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Disulfide-bond formation in the H^+-pyrophosphatase of *Streptomyces coelicolor* and its implications for redox control and enzyme structure

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Abstract

Redox control of disulfide-bond formation in the H^+-pyrophosphatase of *Streptomyces coelicolor* was investigated using cysteine mutants expressed in *Escherichia coli*. The wild-type enzyme, but not a cysteine-less mutant, was reversibly inactivated by oxidation. To determine the residues involved in oxidative inactivation, different cysteine residues were replaced. Analysis with a cysteine-modifying reagent revealed that the formation of a disulfide bond between cysteines 253 and 621 was responsible for enzyme inactivation. This result suggests that residues in different cytoplasmic loops are close to each other in the tertiary structure. Both cysteine residues are conserved in K^+-independent (type II) H^+-pyrophosphatases.

Keywords: H^+-PPase; Proton pump; Redox control; *Streptomyces coelicolor*

List of abbreviations: BM, 3-(*N*-maleimidylpropionyl)biocytin; CuPh, Cu(II)-(1,10-phenanthroline)$_3$; C-less, cysteine-less; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; H^+-PPase, H^+-translocating inorganic pyrophosphatase; PP_i, inorganic pyrophosphate; ScPP, *Streptomyces coelicolor* H^+-PPase; V-ATPase, vacuolar H^+-ATPase

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1. Introduction

H^+-translocating inorganic pyrophosphatases (H^+-PPases) hydrolyze inorganic pyrophosphate (PP_i), generating an electrochemical H^+ gradient across the membrane for the active secondary transport of solutes [1,2]. H^+-PPases are a novel family of H^+-pumps that differ from P-, F-, and V-type ATPases [1,3]. The enzyme monomers have a molecular weight (MW) of ~80 kDa [1-3] and 16 transmembrane domains [4]. H^+-PPases form homo-oligomers in the membrane and are believed to function as dimers [5,6]. As a result of recent biochemical and molecular studies they are divided into two groups according to their K^+ requirements: one requires more than 30 mM K^+ for maximal activity [1], whereas the other does not [2,7]. The former is designated type I and the latter is known as type II [2]. Each type possesses a common motif [8].

H^+-PPases have been found in many organisms [1,2,9-13]. In plant cells, they are responsible for a variety of vacuolar functions (such as cell volume expansion) as well as the homeostasis of cytoplasmic pH and ionic concentrations, and, together with the vacuolar-ATPase (V-ATPase), the accumulation of ions and metabolites. H^+-PPases are mainly regulated at the transcriptional level, and this activity is linked to developmental processes and physiological stresses. Expression and accumulation of the enzymes is extensive in young tissues but reduced in mature tissues [14], and anoxia, chilling, salt and osmotic stresses enhance their transcription [15,16]. Overexpression of H^+-PPases confers salt and drought resistance on transgenic *Arabidopsis thaliana* [17]. Moreover, in the photosynthetic bacterium *Rhodospirillum rubrum*, H^+-PPase expression is up-regulated under conditions of low light intensity, anaerobiosis and salinity [18,19].

In general, covalent and non-covalent modifications are involved in the regulation of enzymes, and phosphorylation is a particularly frequent way of regulating their activity. However, there have been no previous reports on the phosphorylation of H^+-PPases. In the present study, we focused our attention on the possibility of redox regulation of H^+-PPases by the formation of intramolecular disulfide bonds, as the enzymes possess four cysteine residues. Cys^{634} of the *Arabidopsis* enzyme is susceptible to modification by *N*-ethylmaleimide but is not directly involved in H^+-PPase function [20]. In addition, substitution of Cys^{185}, Cys^{222} and Cys^{573} of the *R. rubrum* enzyme with aliphatic residues had no deleterious effect on activity but decreased its sensitivity to mersalyl [21]. These studies strongly suggest that the cysteine residues are not essential for this activity. Recently, we succeeded in expressing wild type and mutant enzymes of *Streptomyces coelicolor* in *Escherichia coli*, and proposed a membrane topology model of the H^+-PPase [4]. In the present work, we prepared H^+-PPase mutants with single and multiple cysteine residues, or lacking cysteines, and oxidized them *in vitro*. Enzyme activity was markedly affected by the mutational changes and by oxidation. On the basis of these results, we discuss the possibility of the redox regulation of H^+-PPase activity.

2. Materials and methods

2.1. Plasmid construction, protein expression and isolation of crude membranes

The synthetic *S. coelicolor* H^+-PPase gene (*sScPP*) constructed previously [4] was used for preparing cysteine mutants. Cysteine-less (C-less) mutants (C178S, C179S, C253A and C621V) and other ScPP mutants were generated from *sScPP* using a QuickChange site-directed mutagenesis kit (Stratagene) according to the method of Kirsch and Joly [22]. A plasmid, pYN309, derived from pET23b (Novagen) by modifying the PstI site, was used for expressing the ScPP proteins. For biotinylation assays, a His_6 tag was added to the carboxyl (C)-terminus to facilitate purification of the ScPPs. *ScPP* constructs in pYN309 were

introduced into *E. coli* strain BLR (DE3) pLysS (Novagene), and transformants were selected on 50 μg/ml ampicillin and 34 μg/ml chloramphenicol.

Protein expression and preparation of crude membrane fractions were conducted as described previously [4]. Cells from a single transformant colony were grown for 16 h at 37°C in Luria-Bertani medium supplemented with antibiotics and diluted 50-fold in the same medium. After a further 4 h at 37°C, isopropyl-1-thio-*β*-D-galactopyranoside was added to a final concentration of 0.4 mM. Then, 2 h later, the cells were disrupted by sonication, centrifuged, and the crude membranes were suspended in 10 mM Mes/Tris (pH 7.2), 0.15 M sucrose, 1 mM $MgCl_2$ and 75 mM KCl.

2.2. Enzyme assays after CuPh and DTT treatment

Oxidation with Cu(II)-(1,10-phenanthroline)$_3$ (CuPh) was carried out essentially as described by Falke and Koshland [23]. Crude membrane fractions were treated with 0.2 mM CuPh at 30°C for 10 min and then washed with 10 mM Mes/Tris (pH 7.2), 0.15 M sucrose, 1 mM $MgCl_2$ and 75 mM KCl (buffer A) supplemented with 1 mM ethylenglycol bis(2-aminoethyl ether)tetraacetic acid (EGTA). After centrifugation at 100,000xg for 15 min, the precipitate was suspended in the buffer A supplemented with 20 mM dithiothreitol (DTT) and incubated for 10 min at 30°C. Membranes were washed again with the buffer A and suspended in the same buffer. As a control, membranes were treated with H_2O, CuPh or DTT alone. Protein content was determined with a Bradford assay kit (Bio-Rad), and both PP_i hydrolysis and PP_i-dependent H^+-transport were measured as described previously [4].

2.3. Immunoblotting analysis

Crude membranes were treated with 0.2 mM CuPh and aliquots mixed with 25 mM Tris/HCl (pH 6.8), 5% sodium dodecyl sulfate (SDS), 20 mM ethylenediaminetetraacetic acid (EDTA), 15% glycerol and 0.2% bromophenol blue in the presence or absence of 5% 2-mercaptoethanol (2-ME) were heated at 70°C for 10 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on 10% gels were transferred to a polyvinylidene difluoride membrane (Millipore Corp.) with a semidry blotting apparatus. Immunoblotting was carried out with a polyclonal antibody against the peptide DVGADLVGKVEC [24], together with horseradish peroxidase-linked protein A and ECL Western blotting detection reagents (Amersham Biosciences).

2.4. Biotinylation assays

Crude membranes (1.5 mg/ml, 100 μl) prepared from *E. coli* expressing His_6-tagged ScPP proteins were treated with 0.2 mM CuPh. The membranes were washed and resuspended in 100 μl of 50 mM Tris/HCl (pH 7.5), 20% (w/v) glycerol, 300 mM KCl and 1 mM $MgCl_2$, before 3-(*N*-maleimidylpropionyl)biocytin (BM) was added to a final concentration of 0.2 mM. After incubation at 30°C for 10 min, the reactions were stopped by dilution with 1 ml of the same buffer containing 5 mM *N*-ethylmaleimide. The membranes were washed with this buffer and suspended in 200 μl of 50 mM Tris/HCl (pH 7.5), 20% glycerol, 300 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride and 5 mM *N*-ethylmaleimide. After centrifugation, the supernatants were mixed with 50 μl of Ni-nitriltriacetic acid-agarose (Qiagen) and incubated at 4°C for 1 h with gentle shaking. The agarose resin was collected by flash centrifugation and washed with 1 ml of 50 mM Tris/HCl (pH 7.5), 20% glycerol, 300 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100 and 50 mM imidazole. The washed resin was treated with 10 μl of 25 mM Tris/HCl (pH 6.8), 5% SDS, 5% 2-ME, 15% glycerol, 0.2% bromophenol blue and 20 mM EDTA, at 70°C for 10 min prior to SDS-PAGE. The separated proteins were transferred to a

polyvinylidene difluoride membrane, and biotinylated proteins were detected by the ECL method using the streptavidin-biotinylated horseradish peroxidase complex (Amersham Bioscience). After the analysis, the labels on the proteins were removed by incubation in 100 mM 2-ME and 62.5 mM Tris/HCl (pH 8.0) at 50°C for 30 min, and they were probed with polyclonal antibodies to H^+-PPase.

3. Results

The previous study on the membrane topology by cysteine scanning demonstrated that ScPP has 17 transmembrane domains and contains four cysteine residues in the transmembrane domain and the cytoplasmic loops (Fig. 1) [4]. To investigate the role of the cysteine residues, we tested the effect of CuPh (an oxidizing agent that stimulates the formation of disulfide bonds) on the enzymes expressed in *E. coli*. Crude membranes prepared in the absence of any reducing reagent were treated with various concentrations of CuPh. PP_i hydrolysis was then measured (Fig. 2A). As a control, a C-less enzyme with cysteine residues replaced by serine, alanine or valine (C178S, C179S, C253A and C621V) was also examined. The C-less enzyme was not affected by CuPh, indicating that the reagent itself had no effect on enzyme activity. Both the PP_i hydrolysis and the PP_i-dependent H^+-transport activities of the C-less enzyme were approximately one-half of those of the wild-type enzyme, as previously reported [4]. CuPh at 0.2 mM markedly inactivated the wild-type enzyme, but not the C-less mutant (Fig. 2A). To distinguish between irreversible inhibition by uncontrolled oxidation with CuPh and reversible oxidation with a possible regulatory significance, membranes oxidized with 0.2 mM CuPh were treated with DTT (Fig. 2B). A substantial proportion of the wild-type activity was restored by reduction with DTT. This result indicates that the inactivation by CuPh is closely related to the formation of disulfide bonds between the cysteine residues of ScPP.

Proton-pump activity was also determined by monitoring the decrease in fluorescence intensity of acridine orange (Fig. 2C). In agreement with the results of PP_i hydrolysis, PP_i-dependent H^+-transport by the wild-type enzyme was inactivated by 0.2 mM CuPh and partially reactivated by 20 mM DTT, whereas the C-less mutant was not affected. These results indicate that ScPP can be functionally modulated by the redox states of its cysteine residues.

To determine which cysteine residues are responsible for the redox effect on enzyme activity, we constructed several cysteine mutants from the C-less mutant and expressed them in *E. coli*. Immunoblots of *E. coli* membranes with anti-H^+-PPase antibodies showed that wild type and mutant enzymes accumulated at roughly equal levels (Fig. 3A). An immunostained band with a molecular mass around 150 kDa was formed by the oxidation of C253A and C178S/C179S/C253A with CuPh (Fig. 3A, upper panel). This band appears to be a dimeric form of the enzyme, as discussed later.

We measured the PP_i hydrolysis and the proton-pump activities of the mutant enzymes after redox treatment (Fig. 3, B and C). Membranes from *E. coli* expressing the various ScPP proteins were treated with CuPh and DTT, or CuPh followed by DTT. C178S, C179S and C178S/C179S behaved like the wild type: CuPh markedly reduced their activity and this inactivation was partially reversed by DTT. The incomplete reactivation by DTT might be due to the irreversible conversion of cysteines to cysteic acid. By contrast, C253A and C621V were relatively insensitive to CuPh. Furthermore, the partial inactivation observed was completely reversed by DTT. In addition, the double mutant C253A/C621V was unaffected by CuPh. We suggest that a disulfide bond between Cys^{253} and Cys^{621} formed by oxidation treatment with CuPh is responsible for inactivating the enzyme.

To examine the formation of a disulfide bond between Cys^{253} and Cys^{621}, ScPP proteins in *E. coli* membranes were biotinylated after treatment with CuPh (Fig. 4). The reagent is somewhat polar but can penetrate membranes at higher concentrations [25]. Thus BM is used a membrane-permeable biotinylation reagent that covalently modifies the sulfhydryl groups of cysteines in hydrophilic regions but not in transmembrane domains [4]. Wild type and mutant proteins (C-less, C178S/C179S, C178S/C179S/C253A and C178S/C179S/C621V) were extracted from *E. coli* membranes, affinity-purified and subjected to Western blotting analysis with streptavidin (Fig. 4, upper panel). The proteins were then detected by immunoblotting with anti-H^+-PPase antibody (lower panel). In the absence of CuPh, the three cysteine-containing mutants were biotinylated, but not in the C-less mutant, as expected. Treatment with CuPh prevented the biotinylation of cysteine residues of the wild type and C178S/C179S, but not of C178S/C179S/C253A and C178S/C179S/C621V. The result suggests that there are no free cysteine residues in the C178S/C179S mutant and, hence, that the remaining two cysteine residues, at positions 253 and 621, form a disulfide bond.

4. Discussion

The present study shows that the activity of ScPP is affected by its redox state as a consequence of disulfide-bond formation between Cys^{253} and Cys^{621} (Fig. 5A). To our knowledge, this is the first demonstration of the functional regulation of an H^+-PPase via cysteine residues. Oxidation of the cysteine residues by CuPh caused a marked reduction of PP_i hydrolysis and H^+-transport by ScPP (Fig. 2), and considerable activity was recovered by reduction with DTT. If redox control of enzyme activity occurs in *S. coelicolor*, there should be natural compounds that reduce the disulfide-bond formation. Actinomycetes, such as *S. coelicolor,* contain mycothiol (a unique low-MW thiol [26,27]), and some mycothiol-dependent enzymes [28-31] along with two functionally different groups of thioredoxins have been identified [32,33]. The target protein molecules of these thioredoxin compounds have not yet been confirmed. We incubated CuPh-oxidized ScPP with natural reducing agents, such as mycothiol and thioredoxin. In our experiments, *E. coli* thioredoxin, which corresponds to one of the two groups in *S. coelicolor,* and *S. coelicolor* mycothiol did not reactivate the oxidized ScPP (data not shown). Further studies using the other type of thioredoxin might provide insight into the possible redox modulation of ScPP in living cells.

Amino-acid sequence alignment of the H^+-PPases of several organisms shows that cysteine residues corresponding to Cys^{253} and Cys^{621} of ScPP are relatively well conserved among type-II enzymes (Fig. 5). The occurrence of cysteine residue at Cys^{222} and Cys^{573} of *R. rubrum* H^+-PPase (type II) was discussed as a common feature of the type II enzymes [20]. This Cys conservation pattern indicates that the redox control of enzyme activity might be unique to type-II H^+-PPases. Cys^{621} is also conserved in type-I enzymes. The oxidation of C253A and C178S/C179S/C253A led to the appearance of high molecular mass complexes under nonreducing conditions accompanied by a decrease of enzyme activity (Fig. 3). This could mean that Cys^{621}, which forms an intramolecular disulfide bond with Cys^{253} under oxidizing conditions, might also form an intermolecular bond between ScPP molecules. The cysteine residue corresponding to Cys^{621} of ScPP is highly conserved in type-I H^+-PPases (Fig. 5). Therefore, it is possible that intermolecular disulfide cross-linking might be a novel redox mechanism for regulating type-I H^+-PPases. This hypothesis could be tested by identifying oxidizing and reducing compounds in living cells, and by further molecular genetic manipulation of H^+-PPases.

There is a good precedent for the redox regulation of a proton pump. Redox control

was reported in the case of the V-ATPase of bovine clathrin-coated vesicles [34]. Disulfide-bond formation between the conserved cysteine residues Cys^{254} and Cys^{532} near the nucleotide-binding site of catalytic subunit A was shown to inactivate the V-ATPase; this inactivation was reversed by disulfide interchange within the catalytic subunit. The identification of genes required for V-ATPase function revealed that a defect in cysteine synthesis resulted in low V-ATPase activity in spite of the accumulation of normal levels of the protein [35]. Moreover V-ATPase activity was restored by adding glutathione, suggesting the existence of *in vivo* redox control of V-ATPase [35]. Recent work on plant V-ATPases has provided *in vitro* support for the idea of reversible redox regulation of V-ATPases. Redox-dependent modulation of the activity affected intramolecular disulfide bonds not only in subunit A but also in the stalk subunit E [36,37]. These observations point to the possibility of cooperative inactivation and reactivation of vacuolar H^+-PPase and V-ATPase under oxidative and reductive conditions, respectively, in plants and protozoa.

Recent studies of functional motifs and residues have provided detailed information on structure-function relationships [38-42]. The present study also yields some information on the structure of ScPP. The finding that Cys^{253} and Cys^{621} in loops *e* and *m* (Fig. 1) form a disulfide bond upon oxidation with CuPh strongly suggests that these two cysteine residues face each other in the tertiary structure. Similarly, Cys^{621} can evidently form an intermolecular disulfide bond in oligomers of ScPP, as C178S/C179S/C253A generated an oligomer (probably a dimmer) upon oxidation (Fig. 3A). Furthermore, it appears that conformational flexibility of loops *e* and *m* is essential for enzyme function, because the formation of an intramolecular disulfide bond between Cys^{253} and Cys^{621} inactivated ScPP (Fig. 3). Loops *e* and *m* are cytoplasmic [4] and contain sequences that are conserved in the H^+-PPases of various organisms, such as GGIFTKAADVGADLVGKVE, EDDPRN and IADNVGDNVGDCA (loop *e*, shared residues are underlined), and EVRRQ (loop *m*). The motifs GGIFTKAADVGADLVGKVE (loop *e*) and EDDPRN (loop *m*) have been demonstrated to form the catalytic site [24,38,43]. The glutamate residues of motif EVRRQ (loop *m*) play a role in maintaining the tertiary structure of the *R. rubrum* enzyme [43]. These observations in conjunction with the present results underline the importance of loops *e* and *m* for substrate hydrolysis.

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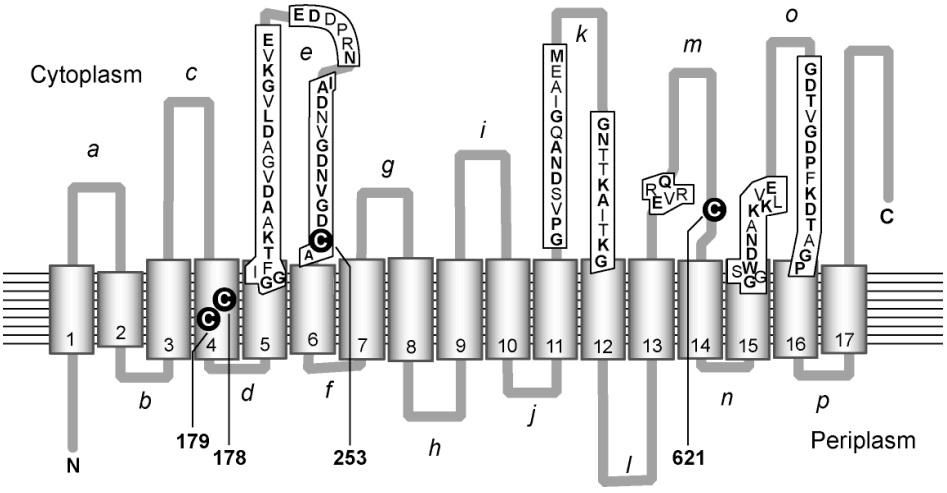

Fig. 1. The four cysteine residues of ScPP. Closed circles indicate the cysteine residues in the membrane topology model [4]. In the present experiments, Cys^{178} and Cys^{179} were substituted with serine, and Cys^{253} and Cys^{621} were substituted with alanine and valine, respectively. Conserved motifs [1-3] are boxed and residues common to various H^+-PPases are marked in boldface.

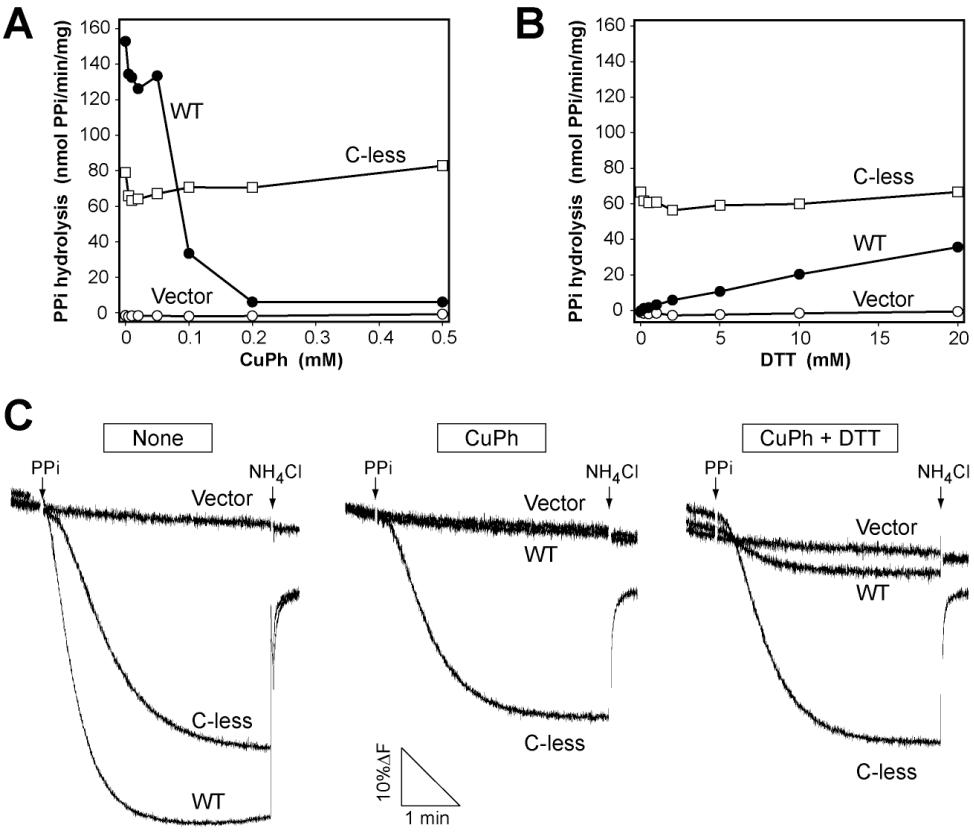

Fig. 2. CuPh-induced inactivation and DTT-mediated partial reactivation of wild-type ScPP. Crude membranes prepared from *E. coli* expressing empty vector, wild-type ScPP and a C-less mutant were used for the assays. (A) The membranes were treated with the indicated concentrations of CuPh at 30°C for 10 min, precipitated and suspended in a CuPh-free buffer. PP_i hydrolysis was then measured. (B) Membranes treated with 0.2 mM CuPh were incubated with the indicated concentrations of DTT at 30°C for 10 min. After washing, PP_i hydrolysis was measured in DTT-free medium. (C) PP_i-dependent H^+-transport activity by membranes (250 μg) was measured by fluorescence quenching of acridine orange. Membranes were treated with H_2O (None) or 0.2 mM CuPh (CuPh). In other case, membranes treated with 0.2 mM CuPh were incubated with 20 mM DTT (CuPh + DTT). The reaction was started by the addition of 0.35 mM PP_i, and the pH gradient was collapsed with the membrane permeable cation NH_4^+ at the end of the assay.

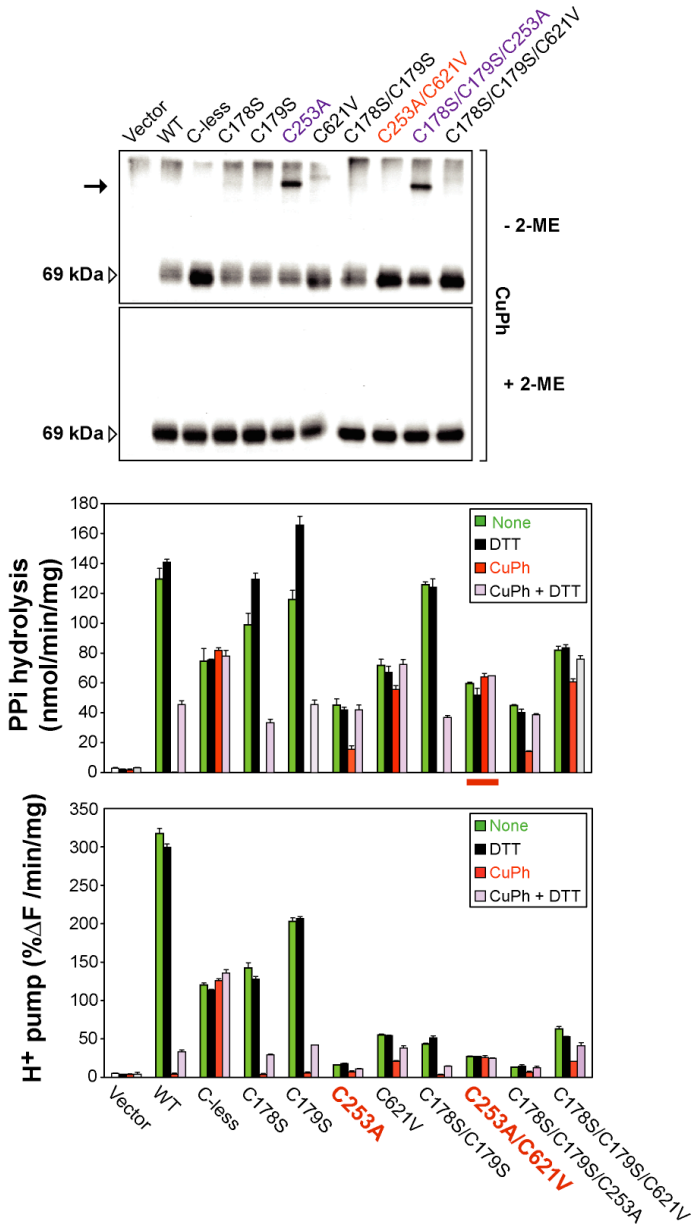

Fig. 3. Analysis of cysteine mutants of ScPP. Crude membranes were prepared from *E. coli* expressing ScPPs. (A) The membranes were treated with 0.2 mM CuPh and aliquots (10 μg) were analyzed by 10% SDS-PAGE after treatment with 2-ME treatment (+ 2-ME) or no treatment (– 2-ME). ScPP proteins were detected by immunoblotting with anti-H^+-PPase. Open arrowheads indicate the 69-kDa band of ScPP and the arrow indicates the position of a complex generated by disulfide cross-linking, (B) PP_i hydrolyzing activity in *E. coli* membranes expressing ScPP was measured after no treatment (open bar), or incubation with 20 mM DTT (black bar), 0.2 mM CuPh (gray bar) or 0.2 mM CuPh followed by 20 mM DTT (light gray bar). (C) PP_i-dependent H^+-transport by membrane vesicles (250 μg) treated as described in (B). The data in (B) and (C) are presented as the mean ± standard deviation (SD) of triplet and duplicate assays, respectively.

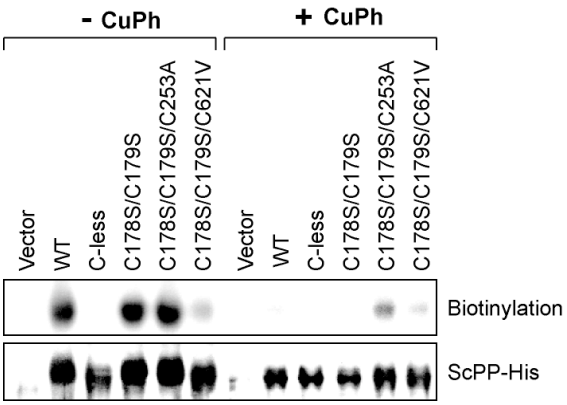

Fig. 4. Biotinylation of cysteine residues after CuPh treatment. Crude membranes containing wild type and mutant ScPP-His proteins were incubated with (+CuPh) or without (–CuPh) CuPh. Biotinylation was carried out as described in Materials and Methods. ScPP-His proteins were solubilized from the membranes, affinity-purified, separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Biotinylation of the ScPP proteins was detected with the streptavidin biotinylated horseradish peroxidase complex (Biotinylation). The same blot membrane was de-probed and immunoblotted with anti-H^+-PPase (ScPP-His).

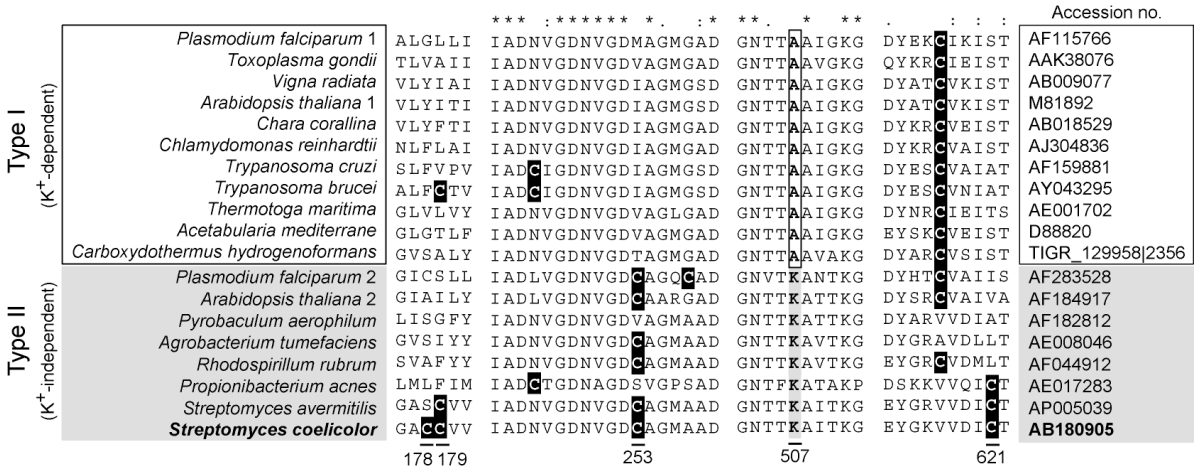

Fig. 5. Alignment of the amino-acid sequences around the cysteine residues of H^+-PPases. Cysteine residues are reversed and the residue numbers are those for ScPP. A sequence (**GNTTA/K**) distinguishing type-I (K^+-dependent) and type-II (K^+-independent) H^+-PPases is present at position 507 of ScPP. Nucleotide sequence accession numbers in GenBank™ are noted, as well as DNA contig numbers for preliminary sequences obtained from the Institute for Genomic Research (www.tigr.org).