrate analogue induced the apparent change in the distances between two entrances of active site cavities on homodimeric subunits. Similar phenomenon was observed as well in V631C mutant, of which the FRET efficiencies after K⁺, IDP, and K⁺/IDP treatments were 0.518 ± 0.118 (n = 76), 0.462 ± 0.110 (n = 98), and 0.532 ± 0.087 (n = 106), respectively (Fig. 6). The corresponding distances between 631–631 pair with K⁺, IDP, and K⁺/IDP treatments were, thus, calculated to be 55.3 ± 4.6z 57.4 ± 4.5, and 54.8 ± 3.3 Å, respectively (Table 1). The presence of ligand or substrate analogue exerted no significant effects on the proximity between two Acidic II motifs on homodimeric subunits in the smFRET measurement. Nevertheless, FRET efficiencies of A191C mutant were 0.297 ± 0.085 (n = 115) and 0.375 ± 0.100 (n = 83) upon the addition of KCl and IDP, respectively (Fig. 6, A and B). Calculated distances between the 191–191 pair for K⁺ and substrate analogue interaction were subsequently estimated to be 64.6 ± 4.2 and 61.0 ± 4.7 Å, respectively (Table 1). In addition, both KCl and IDP treatment of A191C mutant caused a further increase in the efficiency ratio (0.485 ± 0.106, n = 107) (Fig. 6C). The corresponding distance between the
charged residues in the E motif fall within the cavity (33, 139) and 0.719 (135) and 0.719 ± 0.112 (n = 121) (Fig. 6, A and B), indicating no distance variation occurring in Acidic I motif upon K⁺ binding. However, in the presence of IDP, the FRET efficiency decreased to 0.286 ± 0.089 (n = 73) with a corresponding decline in distance of 65.2 ± 4.6 Å (Fig. 6B and Table 1). Similarly for K⁺/IDP treatment, FRET efficiency was brought to 0.257 ± 0.100 (n = 95), yielding a distance of 66.8 ± 5.2 Å between 205–205 pair (Fig. 6C and Table 1). It is conceivable that substrate analogue induces distance change in Acidic I motif, moving these motifs apart from each other. Moreover, chemical modification and mutagenic studies depicted that the K⁺ binding site possibly locates near the His-716 residue of mung bean H⁺-PPase (20). Besides, proteolytic analysis revealed that a conformational change of H⁺-PPase occurred in the region around His-716 as substrate binds to the enzyme (20). The distance between the 622–622 pair of homodimeric CtH⁺-PPase measured in this work is 54.3 ± 3.8 Å in the ligand-free state (Fig. 4B). FRET efficiencies for the H622C mutant was 0.481 ± 0.075 (n = 108) upon the addition of K⁺ (Fig. 6A) with a calculated distance of 56.7 ± 2.9 Å (Table 1), whereas in the presence of IDP was 0.374 ± 0.094 (n = 75) with a distance of 61.0 ± 4.3 Å, respectively (Table 1). Likewise, the major peak in the FRET efficiency histogram of H622C mutant after the addition of both KCl and IDP was 0.374 ± 0.087 (n = 123) (Fig. 6C), giving a distance of 61.0 ± 3.9 Å between the 622–622 pair (Table 1). Binding of substrate analogue to CtH⁺-PPase was accompanied by a separation of about 7 Å between the 622–622 pair. These results suggested that the two His-622 residues on homodimeric subunits are considered to move apart from each counterpart upon binding of the substrate analogue. Furthermore, the energy transfer efficiencies for the 668–668 pair in the presence of KCl and IDP were 0.675 ± 0.079 (n = 135) and 0.719 ± 0.112 (n = 121) (Fig. 6, A and B), resulting in distances of 49.6 ± 3.0 and 47.9 ± 4.5 Å (Table 1), respectively. The addition of both KCl and IDP also produced no significant change in the FRET efficiency between 668–668 dye pairs obtained according to the methods described under “Experimental Procedures” in the presence of 100 mM KCl or 5 mM IDP, if any. A -622–622, 631–631, and 668–668 dye pairs were obtained according to the methods described in the presence of KCl and IDP. FRET efficiency (E) of each single molecule was obtained by Equations 1 and 2. Gaussian fits to the data with the efficiency values (E*) yielded using SigmaPlot software are given in Table 1. Furthermore, energy transfer efficiency for A205C mutant interacting with K⁺ was 0.504 ± 0.095 (n = 90) (Fig. 6A). The distance between the 205–205 pair was then calculated to be 55.9 ± 3.7 Å (Table 1), indicating no distance variation occurring in Acidic I motif upon K⁺ binding. According to x-ray crystallographic studies of soluble PPase, trypsinolysis verified that Lys-261 and Glu-263 in PPi binding motif of mung bean H⁺-PPase are involved in substrate binding (14). Conceivably, interactions with substrate and K⁺ might result in a compact conformation at active site of H⁺-PPase.

FIGURE 6. Ligand effects on the FRET efficiencies. FRET efficiencies at −1−1, 80−80, 191−191, 205−205, 542−542, 622−622, 631−631, and 668−668 dye pairs were obtained according to the methods described under “Experimental Procedures” in the presence of 100 mM KCl or 5 mM IDP, if any. A, shown are FRET efficiencies in the presence of KCl and IDP. B, shown are FRET efficiencies in the presence of KCl and IDP. C, shown are FRET efficiencies in the presence of KCl and IDP. FRET efficiency (E) of each single molecule was obtained by Equations 1 and 2. Gaussian fits to the data with the efficiency values (E*) yielded using SigmaPlot software are given in Table 1.

191–191 pair for KCl/IDP binding was 56.6 ± 4.1 Å (Table 1). Binding of K⁺ and/or substrate analogue led to closer proximity between two PPi binding motifs on homodimeric subunits compared to the ligand-free state (70.8 ± 4.8 Å). Comparison analysis indicated that PPi binding motif is highly conserved among soluble PPase (EX₃KXE) and H⁺-PPase (DX₃KXE) (32). According to x-ray crystallographic studies of soluble PPase, charged residues in the EX₃KXE motif fall within the cavity (33, 34). Moreover, trypsinolysis verified that Lys-261 and Glu-263 in PPi binding motif of mung bean H⁺-PPase are involved in substrate binding (14). Conceivably, interactions with substrate and K⁺ might result in a compact conformation at active site of H⁺-PPase.

Further analysis indicated that PPi binding motif is highly conserved among soluble PPase (EX₃KXE) and H⁺-PPase (DX₃KXE) (32). According to x-ray crystallographic studies of soluble PPase, charged residues in the EX₃KXE motif fall within the cavity (33, 34). Moreover, trypsinolysis verified that Lys-261 and Glu-263 in PPi binding motif of mung bean H⁺-PPase are involved in substrate binding (14). Conceivably, interactions with substrate and K⁺ might result in a compact conformation at active site of H⁺-PPase.
increased the distances between two Acidic I motifs and two His-622 residues on homodimeric subunits. However, two PPi binding motifs on subunits of dimeric Cth+ -PPase move toward its counterpart upon binding of substrate analogue and K+. Nevertheless, the distances between both Acidic II motifs and both Cys-542 residues on subunits still remain the same. This study provides the first quantitative measurements of distances between several essential domains and residues on subunits of dimeric Cth+ -PPase and presumably paves a way for future investigation on its active and H+ translocation reactions.

**Acknowledgment**—We thank Dr. Gerhard Gottschalk (Institute of Microbiology and Genetics, Georg-August University, Germany) for the generous gift of genomic DNA of C. tetani E88.

**REFERENCES**

1. Drozdowicz, Y. M., and Rea, P. A. (2001) *Trends Plant Sci.* 6, 206–211
2. Ginsburg, H. (2002) *Trends Parasitol.* 18, 483–486
3. Maeshima, M. (2000) *Biochim. Biophys. Acta* 1465, 37–51
4. Baltscheffsky, M., Schultz, A., and Baltscheffsky, H. (1999) *FEBS Lett.* 452, 121–127
5. Maeshima, M. (1991) *Eur. J. Biochem.* 196, 11–17
6. Gordon-Weeks, R., Steele, S. H., and Leigh, R. A. (1996) *Plant Physiol.* 111, 195–202
7. Maeshima, M. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 469–497
8. Tzeng, C. M., Yang, C. Y., Yang, S. J., Jiang, S. S., Kuo, S. Y., Hung, S. H., Ma, J. T., and Pan, R. L. (1996) *Biochem. J.* 316, 143–147
9. Sarafian, V., Potier, M., and Poole, R. J. (1992) *Biochem. J.* 283, 493–497
10. Yang, S. J., Ko, S. J., Tsai, Y. R., Jiang, S. S., Kuo, S. Y., Hung, S. H., and Pan, R. L. (1998) *Biochem. J.* 331, 395–402
11. López-Marqués, R. L., Pérez-Castiñeira, J. R., Buch-Pedersen, M. J., Marco, S., Rigaud, J. L., Palmgren, M. G., and Serrano, A. (2005) *Biochim. Biophys. Acta* 1716, 69–76
12. Liu, T. H., Hsu, S. H., Huang, Y. T., Lin, S. M., Huang, T. W., Chuang, T. H., Fan, S. K., Fu, C. C., Tseng, F. G., and Pan, R. L. (2009) *FEBS J.* 276, 4381–4394
13. Yang, S. J., Jiang, S. S., Hsiao, Y. Y., Van, R. C., Pan, Y. J., and Pan, R. L. (2004) *Biochim. Biophys. Acta* 1656, 88–95
14. Nakanishi, Y., Saijo, T., Wada, Y., and Maeshima, M. (2001) *J. Biol. Chem.* 276, 7654–7660
15. Förster, T. (1948) *Annalen der Physik* 2, 55–75
16. Weiss, S. (2000) *Nat. Struct. Biol.* 7, 724–729
17. Tomishige, M., Stuurman, N., and Vale, R. D. (2006) *Nat. Struct. Mol. Biol.* 13, 887–894
18. Roy, R., Hohng, S., and Ha, T. (2008) *Nat. Methods* 5, 507–516
19. Kirsch, R. D., and Joly, E. (1998) *Nucleic Acids Res.* 26, 1848–1850
20. Hsiao, Y. Y., Van, R. C., Hung, S. H., Lin, H. H., and Pan, R. L. (2004) *Biochim. Biophys. Acta* 1608, 190–199
21. Minamisawa, H., Nakaniishi, Y., Hirono, M., and Maeshima, M. (2004) *J. Biol. Chem.* 279, 35106–35112
22. Clerc, S., and Barenholz, Y. (1998) *Anal. Biochem.* 259, 104–111
23. Granier, S., Kim, S., Shafer, A. M., Ratnala, V. R., Fung, J. I., Zare, R. N., and Kolibaba, B. (2007) *J. Biol. Chem.* 282, 13895–13905
24. Lee, N. K., Kapanidis, A. N., Wang, Y., Michalet, X., Mukhopadhyay, J., EBright, R. H., and Weiss, S. (2005) *Biochem. J.* 388, 2939–2953
25. Ishii, Y., Yoshida, T., Funatsu, T., Aizawa, K., and Yanagida, T. (1999) *Chem. Phys.* 247, 163–173
26. Kim, E. J., Zhen, R. G., and Rea, P. A. (1995) *J. Biol. Chem.* 270, 2630–2635
27. Zhen, R. G., Kim, E. J., and Rea, P. A. (1994) *J. Biol. Chem.* 269, 23342–23350
28. Lin, H. H., Pan, Y. J., Hsu, S. H., Van, R. C., Hsiao, Y. Y., Chen, J. H., and
**smFRET Analysis of CtH⁺-PPase**

29. Edstrom, R. D., Meinke, M. H., Yang, X. R., Yang, R., Ellings, V., and Evans, D. F. (1990) *Biophys. J.* **58**, 1437–1448

30. Gaxiola, R. A., Palmgren, M. G., and Schumacher, K. (2007) *FEBS Lett.* **581**, 2204–2214

31. Hsu, S. H., Hsiao, Y. Y., Liu, P. F., Lin, S. M., Luo, Y. Y., and Pan, R. L. (2009) *Bot. Stud.* **50**, 291–301

32. Cooperman, B. S., Baykov, A. A., and Lahti, R. (1992) *Trends Biochem. Sci.* **17**, 262–266

33. Wu, C. A., Lokanath, N. K., Kim, D. Y., Park, H. J., Hwang, H. Y., Kim, S. T., Suh, S. W., and Kim, K. K. (2005) *Acta Crystallogr. D Biol. Crystallogr.* **61**, 1459–1464

34. Pohjanjoki, P., Lahti, R., Goldman, A., and Cooperman, B. S. (1998) *Biochemistry* **37**, 1754–1761