Identification of the Transmembrane Metal Binding Site in Cu⁺-transporting P₁B-type ATPases

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P₁B-type ATPases have an essential role maintaining copper homeostasis. Metal transport by these membrane proteins requires the presence of a transmembrane metal occlusion/binding site. Previous studies showed that Cys residues in the H6 transmembrane segment are required for metal transport. In this study, the participation in metal binding of conserved residues located in transmembrane segments H7 and H8 was tested using CopA, a model Cu⁺-ATPase from Archaeoglobus fulgidus. Four invariant amino acids in the central portion of H7 (Tyr⁶⁸₂ and Asn⁶⁹⁵) and H8 (Met⁷¹¹ and Ser⁷¹⁵) were identified as required for Cu⁺ binding. Replacement of these residues abolished enzyme activity. These proteins did not undergo Cu⁺-dependent phosphorylation by ATP but were phosphorylated by P₁ in the absence of Cu⁺. Moreover, the presence of Cu⁺ could not prevent the enzyme phosphorylation by P₁. Other conserved residues in the H7-H8 region were not required for metal binding. Mutation of two invariant Pro residues had little effect on enzyme function. Replacement of residues located close to the cytoplasmic end of H7-H8 led to inactive enzymes. However, these were able to interact with Cu⁺ and undergo phosphorylation. This suggests that the integrity of this region is necessary for conformational transitions but not for ligand binding. These data support the presence of a unique transmembrane Cu⁺ binding/translocation site constituted by Tyr-Asn in H7, Met and Ser in H8, and two Cys in H6 of Cu⁺-ATPases. The likely Cu⁺ coordination during transport appears distinct from that observed in Cu⁺ chaperone proteins or catalytic/redox metal binding sites.

Cu⁺-ATPases play an essential role maintaining copper homeostasis. For instance, mutations of the human Cu⁺-ATPases, ATP7A and ATP7B, leads to Menkes’ and Wilson’s disease, respectively (1, 2). Similarly, the knockout of Arabidopsis thaliana Cu⁺-ATPase genes produces significant, and in some cases lethal, physiological alterations (3, 4). In bacteria, these proteins appear to be necessary to confer tolerance and viability when exposed to high copper levels (5, 6). Cu⁺-ATPases are members of the P₁B-type family of membrane transport ATPases (7–9). These enzymes couple metal transport to the hydrolysis of ATP through a common catalytic cycle (Fig. 1). Biochemical studies supporting different aspects of this cycle have shown metal-dependent ATPase activity, metal transport capability, interaction of ATP with high and low affinity, phosphorylation of the invariant Asp in the DIKTG motif, distinct E1P and E2P forms, and the typical inhibition by vanadate observed in all P1-type ATPases (6, 10–16). Cu⁺-ATPases have eight transmembrane segments (TMs) with a large cytoplasmic loop located between their sixth (H6) and seventh (H7) TMs and responsible for Cu⁺ ATP hydrolysis (8, 9, 17, 18) (Fig. 2). Many of these enzymes contain cytoplasmic metal binding domains in their N terminus (N-MBD) (2, 9, 19). Although N-MBDs are not essential for metal transport, they appear as central regulatory domains (13, 20, 21). The structure of isolated N-MBDs has been established, and a good understanding of their metal coordination properties has been developed (22–25).

Crucial to their transport mechanism, the catalytic cycle of P₁-type ATPases includes “occluded cation” enzyme conformations (E1P(Cu⁺)) in Fig. 1) (26, 27). In these, occluded cations are bound within the structure of the protein and cannot exchange with cations in the media. Assuming a common mechanism, a transmembrane site transiently binding/coordinating the metal during transport can be postulated as a required component of Cu⁺-ATPases. This Cu⁺ transport site is likely to bind the metal in a distinct manner from those observed in N-MBDs and Cu⁺ chaperones or other proteins where metals play a catalytic or structural function. The current understanding of heavy metal binding by transmembrane transporters is limited. By analogy with organometallic complexes or known metalloprotein structures, it can be hypothesized that histidyl, sulphydryl, hydroxyl, carbonyl, and carboxyl groups might participate in metal coordination during transport. In this direction, the involvement of conserved transmembrane His, Glu, and Ser in Fe²⁺ binding by Arabidopsis IRT1 (a member of the ZIP family of transporters) has been proposed (28). A similar role has been indicated for transmembrane His and carboxylic amino acids in the case of NixA-type Ni²⁺ permeases (29, 30) and the DMT1 Fe²⁺ transporters (31). In contrast, the transmembrane cation binding sites have been well described for the Ca²⁺-ATPase and Na,K-ATPase, extensively characterized P₁⁺ type cation pumps (32–38). Moreover, crystal structures of the cation-bound Ca²⁺-ATPase are available (39, 40). These enzymes coordinate the occluded cations via oxygen atoms in side chains of conserved amino acids located in TMs flanking the large cytoplasmic loop (H4, H5, and H6). These TMs would be equivalent to H6, H7, and H8 in the Cu⁺-ATPases (8, 41). Thus, P₁B-type ATPases provide a relevant framework to understand transmembrane metal binding sites in heavy metal ATPases. The typical “CPX sequences” (CPC, CPS, CPH, TPC, or SPC)

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† The abbreviations used are: TM, transmembrane segment; DDM, dodecyl-β-D-maltoside; DTT, dithiothreitol; N-MBD, N-terminal metal binding domain; WT, wild type.
observed in the H6 of P1b-type ATPases appear as part of a coordinating site required for heavy metal transmembrane transport (9, 10, 13, 42–44). For instance, replacement of Cys in the CPC of Escherichia coli CopA resulted in loss of copper resistance, transport, and phosphoenzyme formation (10). Briefly, membranes were isolated from cells expressing the wild type (WT) CopA or the indicated mutants and treated with 0.1% dodecyl maltoside (DDM). The solubilized His6-tagged proteins were isolated by affinity chromatography using a Ni2+-nitrilotriacetic acid column. All procedures were performed at 0–4 °C. Protein was measured in accordance with Bradford (48). After purification, proteins were stored in 25 mM Tris, pH 8.0, 100 mM sucrose, 50 mM NaCl, 0.01% DDM, 10% glycerol, and 1 mM dithiothreitol (DTT) at −80 °C.

**Functional Assays—Cu**^2+^-dependent ATPase activity and phosphorylation by ATP were assayed as described earlier (11). The ATPase activity assay was performed at 75 °C in a medium containing 50 mM Tris, pH (75 °C) 6.1, 3 mM MgCl2, 3 mM ATP, 20 mM cysteine, 0.01% asolectin, 0.1% DDM, 400 mM NaCl, 50 μM CuSO4, 2.5 mM DTT, and 0.01 mg/ml purified enzyme. Enzyme phosphorylation with ATP was carried out at 37 °C in a medium containing 50 mM Tris, pH (20 °C) 7.5, 1 mM MgCl2, 25 μM [γ-32P]ATP, 0.04 mM EGTA, 20 mM cysteine, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 20% Me2SO, 100 μM CuSO4, 2.5 mM DTT, and 0.05 mg/ml purified enzyme.

**Enzyme phosphorylation with P** was carried out at 37 °C during 10 min in a medium containing 50 mM Tris, pH (20 °C) 7.5, 1 mM MgCl2, 1 μM [γ-32P]P, 0.04 mM EGTA, 20 mM cysteine, 0.01% asolectin, 0.01% DDM, 400 mM NaCl, 20% Me2SO, and 0.1 mM purified enzyme. The effect of Cu vs the E2P formation in the presence of P was tested by including 100 μM CuSO4 and 2.5 mM DTT in the phosphorylation medium. The reactions were initiated by the addition of [γ-32P]P. Phosphorylation was stopped with five volumes of ice-cold 10% trichloroacetic acid, 1 mM P, Samples were centrifuged at 14,000 × g for 10 min, resuspended in acidic SDS-PAGE loading buffer (5 mM Tris–HCl, pH (20 °C) 5.8, 6.7 mM urea, 0.4 M DTT, 5% SDS, and 0.1% bromphenol blue) and resolved by SDS-PAGE in an 8% acetic gel (49). The gels were dried and, radioactivity was monitored and quantified in a PhosphorImager.

**Data Analysis and Structural Modeling—**Curves of ATPase activity versus Cu were fit to v = v_{max} L/(L + K_{i}), where L is the concentration of variable ligand. Data analysis was done using the KaleidaGraph software (Synergy, Reading, PA). The reported S.E. values for Cu/K_{i} are asymptotic S.E. values reported by the fitting program. Homology modeling of CopA TMs was performed using the sarcoplasmic reticulum Ca-ATPase SERC1A structure file, Protein Data Bank code 1EUL (39), as a template and the Deep View Swiss-Pdb Viewer (available on the World Wide Web at us.expasy.org/spdbv/) (50). Sequence alignment was manually modified to align CopA H7 and H8 with the Ca-ATPase TMs H5 and H6.

**RESULTS**

Analysis of Cu^-ATPase sequences indicates that specific amino acids in their last two transmembrane segments are fully conserved and consequently might play important functional roles including metal coordination (9). In the A. fulgidus CopA sequence, these residues are Asn^675, Tyr^682, Asn^683, and Pro^688 in H7 and Pro^704, Met^711, Ser^714, and Ser^715 in H8 (Fig. 2). In addition, position 721 appears to require either Asn or Ser. Interestingly, the hydrophobic Val^718 is also conserved in all Cu^-ATPases, suggesting that it might have an important structural role. The indicated residues were the targets of these studies. They were subjected to conservative (Ser, Thr, Gln, or Cys) and nonconservative (Ala) replacements by site-directed
mutagenesis, and the resulting proteins were functionally characterized. As controls for probable nonspecific functional alterations introduced by structural perturbations in the H7-H8 hairpin, enzymes carrying mutations of residues Ile685 and Leu686 were also included in these studies. In order to simplify data presentation and analysis, results are grouped by the likely position of targeted amino acids with respect to the membrane. In this way, findings associated with the centrally located Tyr682, Asn683, Met711, Ser714, and Ser715 are jointly presented, whereas functional effects of replacing Ile685, Leu694, Pro688, and Pro704 (close to the H7-H8 extracellular end) are shown together, and data corresponding to Asn677 are described alongside those from Val711 and Asn721 (residues adjacent to the H7-H8 cytoplasmic ends).

Functional Role of Amino Acids Located in the Central Region of H7-H8: Tyr682, Asn683, Met711, Ser714, and Ser715—Enzymes carrying replacements of these amino acids, with the exception of S714A- and S714T-substituted proteins, were largely inactive (Fig. 3A). Further analysis of Ser714 mutants indicated that the replacements did not affect the enzyme apparent affinity for Cu⁺, assessed as the Cu⁺ K₅₀ for ATPase activation (WT, 2.7 ± 0.3 μM; S714A, 2.3 ± 0.3 μM; S714T, 1.0 ± 0.5 μM; see Supplemental Data). These data suggest that Ser714 is not required for Cu⁺ binding. On the other hand, considering their locations it can be proposed that Tyr682, Asn683, Met711, and Ser715 are probably responsible for Cu⁺ binding. Toward testing this hypothesis, it was relevant to determine the ability of the corresponding enzymes to interact with ligands and undergo catalytic conformational transitions, although they were unable to hydrolyze ATP at a measurable rate. To explore these characteristics, partial reactions that are either Cu⁺-dependent (phosphorylation by Pi) (Fig. 3C) were measured. Fig. 3B shows that enzymes carrying mutations of Tyr682, Asn683, Met711, and Ser715 were not significantly phosphorylated by ATP in the presence of 100 μM Cu⁺, a concentration 25 times higher than the Cu⁺ K₅₀ for this partial reaction (11). As a control, enzymes mutated at position Ser714 were largely capable of forming a phosphorylated intermediary in the presence of Cu⁺ and ATP (Fig. 3B). Although the lack of activity and catalytic phosphorylation are probably related to the inability of these proteins to bind Cu⁺ at the transmembrane binding site, nonspecific structural alterations preventing the formation of stable phosphointermediaries might yield similar observations. Contrary to this latter possibility, enzymes mutated at positions Tyr682, Asn683, Met711, and Ser715 were able to undergo Cu⁺-independent phosphorylation by P₁ (Fig. 3C). Most of these proteins, including Ser714 mutants, reached phosphoenzyme levels that were 35–60% of those in the WT enzyme. Moreover, Met711-substituted proteins yielded phosphoenzyme levels similar to or even higher (60%) than those of WT. Mutation Y682S appeared fully inactive, and no enzymatic characteristic could be tested in this mutant.

The capacity to undergo phosphorylation by P₁ provided an additional tool to test Cu⁺ binding to transport sites in these enzymes. Cu⁺, by shifting the conformational equilibrium toward E₁, prevents enzyme phosphorylation by P₁ (Fig. 1). Fig. 3D shows that in contrast to the WT and Ser714 mutants, Tyr682-, Asn683-, Met711-, and Ser715-substituted proteins were largely insensitive to 100 μM Cu⁺; i.e. similar phosphoenzyme levels (within S.E. values) were observed in the absence and presence of the metal. These results further support the postulated role of Tyr682, Asn683, Met711, and Ser715 probably participating in Cu⁺ coordination during transport.

Functional Effects of Replacing Amino Acids Close to the Extracellular End of H7-H8: Ile685, Leu694, Pro688, and Pro704—Mutation of these residues led to relatively minor effects on enzyme function. For instance, their overall Cu⁺-ATPase activity was similar to that of the WT enzyme, except in the case of Ile685 substitutions, where significant reductions in activity were observed (76–48%) (Fig. 4A). Similarly, these mutations had small effects on the apparent affinity of the enzyme for Cu⁺ (Cu⁺ K₅₀ for ATPase activation: WT, 2.7 ± 0.3 μM; Ile685E, 1.3 ± 0.4 μM; Ile685T, 2.3 ± 0.8 μM; Leu694E, 3.8 ± 0.6 μM; P688A, 2.3 ± 0.5 μM; P704A, 0.9 ± 0.4 μM; see Supplemental Data). Considering that conserved Pro might be required for conformational stabilization or have a role not evident in the structural alterations preventing the formation of stable phosphointermediaries might yield similar observations. Contrary to this latter possibility, enzymes mutated at positions Tyr682, Asn683, Met711, and Ser715 were able to undergo Cu⁺-independent phosphorylation by P₁ (Fig. 3C). Most of these proteins, including Ser714 mutants, reached phosphoenzyme levels that were 35–60% of those in the WT enzyme. Moreover, Met711-substituted proteins yielded phosphoenzyme levels similar to or even higher (60%) than those of WT. Mutation Y682S appeared fully inactive, and no enzymatic characteristic could be tested in this mutant.

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overall ATPase activity determinations, enzyme phosphorylation was measured. Fig. 4B shows that I685T, L686A, and P688A mutations allowed significant levels of phosphoenzyme formation by ATP in the presence of Cu++. Under similar conditions, low phosphoenzyme levels were detected in the case of I685E and P704A proteins. However, I685E- and P704A-substituted proteins, as the other mutants, were largely phosphorylated by P1 (Fig. 4C). Considering the different phosphoenzyme levels observed for I685E and P704A proteins, it is apparent that these mutations might lead to unstable E1P/Cu++/E2P forms under the forward phosphorylation assay conditions and a larger formal turnover if calculated as Vmax/EP. However, most importantly, the data indicate that amino acids close to the H7-H8 joining loop are not required for ligand binding to the enzyme.

Functional Roles of Amino Acids Proximal to the Cytoplasmic End of H7-H8: Asn675, Val718, and Asn721—Replacement of these residues had significant effects on enzyme function, since proteins carrying substitutions N675A, V718E, and N721A lacked ATPase activity. However, these enzymes showed phosphoenzyme levels comparable with the WT when the phosphorylation in the presence of ATP and Cu+ was tested (Fig. 5A). Although this suggests that the lack of function is not associated with alterations in Cu+ or ATP binding or disruption of the phosphorylation step (Fig. 1), the capability of the Asn675-, Val718-, and Asn721-substituted enzymes to interact with substrates was further tested by analyzing phosphorylation of the E2 form by P1 (Fig. 5B). In these conditions, V718E protein was not able to undergo phosphorylation, whereas N675A and N721A mutants yielded phosphoenzyme levels much lower than that of WT enzyme. These observations might be due to a preference of the mutated enzymes to remain in E1 conformations, consequently preventing “backdoor” phosphorylation without largely affecting phosphorylation by ATP. This was corroborated when the sensitivity of the E2 + P1 → E2P partial reaction in the presence of Cu+ was analyzed. Fig. 4C shows that despite their modest “backdoor” phosphorylation, N675A and N721A enzymes presented high ratios for the phosphorylation in the absence and presence of Cu+. The absence of significant “backdoor” phosphorylation in V718E mutants prevented this particular analysis. The characteristics of these mutants suggest that whereas Asn675, Val718, and Asn721 do not participate in metal coordination, their replacements “lock” the resulting enzymes in an E1 (or E1P) conformation, which in turn prevents turnover at a measurable rate.

**DISCUSSION**

We have previously proposed that conserved residues in TMs H7 and H8 of Cu++-transporting ATPases are required for metal binding during metal translocation across the membrane (9). In this work, site-directed mutagenesis in combination with functional characterization was used to test this hypothesis. Four amino acids (Tyr682, Asn683, Met711, and Ser715) that probably participate in metal coordination were identified. These residues, together with two Cys in H6, probably form a transmembrane site with a distinct Cu++ coordination required for the transient binding and subsequent vectorial release. Various findings support these ideas. Tyr682, Asn683, Met711, and Ser715 are fully conserved in Cu++-transporting ATPases and Cu++-transporting ATPases (in the latter, a His replaces a Cys in H6) (51). Replacement of any of these four residues led to inactive CopA enzymes. Similarly, mutation of the relevant Ser in H8 of the human ATP7B (S1363F) has been identified in Wilson’s disease patients (52). These mutant CopA proteins were unable to perform Cu++-dependent partial reactions, although their structural integrity was apparent from their capacity to undergo “backdoor” phosphorylation by P1. These specific effects of Tyr682, Asn683, Met711, and Ser715 mutations, since they were not observed in proteins mutated in neighboring positions. Moreover, mutagenesis studies targeting polar amino acids conserved in several eukaryote Cu++-ATPases (but not in all Cu++-ATPases if noneukaryotes are also considered) and located close to the borders of TMs H3–H6 did not observe alterations in Cu++-dependent functions (44). Considering alternative explanations for the observed results, it could be speculated that while binding Cu++, Tyr682, Asn683, Met711, and Ser715-mutated proteins might be incapable of undergoing the subsequent confor-
nitional transitions and thus unable to sustain catalytic phosphorylation by ATP or prevent phosphorylation by P. However, the most parsimonious model to rationalize our findings is one in which these four amino acids participate in metal coordination and consequently yield mutants unable to bind Cu⁺.

The position of putative Cu⁺ binding residues in the center of H6, H7, and H8 has remarkable similarity with the cation binding sites in the Na,K-ATPase and Ca-ATPase (32–40). In both cases, amino acids in the central portion of the TMs flanking the large cytoplasmic loop contribute side chain atoms for metal coordination. Expanding this hypothesis, Fig. 6 shows a model of CopA H6, H7, and H8 based on one of the described Ca-ATPase structures, 1EUL (39). The low homology of CopA and SERCA1α in the region of interest limits the specific information to be inferred from the model; nevertheless, it suggests similar transport mechanisms in P₁B and P₁H-like ATPases, involving comparable conformational changes influencing the geometry of these TMs (40, 53–56). Whereas it is tempting to speculate on the arrangement of Cys380, Tyr682, Asn683, Met711, and Ser715 forming a metal binding site, key information is still missing. In particular, it might be considered that the stoichiometry of transport remains to be established. The distinct requirements for coordination of one or two Cu⁺ are clear. In addition, whereas some of the identified residues might be required for ion occlusion, others might be necessary for metal access to its binding site or alternatively required for metal delivery to the trans-side of the membrane.

Fig. 6 also shows the spatial relation of the putative Cu⁺ binding residues with other conserved amino acids in H7-H8 and provides a framework for interpreting the similar findings observed in proximal residues located in different TMs. For instance, Pro⁶⁰⁸ and Pro⁷⁰⁴ are probably located at the ends of the H7 and H8 helices, respectively. Replacement of these Pro residues has no major effect on enzyme function, and clearly, they do not participate in Cu⁺ binding. Pro are frequently present in the equivalent loop of other P-type ATPases. Mutation of Pro in the same region of the Na,K-ATPase has little functional effect (55). However, these seem necessary for proper insertion of the corresponding hairpin into the membrane (57), supporting a structural role introducing bends required for the joining loop. The model presented in Fig. 6 also reinforces the importance of residues in the cytoplasmic ends of H7 and H8. In the case of CopA, Asn⁶⁷⁵, Val⁷¹⁸, and Asn⁷²¹ substitutions lead to alterations compatible with the mutant enzymes being “locked” in an E₁ conformation and thus largely inactive and undergoing little or none “backdoor” phosphorylation. Significant functional alterations have also been shown by previous work characterizing mutations of conserved polar amino acids located in the equivalent region of the Na,K-ATPase (58), Ca-ATPase (59, 60), and H,K-ATPase (61). Depending on the model protein and the introduced mutation, lack of activity, high activity in the absence of contra-ion (Na⁺-ATPase), enzyme uncoupling, and reduced phosphoenzyme levels have been reported. The stringent structural requirements of H5-H6 (P₁H-like ATPases) and H7-H8 (CopA) cytoplasmic ends are perhaps not surprising, considering that the corresponding hairpins participate in critical conformational transitions (53–55).

In summary, amino acids Tyr⁶⁸², Asn⁶⁸³, Met⁷¹¹, and Ser⁷¹⁵, together with Cys⁶⁸⁰ and Cys⁷²², probably participate in Cu⁺ coordination during transport by a Cu⁺-ATPase. These findings advance our understanding of heavy metal binding sites not only in P₁H-type ATPases but also in other heavy metal transmembrane transporters. In addition, they support our previous hypothesis (9) concerning metal selectivity mechanisms and provide a basis to test alternative ideas on the transient binding heavy metals during their transport.

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