The Cadmium Transport Sites of CadA, the Cd\textsuperscript{2+}-ATPase from Listeria monocytogenes

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CadA, the Cd\textsuperscript{2+}-ATPase from Listeria monocytogenes, belongs to the Zn\textsuperscript{2+}/Cd\textsuperscript{2+}/Pb\textsuperscript{2+}-ATPase bacterial subfamily of P\textsubscript{1B}-ATPases that ensure detoxification of the bacteria. Whereas it is the major determinant of Listeria resistance to Cd\textsuperscript{2+}, CadA expressed in Saccharomyces cerevisiae severely decreases yeast tolerance to Cd\textsuperscript{2+} (Wu, C. C., Bal, N., Pérard, J., Lowe, J., Boscheron, C., Mintz, E., and Catty, P. (2004) Biochem. Biophys. Res. Commun. 310, 1034–1040). This phenotype, which reflects in vivo Cd\textsuperscript{2+}-transport activity, was used to select from 33 point mutations, shared out among the eight transmembrane (TM) segments of CadA, those that affect the activity of the protein. Six mutations affecting CadA were found: M149A in TM3; E164A in TM4; C354A, P355A, and C356A in TM6; and D692A in TM8. Functional studies of the six mutants produced in SF9 cells revealed that Cys\textsuperscript{354} and Cys\textsuperscript{356} in TM6 as well as Asp\textsuperscript{692} in TM8 and Met\textsuperscript{149} in TM3 could participate at the Cd\textsuperscript{2+}-binding site(s). In the canonical Cys-Pro-Cys motif of P\textsubscript{1B}-ATPases, the two cysteines act at distinct steps in the transport mechanism, Cys\textsuperscript{354} being directly involved in Cd\textsuperscript{2+} binding, while Cys\textsuperscript{356} seems to be required for Cd\textsuperscript{2+} occlusion. This confirms an earlier observation that the two equivalent Cys of Ccc2, the yeast Cu\textsuperscript{2+}-ATPase, also act at different steps. In TM4, Glu\textsuperscript{164}, which is conserved among P\textsubscript{1B}-ATPases, may be required for Cd\textsuperscript{2+} release. Finally, analysis of the role of Cd\textsuperscript{2+} in the phosphorylation from ATP and from Pi of the mutants suggests that two Cd\textsuperscript{2+} ions are involved in the reaction cycle of CadA.

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P-type ATPase is the generic name for a class of membrane proteins that pump cations against their electrochemical gradient at the expense of ATP. Whatever their origin, location, and ionic selectivity, P-type ATPases are thought to share the same overall fold and reaction mechanism. The P-type ATPase fold, determined to a few angstroms resolution in the Ca\textsuperscript{2+}-ATPase SERCA1a (1), is made of two domains, one cytoplasmic, bearing the catalytic site, the other membranous, comprising the cation transport site(s). The P-type ATPase reaction mechanism can be reduced to a four-step process (Scheme 1) where vectorial events (cation binding (step 1) and cation release (step 3)) and chemical events (phosphorylation (step 2) and dephosphorylation (step 4)) alternate (for recent reviews, see Refs. 2–4).

The P\textsubscript{1B}-ATPases (nomenclature from data base maintained by K. B. Axelsen) constitute a subfamily that assures the transport of heavy metals like Cu\textsuperscript{2+}, Ag\textsuperscript{+}, Zn\textsuperscript{2+}, Pb\textsuperscript{2+}, or Co\textsuperscript{2+} (5). They differ from most of the P\textsubscript{1B}-ATPases by a smaller membranous domain made of 8 transmembrane helices instead of 10 and the presence at their NH\textsubscript{2} terminus of 1–6 metal-binding domains (6, 7). Most of these NH\textsubscript{2}-terminal metal-binding domains are made of 70 amino acids comprising a Cys-X-X-Cys motif whose cysteines are directly involved in metal binding. In some cases, represented by the subgroup P1B-3, the NH\textsubscript{2} terminus consists of a histidine-rich sequence (8).

P-type ATPases possess in their membranous domain one or more sites which bind the cation(s) to be transported. These so-called transport sites have been mainly studied on Ca\textsuperscript{2+}-, Na\textsuperscript{+}/K\textsuperscript{+}-, and H\textsuperscript{+}/K\textsuperscript{+}-ATPases (P\textsubscript{2} subfamily) and H\textsuperscript{+}-ATPases (P\textsubscript{3} subfamily). It emerged from these studies that whatever the ionic selectivity of the pump, the transport site(s) involved at least three transmembrane helices, including those two that are directly linked to the catalytic loop. For instance, in SERCA1a, the Ca\textsuperscript{2+}-transport sites involve amino acids from four trans-membrane helices (1). In the transport sites of P\textsubscript{2} and P\textsubscript{3} ATPases, Ca\textsuperscript{2+}, Na\textsuperscript{+}, K\textsuperscript{+}, or H\textsuperscript{+}, which are all hard Lewis acids, are bound to carbonyl, carboxyl, or hydroxyl groups, all hard Lewis bases, thereby verifying the principle of hard and soft acids and bases (9). For instance, in SERCA1a, the Ca\textsuperscript{2+}-transport sites consist of Glu, Asp, Asn, and Thr, which bind Ca\textsuperscript{2+} through their side chain (10), plus some hydrophobic amino acids, which participate through their backbone carboxyl group, and also a water molecule.

Much less is known about the transport site of P\textsubscript{1B}-ATPases. Nevertheless, various early studies on Cu\textsuperscript{2+}/Ag\textsuperscript{+}-ATPases suggested a key role of the cysteines of the Cys-Pro-Cys motif located in TM6, the upstream transmembrane helix linked to...
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\[
\begin{array}{ccc}
\text{ATP} & \text{Cd₄E} & \text{ADP} \\
\text{nCd²⁺_{cyt}} & \text{E} & \text{nCd²⁺_{extracyt}} \\
\text{Cd₄E-P} & \text{Pi} & \text{E-P} \\
\text{H₂O} & \text{H₂O} & \text{H₂O}
\end{array}
\]

**SCHEME 1**

The catalytic loop (11–19). This exemplifies once again the hard and soft acids and bases principle, soft Lewis acids (\(\text{Cu}^+\), \(\text{Ag}^+\)) interacting with a soft Lewis base (S) (9). However, as described for the yeast \(\text{Cu}^+\)-ATPase Ccc2p (19) and for model peptides of the human \(\text{Cu}^+\)-ATPase ATP7B (20), the two cysteines of the Cys-Pro-Cys motif are likely to play distinct roles in ion transport. In ATP7B, two mutations flanking the Cys-Pro-Cys motif were found to be responsible for Wilson disease, an autosomal recessive defect of copper transport. Expressed in *Saccharomyces cerevisiae*, the ATP7B mutants T977M and P922L were unable to complement a ccc2 mutant and had a reduced Fet3p activity, suggesting a defect in copper transport (11).

Not only TM6 but also TM8 is likely to be involved in the transport site of \(\text{P}_{1B}\)-ATPases. Indeed, the toxic milk mouse considered as an animal model for Wilson disease was shown to bear a mutation in the murine ATP7B homologue. Located in TM8, this mutation, the replacement by valine of a methionine highly conserved in the \(\text{P}_{1B}\)-1 subgroup, was shown to abolish \(\text{Cu}^+\)-transport (21, 22).

A recent study used sequence alignments to predict which amino acids from TM6, TM7, and TM8 could constitute the transport site of \(\text{P}_{1B}\)-ATPases, assuming that these transmembrane helices can play the same role as those forming the transport sites in \(\text{P}_{1R}\) and \(\text{P}_{3}\)-ATPases. Alignment of over 200 sequences revealed some amino acids in TM7 and TM8 that are conserved and were therefore used to define five \(\text{P}_{1B}\)-ATPase subgroups. As these subgroups display different ionic selectivity, these amino acids were predicted as putative components of the transport site of \(\text{P}_{1B}\)-ATPases (8). The prediction has been verified for the \(\text{Cu}^+/\text{Ag}^+\)-ATPase CopA from *Archeoglobus fulgidus*, a member of subgroup IB-1, whose mutations at Tyr⁶₈₂ (TM7), Asn⁶₈₃ (TM7), Met⁷¹₁ (TM8), and Ser⁷¹⁵ (TM8) were shown to severely affect ATPase activity and phosphoenzyme formation from ATP (23).

The inventory of the ATP7B mutations causing Wilson disease suggests that transmembrane segments other than TM6, TM7, or TM8 can participate in the transport site of the \(\text{P}_{1B}\)-type ATPases. Indeed, among the 33 mutations of the transmembrane domain of ATP7B found in patients with Wilson disease, only 11 are located in TM6, TM7, or TM8 (see the Wilson disease data base).

The experimental studies carried out so far on the transport site of \(\text{P}_{1B}\)-ATPases have all focused on \(\text{Cu}^+/\text{Ag}^+\)-ATPases, which constitute the largest subgroup of heavy metal pumps known today. In the present study, we have searched for the amino acids involved in the transport site of CadA, the \(\text{Cd}^{2+}\)-ATPase from *L. monocytogenes*, a member of subgroup P1B-2. To do so, we first used a phenotypic test in yeast to identify among 33 point mutations of the transmembrane domain of CadA those that affect \(\text{Cd}^{2+}\)-transport. We then produced the selected mutants in *S. cerevisiae* cells to assess how \(\text{Cd}^{2+}\) transport was affected. We found that (i) the two cysteines of the Cys-Pro-Cys motif (TM6) act at distinct steps of the transport process, Cys³⁵⁴ being directly involved in \(\text{Cd}^{2+}\) binding, whereas Cys³⁵⁶ is required for \(\text{Cd}^{2+}\) occlusion; (ii) Asp⁶⁹² in TM8 would be directly involved in \(\text{Cd}^{2+}\) binding; (iii) Glu¹⁶⁴ in TM4 would be required for \(\text{Cd}^{2+}\) release. In addition, we propose that two \(\text{Cd}^{2+}\) ions are involved in the reaction cycle of CadA.

**MATERIALS AND METHODS**

**Molecular Biology Procedures**—JM109 *Escherichia coli* strain was used for DNA subcloning and amplification. Restriction and modification enzymes were from Fermentas or Invitrogen. DNA fragments were isolated using the Gene Clean Spin kit (Q-Biogene). Plasmids were purified by anion exchange chromatography (Qiagen). Site-directed mutagenesis was done using the QuickChange method (Stratagene). DNA sequencing was performed by Genome Express Inc. (Meylan, France).

**Yeast Manipulations**—Yeast procedures are detailed in Ref. 24. The yeast strain used in the present study, W303-1A (25), was transformed following the method of Kuo and Campbell (26) and grown at 30 °C, with continuous stirring at 200 rpm for liquid cultures. Yeast transformants were grown in synthetic minimal DOB medium (2% glucose, 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, Q-Biogene) supplemented with dropout powder without uracil (CSM-URA, Q-Biogene). HA-tagged CadA mutants were expressed from a centromeric vector derived from pRS316 (27), under the control of the strong constitutive PMA1 promoter. The sensitivity of the different mutants was determined by drop tests, and their expression level was assessed by immunodetection of the HA epitope on a membrane preparation.

**Protein Preparation and Detection**—The wild-type and mutant CadA proteins were produced using the Bac-to-Bac™ Baculovirus Expression System (Invitrogen). After 3 days of culture at 27 °C, 100 ml of cells (2 × 10⁶ cells/ml) were harvested by centrifugation (10 min, 500 × g, 4 °C). The pellet was suspended in 18 ml of 10 mM MOPS/Tris (pH 7), 100 mM KCl, 0.1 mM MgCl₂, 300 mM sucrose. The sample was centrifuged (1 h, 100,000 × g, 4 °C), and the P100 pellet corresponding to a crude membrane fraction was suspended in 3 ml of 50 mM MOPS/Tris (pH 7), 100 mM KCl, 1 mM MgCl₂, 300 mM sucrose. Aliquots were homogenized by potterization, rapidly frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined using the DC Protein Assay (Bio-Rad), with bovine serum albumin as standard. Immunodetection was carried out using the monoclonal antibody anti-HA-peroxidase (3F10) and the BM Chemiluminescence Western blotting kit from Roche Applied Science.
ATPase Activity Measurements—ATPase activities were measured at 28 °C with continuous stirring, in a medium containing 50 mM MOPS/Tris (pH 7), 100 mM KCl, 300 mM sucrose, 5 mM MgCl₂, 4 mM ATP, and 0.5 mg/ml of Sf9 membrane preparations. ATP hydrolysis was measured following NADH absorbance changes at 340 nm, using a coupled enzyme assay as described in Ref. 28.

Phosphorylations—The phosphoenzyme intermediates formed in the presence of 32P, or [γ-32P]ATP were quantitated as a function of Cd²⁺ concentration. In the two types of reactions, the acid-quenched sample was submitted to an acidic SDS-PAGE as described by Weber and Osborn (29). The phosphorylation signal was revealed by autoradiography and analyzed using the Optiquant Software (Cyclone, PerkinElmer Life Sciences). Later, polycrylamide gels were stained with Coomassie Blue to check the amounts of loaded protein. Phosphorylations from P₃ were performed at 25 °C in a medium containing 0.5 mg/ml of S9 membrane preparations, 50 mM MES pH 6, 10 mM MgCl₂, 20% Me₂SO, in the presence of CdCl₂ or EGTA as indicated in the figure legend to Fig. 5. After 10-min incubation at 25 °C, the reaction was started by addition of 100 μM 32P, at 10–100 μCi/nmol and stopped 10 min later by addition of 1 ml of ice-cold 1 mM KH₂PO₄ in 7% trichloroacetic acid. Phosphorylations from ATP were performed at 0 °C in a medium containing 0.5 mg/ml of S9 membrane preparations, 50 mM MOPS/Tris (pH 7), 100 mM KCl, 5 mM MgCl₂, 300 mM sucrose, and CdCl₂ as indicated. The reaction was started by addition of 1 μM [γ-32P]ATP at 50–500 μCi/nmol and stopped 15 s later by addition of 1 ml of ice-cold 1 mM KH₂PO₄ in 7% trichloroacetic acid.

RESULTS

The present work aims to identify the amino acids of the CadA transmembrane domain that are involved in Cd²⁺ binding and release during the transport cycle.

CadA Topology—The initial step of this study consisted of the proper delimitation of the transmembrane segments of CadA, using the results of 10 prediction programs and a comparison with the experimentally determined topology of a P₁₆⁶ ATPase from Helicobacter pylori (30). Eight transmembrane segments were defined, hereafter named TM1 to TM8, and we must emphasize the uncertainty concerning the delimitation of TM3 and TM4 as well as TM7 and TM8 (Fig. 1). Point mutations were performed on negatively charged (Asp, Glu), polar (Ser, Thr, and Asn), and sulfur-containing amino acids (Cys, Met) liable to coordinate Cd²⁺, and sulfur-containing amino acids (Cys, Met) liable to coordinate Cd²⁺ or to participate in its interaction with CadA (Fig. 1).

Phenotypic Sorting of CadA Mutants—Expression of CadA in S. cerevisiae was shown to strongly increase yeast sensitivity to Cd²⁺. As CadA was found inserted in the membrane of the endoplasmic reticulum, this phenotype was explained by the toxicity of Cd²⁺ accumulated in this compartment (24). In the following, we used this phenotype as an assessment of the transport activity to sort CadA mutants produced by site-directed mutagenesis.

Three classes of mutants were defined (Fig. 1). Class 1 comprises all the mutants (27 of the 33 tested) that induce growth arrest at 1 μM Cd²⁺ as does the wild-type protein (24). Class 3 comprises mutants that do not alter yeast response to Cd²⁺. The reference in this class is D398A, a mutant of the phosphorylation site (DKTGT) that does not transport Cd²⁺ (24). To this class belong the mutants C354A and C356A of the Cys-Pro-Cys motif cysteines in TM6 and the mutant D692A in TM8. Class 2 was defined as intermediate between class 1 and class 3, as some mutants induce yeast growth arrest at 10 μM Cd²⁺. In this class are found the mutants M149A, E164A, and P355A, which belong to TM3, TM4, and TM6, respectively. The expression level of the mutants was assessed by immunodetection of the HA-epitope added at the COOH-terminal end of the protein. The mutants were all equally produced in yeast, whatever the class they belong to (Fig. 2).

Functional Study of the Selected CadA Mutants—Yeast was a very efficient system for sorting 6 mutants from 33 and, according to the phenotype, C354A, C356A, and D692A (class 3) are expected to be unable to transport Cd²⁺, whereas in M149A, E164A, and P355A (class 2) lower transport efficiency is expected. To assess the role of each of these 6 amino acids in the
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Limited Proteolysis—Limited proteolysis by trypsin was used to compare the conformational states of the mutants with that of CadA. As shown in Fig. 3 (lane D), the digestion pattern resulting from CadA proteolysis in the absence of ATP displays two major bands of about 46 and 10 kDa, as well as a doublet around 27 kDa. Considering the location of the HA tag at the COOH-terminal end of CadA, these bands could result from cleavages at the end of the A domain, at the end of the large COOH-terminal end of CadA, these bands could result from a cleavage in the middle of the A domain. The presence of 5 mM ATP clearly modifies the proteolysis profile of CadA in vitro biochemical studies of the mutants were necessary, and yeast was not well suited anymore. The main limitations were the low expression level of the proteins and the high endogenous ATPase activity of yeast membranes. This is the reason why the six mutants selected from the phenotypic test were produced in Sf9 cells for functional studies. In this system, all the mutants exhibited the same expression level and they were all studied in a crude membrane preparation. Four methods were used to assess the functionality of the mutants: limited proteolysis, activity measurements, phosphorylation from ATP, and phosphorylation from pH 7. In all these experiments, the non-functional D398A mutant was used to measure the endogenous activity of membranes from transfected Sf9 cells.

**Limited Proteolysis**—Limited proteolysis by trypsin was performed on 50 µg of Sf9 membranes containing CadA bearing an HA epitope at its COOH-terminal end. The samples were incubated at 30 °C for 5 min in a medium containing 20 mM Tris-HCl (pH 7), 0.1 mM KCl, and 5 mM MgCl₂, without trypsin (lane N), with trypsin (trypsin/protein = 10 (w/w)) (lane D), or with trypsin plus 5 mM ATP (lane A). HA-tagged fragments were immunodetected as described for Fig. 2. Experiments were reproducibly obtained three times on two different membrane preparations. Limited proteolysis of CadA mutants is displayed in supplemental Fig. S1.

ATPase Activity Measurements—A membrane fraction from Sf9 cells expressing CadA displays a $\text{Cd}^{2+}$-dependent ATPase activity of about 50 nmol of ATP hydrolyzed per min and mg of protein. This value results from two effects of Cd²⁺, which on the one hand inhibits the endogenous ATPase activity, as illustrated in the membrane fraction containing the non-functional D398A mutant, and on the other hand activates CadA (Ref. 31 and Fig. 4).

ATPase activity measurements on membrane fractions from Sf9 cells expressing the various mutants showed that two of the mutants, C354A and D692A, were inactive. The other four mutants M149A, E164A, P355A, and C356A all displayed an ATPase activity in the same Cd²⁺ concentration range as CadA. The maximal activities were lower than that of CadA and ranged from 10 nmol of ATP hydrolyzed per min and per mg of protein for M149A and P355A to 20 for C356A and 40 for E164A.

ATPase activity measurements (in vitro) were in complete agreement with the phenotype-based classification (in vivo), for five of the six mutants. As expected, the class 3 mutants C354A and D692A are inactive, and the class 2 mutants M149A, E164A, and P355A partially active. The fact that C356A, a class 3 mutant, displayed a Cd²⁺-dependent ATPase activity in the same Cd²⁺ concentration range as CadA. The maximal activities were lower than that of CadA and ranged from 10 nmol of ATP hydrolyzed per min and per mg of protein for M149A and P355A to 20 for C356A and 40 for E164A.

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The Phosphorylated Intermediates—CadA can be phosphorylated from ATP in the forward direction (Scheme 1, step 2) and from P, in the backward direction (Scheme 1, step 3). These two reactions depend on Cd²⁺. The presence of Cd²⁺ at the transport site of CadA (Scheme 1, Cd₆E) is required.
for the phosphorylation from ATP (Scheme 1, step 2), whereas Cd2⁺ binding at that site (Scheme 1, step 1) competes with the phosphorylation from Pᵢ (Scheme 1, reverse of step 4). Hence, both phosphorylation reactions give access to an apparent affinity of the Cd2⁺-transport site.

**Phosphorylation from Pᵢ—**Membrane fractions from S9 cells expressing CadA are phosphorylated from Pᵢ, at equilibrium (Fig. 5A). In the absence of Cd2⁺, full phosphorylation is reached, hence the 100% value. Cd2⁺ inhibits CadA phosphorylation from Pᵢ with an apparent affinity of 0.5 μM (Fig. 5A). It is commonly admitted for P-type ATPases that such an inhibition reflects the binding of the transported ion to the transport site (see Ref. 33 for SERCA1a). However, like most of the P₁B-ATPases, CadA possesses an additional metal-binding domain in its cytoplasmic NH₂-terminal end (34) that was shown to regulate CadA activity (31). To verify whether such a domain could participate in the inhibition of the phosphorylation from Pᵢ, we performed similar experiments on two CadA mutants, one truncated from its NH₂ terminus (ΔMBD), the other mutated at the two cysteines of the Cys-X-X-Cys motif (CadA-AA). Cd2⁺ inhibits CadA, ΔMBD, and CadA-AA phosphorylation from Pᵢ at the same concentration, reflecting its binding to the transmembrane domain (Fig. 5B).

When the same experiments were performed on the class 2 and class 3 mutants, the same phosphorylation level was measured in the absence of Cd2⁺ (Fig. 5A). The apparent affinity for Cd2⁺ is similar to that of CadA for M149A (0.5 μM) and P355A (0.7 μM), suggesting that Cd2⁺ binding is not altered in these two mutants. On the other hand, D692A (5.1 μM) and C354A (7.5 μM) displayed more than 10-fold decrease in their apparent affinity for Cd2⁺, suggesting an essential role of Cys⁵⁵⁴ and Asp⁶⁹² in Cd2⁺ coordination, in agreement with the finding that these two mutants do not have any ATPase activity (Fig. 4).

To a lesser extent, C356A (2 μM) and E164A (2.3 μM) also exhibited a reduced apparent affinity for Cd2⁺.

**Phosphorylation from ATP—**In the presence of Cd2⁺, addition of ATP to membrane fractions from S9 cells expressing CadA triggers the enzymatic cycle (Scheme 1). The phosphoenzyme level, measured 15 s later, reflects the ratio between the phosphorylation and dephosphorylation rates of the cycle (Fig. 6A). The effect of Cd2⁺ on CadA phosphorylation from ATP displayed two phases. First, the phosphoenzyme increased from 0 to 20 μM Cd2⁺ and stabilized at a level corresponding to 25% of the maximum level reached by CadA, until 100 μM Cd2⁺ was reached. It should be emphasized that the apparent affinity for Cd2⁺ in this first phase (0.75 μM) is similar to that previously estimated from Pᵢ phosphorylation (Fig. 5A). The second phase of the phosphorylation peaked at 1 mM Cd2⁺ (the phosphorylation level decreased at higher Cd2⁺ concentrations). Referring to Scheme 1, the second phase could reflect an inhibitory effect of Cd2⁺ on Cd2⁺ release (step 3), leading to the accumulation of CdₐE=⁻P. Hence, the first phase results from Cd2⁺ binding to

![Figure 4. ATPase activity measurements.](image-url)
The Membranous Cd\textsuperscript{2+}-transport Site(s) of CadA

**FIGURE 6.** Phosphorylation from ATP. A, phosphorylation from ATP was performed at 0°C in a medium containing 0.5 mg/ml S9 membrane preparations, 50 mM MOPS/Tris (pH 7), 100 mM KCl, 5 mM MgCl\textsubscript{2}, 300 mM sucrose. The reaction was started by the addition of 1 μM [γ\textsuperscript{32}P]ATP at 50–500 μCi/nmol and stopped 15 s later by addition of 1 ml ice-cold 1 mM KH\textsubscript{2}PO\textsubscript{4} in 7% trichloroacetic acid. The samples were treated as described for the phosphorylation from P\textsubscript{i}; 100% phosphorylation was taken as the phosphorylation level for the wild-type protein authorizes “over”-phosphorylation levels such as those shown below for the mutants C356A and E164A (Fig. 6B).

As shown in Fig. 6A, C356A and D692A, two class 3 mutants characterized by a low apparent affinity for Cd\textsuperscript{2+} and no ATPase activity, do not produce any phosphorylated intermediates from ATP, a result that seems to contradict the inhibition of the phosphorylation from P\textsubscript{i} by Cd\textsuperscript{2+} shown in Fig. 5A. Unlike C354A and D692A, C356A, the third class 3 mutant, is phosphorylatable from ATP (Fig. 6, A and B). However, the phosphorylation profile of C356A has two main differences from that of CadA, a shift of the first phase toward higher Cd\textsuperscript{2+} concentrations and an increase in the phosphoenzyme level of the second phase. Bearing in mind the origin of the two phases, the enhancement of the second phase suggests that the slow-down of the Cd\textsubscript{E}P to E-P conversion at high Cd\textsuperscript{2+} concentrations is more pronounced in C356A than in the wild type. The shift of the first phase suggests a reduced affinity of C356A for Cd\textsuperscript{2+}, in agreement with the P\textsubscript{i} phosphorylation experiments (Fig. 5A). The phosphorylation profile of E164A is different from that of CadA as it displays only one phase, whose maximum is reached at 10 μM Cd\textsuperscript{2+} (Fig. 6, A and B). To gain more information on this phosphoenzyme, we assessed its reactivity toward ADP in the presence of 50 μM Cd\textsuperscript{2+} and compared it to the reactivity of CadA (Fig. 6D). ADP allows discrimination between the first phosphoenzyme, Cd\textsubscript{E}P, which is able to donate the phosphoryl group back to ADP to synthesize ATP, and the second phosphoenzyme, E-P (Scheme 1). Therefore, EGTA was added to the phosphoenzyme to stop cycling, and the existence of Cd\textsubscript{E}P was assessed from the effect of ADP on the phosphoenzyme level (compare lanes 2 and 3). E164A phosphoenzyme totally disappeared upon addition of ADP, suggesting it is Cd\textsubscript{E}P, as found for CadA (Fig. 6D). As suggested for CadA at high Cd\textsuperscript{2+} concentration, the overphosphorylation observed with E164A at 10 μM Cd\textsuperscript{2+} could result from a direct effect of the mutation of Glu\textsuperscript{164} on Cd\textsuperscript{2+} release.

The two other class 2 mutants, M149A and P355A, are phosphorylatable from ATP but their phosphorylation level is so low that it needs 1 mM Cd\textsuperscript{2+} to be measurable (Fig. 6A). Such a low phosphorylation level is in good agreement with the low ATPase activity of M149A and P355A (Fig. 4) but seems to contradict the fact that the two mutants display the same apparent affinity for Cd\textsuperscript{2+} as CadA (Fig. 5A). This means that in both mutants, as for C354A and D692A, the mutation has affected an event occurring after Cd\textsuperscript{2+} binding and that is necessary for phosphorylation from ATP.

**DISCUSSION**

In this study, we used a phenotypic test in *S. cerevisiae* to detect amino acids critical for Cd\textsuperscript{2+} transport by CadA, the Cd\textsuperscript{2+}-ATPase from *L. monocytogenes*. Thirty-three acidic, polar, or sulfur-containing amino acids belonging to the transmembrane segments of CadA were mutated to alanine and expressed in yeast. Mutants were classified in one of the three following classes based upon the sensitivity to Cd\textsuperscript{2+} they confer on yeast cells. The class 1 mutants (27 of the 33 mutants tested) induce cell growth arrest at 1 μM Cd\textsuperscript{2+} as does CadA and thereby correspond to functional proteins. The class 3 mutants,
C354A and C356A of the Cys-Pro-Cys motif in TM6 and D692A in TM8, do not affect yeast sensitivity to Cd\(^{2+}\), similar to the phosphorylation site mutant D398A, and thereby correspond to non-functional proteins. The class 2 mutants, M149A in TM3, E164A in TM4, and P355A in TM6, induce cell growth arrest at 10 \(\mu\)M Cd\(^{2+}\). This intermediate phenotype suggests that these mutants are functional but less efficient than CadA.

The Class 3 Mutants—The critical role of the Cys-Pro-Cys motif cysteines has been well established in P\(_{1b}\)-ATPases through numerous studies of single and double mutants (11, 12, 15–17, 19, 35). In addition, the mutated motifs Arg-Pro-Cys in ATP7A (14) and Cys-Pro-Tyr in ATP7B (13) were found in patients with Menkes and Wilson diseases, respectively.

As revealed by sequence analysis, not only thiolate but also imidazolyl groups as, those found in the Cys-Pro-His motif of P1B-3 ATPases, are likely to participate in metal binding (8). Sequence analysis also showed that the TM6 motif did not necessarily contain two metal ligand groups as illustrated by the Ser-Pro-Cys motif of P1B-4 ATPases, the Thr-Pro-Cys motif of P1B-5 ATPases, and the Ala-Pro-Cys or Cys-Pro-Gly motifs found in some P1B-6 ATPases. This suggests that the cysteines may have distinct roles in metal transport as shown with the yeast Cu\(^{2+}\)-ATPase Ccc2p whose first cysteine, Cys\(^{583}\), seems to be essential for copper release, whereas the second one, Cys\(^{585}\), is apparently required for copper binding (19).

Our results suggest that the Cys-Pro-Cys motif cysteines of CadA also have distinct roles in metal transport. The first one, Cys\(^{354}\), whose mutation severely affects the apparent affinity for Cd\(^{2+}\), appears to be essential for metal binding and would therefore be the equivalent of Ccc2p Cys\(^{583}\). Interestingly, the two mutants C354A for CadA and C358S for Ccc2p are both unable to be phosphorylated from ATP and have no ATPase activity.

The data concerning C354A reveal a step that is not described in Scheme 1, in between Cd\(^{2+}\) binding and the ATP-phosphorylation reaction. The fact that in this mutant Cd\(^{2+}\) binding competes with phosphorylation from P\(_i\) but does not promote phosphorylation from ATP is puzzling. If only one Cd\(^{2+}\) is involved in Scheme 1, inhibition by Cd\(^{2+}\) of the phosphorylation from P\(_i\) implies that the enzyme reaches the Cd\(_d\)-E state, which is normally phosphorylatable from ATP. As deduced from Fig. 5A, C354A adopts, in the presence of Cd\(^{2+}\), a conformation that forbs any phosphorylation from P\(_i\), but obviously this conformation is not phosphorylatable from ATP. One could argue that the low temperature used for the phosphorylation with ATP could slow down Cd\(^{2+}\) binding and thereby prevent the phosphorylation. However, neither the increase in the reaction time nor in the temperature (up to 10 min at 25 °C as for phosphorylation from P\(_i\)) allows C354A to be phosphorylated from ATP (data not shown). Our results mean that in C354A the mutation has affected an event which occurs after Cd\(^{2+}\) binding and is necessary for phosphorylation from ATP. This event could be a conformational change normally induced by Cd\(^{2+}\) and required for phosphorylation from ATP. Alternatively, this event could be the binding of a second Cd\(^{2+}\) ion, impaired by the mutation. In that case, Cd\(^{2+}\) binding to the remaining site would prevent phosphorylation from P\(_i\) without promoting phosphorylation from ATP. What we observed with C354A has been described earlier for the mutations of Glu\(^{309}\), Asn\(^{796}\), and Asp\(^{800}\), the major constituents of the Ca\(^{2+}\)-binding site II of SERCA1a. As SERCA1a transports two Ca\(^{2+}\) ions per reaction cycle, it was suggested that the binding of one Ca\(^{2+}\) was sufficient to inhibit the phosphorylation with P\(_i\) (10).

Taking into account the sequence and functional similarities between CadA and ZntA, it is unlikely that these two proteins follow different enzymatic mechanisms. However, our hypothesis regarding the CadA mechanism seems to disagree with the recent finding that the transmembrane domain of ZntA binds one metal ion (35). An explanation for such a discrepancy could be that the repeated cycles of dilution and concentration performed before inductively coupled plasma mass spectroscopy measurements have removed one of the two ions bound to ZntA. This would not be unexpected, since the binding of an ion to a transport site is supposed to follow a reversible equilibrium, as illustrated with SERCA1a (36).

The mutation of the second Cys-Pro-Cys motif cysteine of CadA, Cys\(^{356}\), significantly affects the apparent affinity for Cd\(^{2+}\), but above all uncouples ATP hydrolysis and Cd\(^{2+}\) transport. As C356A is phosphorylatable from ATP, a question arises on the nature of the defect induced by the mutation. Does C356A follow a reaction cycle where only Cd\(^{2+}\) release is impaired? This would imply that C356A still reaches the E-P state, as observed for the uncoupled Y763G mutant of SERCA1a (37). Alternatively, does C356A follow an aborted cycle where Cd\(^{2+}\) release and, therefore, E-P formation, do not occur, as observed for the uncoupled D684N mutant of the H\(^{+}\)-ATPase AHA2 (38)? Although we do not know yet the reasons for this uncoupling, it is remarkable that Cys\(^{356}\) is the equivalent of Glu\(^{309}\) in SERCA1a, whose essential role in Ca\(^{2+}\) gating and occlusion has been recently demonstrated (39).

D692A in TM8 displays the same biochemical characteristics as C354A and is likely to be directly involved in metal coordination. Sequence alignments reveal that an aspartate is present at the place of Asp\(^{692}\) in all the P1B-2 ATPases and that a conserved His-Glu motif is found at the same location in P1B-4 ATPases (8). As P1B-1 and P1B-3 ATPases all have a methionine at this position, the presence of carboxylic acids in TM8 could be required for the transport of divalent metals. An interesting parallel can be drawn between this assumption and the results obtained for the NH\(_2\)-terminal metal-binding domains of P\(_{1b}\)-ATPases showing that in addition to the two cysteines of the Cys-X-X-Cys motif, which are sufficient to coordinate Cu\(^{+}\) in P1B-1 ATPases, an aspartate in ZntA (40) and a glutamate in CadA (34) are likely to participate in metal coordination. The functional importance of TM8 of P\(_{1b}\)-ATPases has been previously illustrated with the toxic milk mouse, a murine model of Wilson disease (21, 22, 41), and more recently with the Cu\(^{+}\)-ATPase CopA from Archeoglobus fulgidus (23) and the Zn\(^{2+}\)-ATPase ZntA from E. coli (42). In P\(_{2}\)- and some P\(_{3A}\)-ATPases, a conserved aspartate was shown to be an essential constituent of the ionic transport site (38, 43–45). As an example, Asp\(^{800}\) in SERCA1a is involved in both Ca\(^{2+}\) transport sites and its mutation in Asn abolishes Ca\(^{2+}\) occlusion, thereby impairing transport (10, 46).

Assuming a reaction cycle involving two Cd\(^{2+}\) ions, Cys\(^{354}\) and Asp\(^{692}\) appear as indispensable constituents of one Cd\(^{2+}\)
binding site (site 2), their mutation preventing phosphorylation from ATP. They are likely to participate also at the other Cd²⁺-binding site (site 1), as suggested by the low efficiency of Cd²⁺ in inhibiting C354A and D692A phosphorylation by P₁. Following the same reasoning, Cys³⁵⁶ may participate at site 1 but above all could have an important role in Cd²⁺ occlusion.

The Class 2 Mutants—Sequence analysis revealed that Met¹⁴⁹ is highly conserved in P₁B-2 (present in 115 of 123 sequences) and P₁B-4 ATPases (present in the 39 sequences analyzed). As no such conserved amino acid is found at this position in P₁B-1 or in P₁B-3 ATPases, this methionine could be specific to Cd²⁺-, Zn²⁺-, Pb²⁺-, and Co²⁺-ATPases. Since the apparent affinity of M149A for Cd²⁺ is similar to that of CadA, Met¹⁴⁹ is probably not involved at site 1. On the other hand, it is difficult for M149A to be phosphorylated from ATP, suggesting that Met¹⁴⁹ somehow participates at site 2.

The proline Pro³⁵⁵ in TM6 corresponds to the highly conserved proline in the upstream transmembrane helix linked to the catalytic loop of P₂-, P₃-, and P₄-ATPases. Except in P₄-ATPases (aminophospholipid translocases), this residue is located at a conserved distance from the phosphorylation site aspartate. In SERCA1a, Pro³⁰⁸ and Pro³¹⁰ introduce unwinding of the transmembrane helix by twisting the backbone and depriving the carbonyl groups of their natural hydrogen-bonding partners. This allows Val³⁰⁴, Ala³⁰⁵, and Ile³⁰⁷ to participate in Ca²⁺-coordinated at site II, by their backbone carbonyl groups. Other P₂-ATPases like the H⁺-ATPases AH₂ from Arabidopsis thaliana and PMA1 from S. cerevisiae contain prolines at equivalent positions (Pro²⁸⁶ and Pro²⁹⁰ for AH₂, Pro³⁵⁵ and Pro³⁵⁹ for PMA1), suggesting that the transmembrane helix linked to the catalytic loop is also unwound in these pumps. As suggested by homology modeling, unwinding of this transmembrane helix in AH₂ and PMA1 would expose some backbone carboxyls (Ile²⁸², Gly²⁸³, and Ile²⁸⁵ for AH₂ and Ile³³¹, Ile³⁳², and Val³³⁴ for PMA1) to the hydronium ion-binding site (47). In CadA, the mutation of Pro³⁵⁵ does not affect the apparent affinity for Cd²⁺, as assessed by phosphorylation from P₁, but strongly reduces the phosphoenzyme level from ATP. This suggests that Pro³⁵⁵, which cannot by itself provide any coordination to Cd²⁺, could ensure the proper arrangement of the amino acids participating at site 2.

The glutamate Glu¹⁶⁴ in TM4 is a highly conserved amino acid of P₁B-ATPases, sometimes substituted by an aspartate or shifted backward by one amino acid as in P₁B-1 ATPases. In CadA, we propose that mutation of Glu¹⁶⁴ affects the dephosphorylation rate of the reaction cycle (step 3). Interestingly, mutation of the equivalent amino acid in the yeast Cu⁺-ATPase Ccc2p, namely Asp⁷⁷⁵, was also shown to affect this step (19). As mutation of Glu¹⁶⁴ also affects the apparent affinity for Cd²⁺ as assessed by the phosphorylation from P₁, it is likely that this residue somehow participates at site 1. However, it is probably an indirect effect, since Glu¹⁶⁴ is predicted to be located at the membrane/extracellular medium interface.

Our work has pointed out 6 amino acids from the transmembrane domain of CadA as being involved in the transport of Cd²⁺. These amino acids are either highly conserved in P₁B-ATPases like Cys³⁵⁴, Pro³⁵⁵, Cys³⁵⁶, and Glu¹⁶⁴ or specific for the Cd²⁺/Zn²⁺/Pb²⁺-ATPases like Met¹⁴⁹ and Asp⁶⁹². We have also proposed that two Cd²⁺ ions could be involved in the reaction cycle of CadA. One of them would be located at a site comprising Cys³⁵⁴, Cys³⁵⁶, and Asp⁶⁹² (site 1), whereas the other would be located at a site comprising Met¹⁴⁹, Cys³⁵⁶, and Asp⁶⁹² (site 2). In that configuration Asp⁶⁹² would be shared by the two sites, a situation similar to that encountered in SERCA1a for Asp⁸⁰⁰. It is plausible that Cys³⁵⁴ belongs to the two sites, when referring to the coordination of Cd²⁺ in metallothioneins (48). Obviously, more in-depth investigations will be required to test these assumptions.

The 6 amino acids involved in Cd²⁺ transport by CadA belong to four transmembrane segments. Interestingly, two of these segments are located upstream from the A domain in the primary sequence, a location that differs from P₂- and P₃-ATPases in which the transmembrane segments constituting the ion transport site are all located downstream from the A domain. This probably results from the specific topology of P₁B-ATPases and suggests, given the role of the A domain in the cycle of P₂-ATPases, that the detailed mechanism of ion transport in P₁B-ATPases could differ from that of SERCA1a, the best known P-type ATPase, which is often taken as the reference for all of them.

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