Cd²⁺ and the N-terminal metal-binding domain protect the putative membranous CPC motif of the Cd²⁺-ATPase of *Listeria monocytogenes*

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CadA, the Cd²⁺-ATPase of *Listeria monocytogenes*, contains four cysteine residues: two in the CTNC (Cys-Thr-Asn-Cys) sequence in the cytoplasmic metal-binding domain (MBD), and two in the CPC (Cys-Pro-Cys) sequence in the membrane domain. Taking advantage of ΔMBD, a truncated version of CadA that lacks the MBD but which still acts as a functional Cd²⁺-ATPase [Bal, Mintz, Guillain and Catty (2001) FEBS Lett. 506, 249–252], we analysed the role of the membrane cysteine residues (studied using ΔMBD) separately from that of the cysteine residues of the MBD, which were studied using full-length CadA. The role of the cysteines was assessed by reacting ΔMBD and CadA with *N*-

ethylmaleimide (NEM), an SH-specific reagent, in the presence or absence of Cd²⁺. We show here that (i) in both ΔMBD and CadA, the cysteine residues in the CPC motif are essential for phosphorylation; (ii) in both proteins, Cd²⁺ protects against alkylation by NEM; and (iii) in the absence of Cd²⁺, the MBD of CadA also protects against alkylation by NEM. Our results suggest that the CPC motif is present in the membrane Cd²⁺ transport site(s) and that the MBD protects these site(s).

Key words: CadA, CXXC, heavy metal, P-type ATPase, transport site.

INTRODUCTION

The P-type ATPases are widespread membrane proteins that are found in all living cells from bacteria to humans. They occur in the plasma membrane and in many subcellular compartment membranes, and transport a broad variety of ions against their own electrochemical gradient at the expense of ATP hydrolysis. During their catalytic cycle, P-type ATPases are transiently phosphorylated, hence their name.

Prediction programs suggest that P-type ATPases share the same spatial organization as that elucidated recently for Serca1a, the sarcoplasmic reticulum Ca²⁺-ATPase [1]. In Serca1a, the part embedded in the lipid bilayer is a bundle of 10 transmembrane helices, four of which are organized to create the ion pathway. The part exposed to the cytoplasm, which contains the major amino acid sequence signatures [2], is composed of two domains, the larger of which comprises the nucleotide-binding and phosphorylation sites. P-type ATPases have been classified according to their topology [3] or their primary sequence homology [4].

The P1-type ATPases (also called the CPX-ATPases [5] or the soft-metal-ion-transporting-ATPases [6]) represent a newly emerging subfamily of P-type ATPases found in both prokaryotic and eukaryotic organisms. In bacteria, P1-type ATPases behave as Zn²⁺, Cd²⁺ or Pb²⁺ detoxification pumps [7], or are involved in copper homeostasis [8]. In eukaryotes, copper is the only transition metal for which homeostasis is regulated by a P1-type ATPase: ATP7A (or Menkes) and ATP7B (or Wilson) in humans [9], and Ccc2p in *Saccharomyces cerevisiae* [10].

P1-type ATPases have two consensus sequences – CXXC and CPX – that contain cysteine residues, an amino acid that is frequently found in heavy-metal-binding sites. The predicted spatial organization of P1-type ATPases differs from the known structure of Serca1a in two major features. The first difference is in the membrane domain, which comprises only eight trans-

membrane helices, with the sixth helix containing the CPX sequence. The second difference is in the N-terminal cytoplasmic domain, which is made up of between one and six repeats of a so-called metal-binding domain (MBD) containing the CXXC sequence (see Figure 1). Since there is still little information about the heavy-metal-binding sites in these P1-type ATPases, all information gathered on one of these proteins is useful for the others.

Regardless of the organism, copper is delivered to the P1-type ATPases by a soluble cytoplasmic metallochaperone [11–14].

Figure 1 Schematic representation of CadA

On the cytoplasmic (cyt) side, the N-terminal domain contains the P1-type ATPase consensus sequence CXXC, here C¹⁴TNC, which binds Cd²⁺. The large loop contains the P-type ATPase consensus sequence DKTGT, where D³⁹⁸ is the phosphorylated amino acid. In the membrane, the P1-type ATPase consensus sequence CPX is shown, here C³⁵⁴PC. In ΔMBD, I⁸⁵ (which is thought to be at the membrane interface) becomes M¹.

Abbreviations used: MBD, metal-binding domain; ΔMBD, truncated version of CadA that lacks the MBD but which still acts as a functional Cd²⁺-ATPase; NEM, *N*-ethylmaleimide.

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Scheme 1 CadA enzymic cycle

E denotes the Cd^{2+} -ATPase, and -P denotes a phosphorylated protein. cyt, cytoplasmic; ext, extracellular.

Copper delivery involves an exchange of copper between two MBDs of approx. 70 amino acids. The first MBD is the metallochaperone itself, which transfers Cu^+ to a second MBD in the N-terminal domain of the ATPase. Both MBDs contain the CXXC cysteine residues that are directly involved in heavy metal binding [15,16]. Functional studies of various P1-type ATPases have shown that truncation of the MBD or mutation of the CXXC cysteines altered some of their enzymic properties [17–19]. In a previous study on CadA, the *Listeria monocytogenes* Cd^{2+} -ATPase, we showed that ΔMBD , an N-terminally truncated form that does not contain the CXXC motif, is still a functional Cd^{2+} -ATPase (Scheme 1), since: (i) it is able to transport Cd^{2+} in the presence of ATP, (ii) its phosphorylation by ATP requires Cd^{2+} (step 2 in Scheme 1), and (iii) its phosphorylation by P_i requires the absence of Cd^{2+} (step 4 in Scheme 1). However, ΔMBD had lower initial transport velocity and maximal ATPase activity, and a 3-fold higher apparent affinity for Cd^{2+} , which leads us to propose a regulatory role for the MBD [20].

Although no precise location of the membrane transport site(s) in P1-type ATPases has been proposed, site-directed mutagenesis combined with complementation assays in yeast have demonstrated the essential role of the CPC motif in protein function [21–23]. By analogy with the Ca^{2+} -transport sites in Sercala, and considering the chemical reactivity of SH groups towards soft metals, it can be assumed that the cysteine residues of the CPC motif participate in the metal membrane transport site(s).

The aim of the present study was to assess the role of the cysteine residues of the CPC motif in the transport cycle of CadA. CadA possesses only the four cysteines described above, i.e. one pair in the MBD (CTNC motif) and another in the sixth putative transmembrane helix (CPC motif). Secondary structure prediction and the location of the cysteine residues in CadA are shown in Figure 1. Taking advantage of the fact that ΔMBD is a functional Cd^{2+} -ATPase, we analysed first the role of the membrane-domain cysteines (using ΔMBD), and secondly the role of the MBD cysteines (using full-length CadA). The role of the cysteine residues was assessed by reacting CadA or ΔMBD with *N*-ethylmaleimide (NEM), an SH-specific reagent, in the presence or absence of Cd^{2+} . We show here that (i) the CPC motif cysteines are essential for protein phosphorylation; (ii) Cd^{2+} protects the protein against alkylation by NEM, and (iii) MBD also protects against alkylation. Our results thus suggest that the CPC motif belongs to the Cd^{2+} -transport site(s), and that the MBD interacts with the rest of the protein.

EXPERIMENTAL

DNA manipulation, cloning procedures and CadA expression in Sf9 cells

CadA subcloning and modification procedures were as described previously [20]. Wild-type and mutant CadA proteins were produced using the BAC-TO-BAC™ Baculovirus Expression System (Invitrogen, Cergy, France). Protein preparation and detection were as described previously [20]. Functional assays were all performed on the 100 000 *g* pellet (P100), i.e. the total membrane fraction.

Phosphorylation from P_i

Formation of a phosphoenzyme intermediate due to phosphorylation by P_i was carried out at 25 °C. Aliquots of 70 μg of P100 membrane fraction were incubated for 10 min in 100 μl of 50 mM Mes/Tris (pH 6), 10 mM MgCl_2 , 50 μM thapsigargin and 20 % (v/v) DMSO, plus either 500 μM EGTA or 30 μM CdCl_2 . Phosphorylation was initiated by the addition of 100 μM $[\text{P}_i]$ to the mixture. After 10 min, the reaction was stopped by the addition of ice-cold buffer S [7 % (w/v) trichloroacetic acid, 1 mM KH_2PO_4]. Precipitated proteins were collected by centrifugation, washed twice with buffer S, suspended in 40 μl of 18 mM lithium dodecyl sulphate, 0.1 mM SDS, 2.5 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 6, 1 % (v/v) β -mercaptoethanol, 0.25 mg/ml Bromophenol Blue and 50 % (v/v) glycerol, and loaded on to an acidic polyacrylamide gel as described by Weber and Osborn [24]. The gel was exposed to Phosphor Screen and analysed on a PhosphorImager (Molecular Dynamics) to measure the intensity of the phosphorylated bands. It was later stained with Coomassie Blue in order to check the amounts of loaded protein.

Phosphorylation from ATP

Formation of phosphoenzyme intermediates due to phosphorylation by ATP was carried out at 0 °C by incubating 70 μg of P100 membrane fraction in 100 μl of 44 mM Mops/Tris (pH 7), 88 mM KCl, 1 mM MgCl_2 , 264 mM sucrose and 50 μM thapsigargin, plus either 30 μM CdCl_2 or 500 μM EGTA. The phosphorylation reaction was started by the addition of 1 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and stopped 15 s later by the addition of ice-cold buffer S. The subsequent steps were the same as those described above for phosphorylation from P_i .

Treatment with NEM

Prior to phosphorylation, P100 membrane fractions (1 mg/ml) were incubated at room temperature for 30 min in 50 mM Mops/Tris (pH 7), 100 mM KCl and 300 mM sucrose, plus the following additions, depending on the experiment: 500 μM EGTA and no NEM for control experiments, 500 μM EGTA and 50 μM –5 mM NEM for NEM+EGTA experiments, or 100 μM CdCl_2 and 1 mM NEM for NEM+ Cd^{2+} experiments. The suspension was then centrifuged for 30 min at 100 000 *g* and 4 °C, and the pellet was suspended in 200 μl of 50 mM Mes/Tris (pH 6) and centrifuged again. The washed pellet was then suspended in 100 μl of the buffer described above for phosphorylation along with either 500 μM EGTA or 100 μM CdCl_2 .

RESULTS

CadA possesses four cysteine residues: two in the CTNC motif in the cytoplasmic N-terminal MBD, and two in the CPC motif which is present in the sixth putative transmembrane helix. SH groups are known to be the preferential ligands for soft metals such as Cd^{2+} . We therefore investigated the effects of cysteine

Figure 2 Effects of NEM on the phosphorylation of CadA and ΔMBD from P_i

CadA (upper panel) and ΔMBD (lower panel) were phosphorylated from P_i as described in the Experimental section in the presence of either 500 μM EGTA or 30 μM CdCl₂, as indicated. NEM (1 mM) treatment was performed as described in the Experimental section prior to the phosphorylation assay, in the presence of either 500 μM EGTA or 100 μM CdCl₂. R, radioactivity measured in each band corresponding to CadA or ΔMBD, as analysed on the PhosphorImager and normalized to the radioactivity measured in the control experiment (lane 1; no NEM and assay in the presence of EGTA); P, total amount of protein in each lane revealed by Coomassie Blue staining, analysed using the Scion Image Beta 4.0.2 program and normalized to the total amount of protein in the control experiment (lane 1).

modification on both CadA and ΔMBD. This was achieved by reacting both proteins with NEM, an SH-specific reagent, in the presence or absence of Cd²⁺. Comparison between CadA and ΔMBD allowed us to discriminate between MBD cysteines, which are present in CadA but not in ΔMBD, and the CPC motif cysteines, which are present in both CadA and ΔMBD (see Figure 1).

As shown previously [20], both CadA and ΔMBD can be phosphorylated from P_i, providing that there is no Cd²⁺ present during phosphorylation assay (Figure 2; no NEM). Such inhibition of phosphorylation from P_i in the presence of Cd²⁺, the cation that induces phosphorylation from ATP, is a well known characteristic of P-type ATPases [25]. These experiments were performed on Sf9 cell membrane fractions, prepared as described in the Experimental section. Because there were almost no endogenous Sf9 proteins of the size of CadA (77 kDa) or ΔMBD (69 kDa) that were phosphorylated by P_i, this method allowed the individual evaluation of CadA and ΔMBD functionality among the 1000 or so proteins found in Sf9 membrane fractions. Given the quality of these data, we chose phosphorylation from P_i in order to monitor the effects of NEM on the two proteins. Reaction with NEM was achieved under two different sets of conditions designed to result in either empty transport site(s) (i.e. the presence of EGTA) or saturated transport site(s) (i.e. the presence of CdCl₂). These experimental conditions were chosen according to the Cd²⁺-dependence of the ATPase activity of both CadA and ΔMBD, as measured previously [20].

In a first set of experiments, CadA and ΔMBD were reacted with 1 mM NEM in the presence of 500 μM EGTA prior to phosphorylation from P_i (Figure 2; NEM + EGTA). With both proteins, phosphoenzyme formation was inhibited by NEM treatment. However, the level of inhibition was not the same for

Figure 3 Quantitative analysis of the effects of NEM on the phosphorylation of CadA and ΔMBD from P_i

Three independent experiments were performed under the same conditions as described in the legend to Figure 2. In each experiment, the data were quantified as in Figure 2 and the R/P ratio (see Figure 2) was calculated for each condition. E-P represents phosphorylated enzyme. Average values are reported as a percentage of that in the control experiment (P_i phosphorylation in the presence of EGTA with no NEM treatment).

the two proteins, with ΔMBD being significantly more sensitive to 1 mM NEM than was CadA (see below).

In a second set of experiments, CadA and ΔMBD were reacted with NEM in the presence of 100 μM CdCl₂ prior to phosphorylation from P_i (Figure 2; NEM + Cd²⁺). Under these conditions, NEM did not inhibit phosphoenzyme formation in either CadA or ΔMBD, demonstrating a protective effect of the metal. Phosphoenzyme formation did not occur in the presence of Cd²⁺, as observed under control conditions (Figure 2). Therefore, after treatment with NEM in the presence of Cd²⁺, CadA and ΔMBD had the same ability to be phosphorylated by P_i as if they had not been reacted with NEM, i.e. as under control conditions.

Quantitative analysis of such experiments required scanning of both the amount of ³²P labelling (R) and the amount of protein (P) under each set of conditions. An example of such quantification is shown at the bottom of Figure 2, where R and P are normalized to the values obtained under control conditions, i.e. no NEM treatment and P_i phosphorylation in the presence of EGTA. The results from three independent experiments are shown in Figure 3. When NEM treatment was performed in the presence of EGTA, phosphoenzyme levels decreased to 52% and 13% of the control for CadA and ΔMBD respectively. Interestingly, when NEM treatment was performed in the presence of Cd²⁺, phosphoenzyme levels reached approx. 120%. Such an enhancement was observed for both CadA and ΔMBD, which do not migrate to the same place, ruling out a non-specific reaction occurring on another protein. In addition, this effect was not due to incubation with Cd²⁺ itself, since the enhancement was only observed after incubation with both NEM and Cd²⁺.

The greater effect of 1 mM NEM on ΔMBD than on CadA in the presence of EGTA suggests that the presence of the MBD in CadA protects the rest of the protein from alkylation. This was explored further by repeating these experiments using a broad concentration range of NEM. Thus ΔMBD and CadA were reacted with 50 μM–5 mM NEM in the presence of 500 μM EGTA prior to phosphorylation from P_i, as described above. The results obtained after quantification showed that there was indeed protection against alkylation in CadA; for example,

Figure 4 Effects of NEM concentration on phosphorylation of CadA and ΔMBD from P_i

Three independent experiments were performed, with EGTA present during both NEM treatment and the P_i phosphorylation assay. The concentration of NEM was as indicated. The data were quantified as in Figure 3: ●, CadA; ○, ΔMBD. Controls without NEM: 100% phosphorylation was obtained in the presence of 500 μ M EGTA, and minimum phosphorylation (■, CadA; □, ΔMBD) was obtained in the presence of 30 μ M Cd^{2+} .

500 μ M NEM inhibited phosphoenzyme formation from P_i much more efficiently in ΔMBD than in CadA (Figure 4). The protection disappeared at 5 mM NEM, the highest concentration used.

DISCUSSION

NEM inhibits phosphorylation of CadA and ΔMBD

Our present results show that both CadA and ΔMBD were sensitive to NEM in EGTA-treated membrane fractions. This is illustrated in Figures 1 and 2 for phosphorylation from P_i . As both CadA and ΔMBD were shown to be phosphorylatable by ATP [20], phosphorylation from ATP was also assayed after NEM treatment. As with P_i , reacting CadA or ΔMBD with NEM in the presence of EGTA resulted in inhibition of phosphoenzyme formation from ATP (results not shown). Therefore it is suggested that, upon alkylation by NEM, Cd^{2+} -depleted CadA and ΔMBD undergo a chemical change that impairs phosphorylation by either ATP or P_i .

In terms of protein functionality, our results provide an explanation for previous observations on Cu^+ -ATPases, whereby NEM was shown to inhibit the ATP-mediated phosphorylation of ATP7A and *Enterococcus hirae* CopA [26,27], and also to inhibit ATP7A Cu^+ transport [28].

The CPC cysteines of CadA are essential for its phosphorylation

Given the specificity of NEM for cysteine residues, the fact that phosphorylation of ΔMBD is sensitive to NEM suggests that the alkylated SH group(s) in CadA are located in the protein, anywhere but in the MBD. Therefore the cysteines in the CPC motif are likely to be the residues that are alkylated after treatment with NEM. This also suggests that these cysteines are key residues for the phosphorylation reactions, although these reactions occur at the catalytic site, in the large cytoplasmic loop of the proteins (see Figure 1).

The CPC cysteines are present in the Cd^{2+} transport site(s)

When Cd^{2+} was present during the reaction with NEM, no subsequent phosphorylation of CadA or ΔMBD was observed, either from P_i (Figures 2 and 3) or from ATP (results not shown). According to the Lewis classification, Cd^{2+} is a soft metal that binds preferentially to cysteine SH groups. Therefore the fact that Cd^{2+} suppresses the effect of NEM suggests that the CPC motif is not only the target for NEM-induced alkylation, but also part of the Cd^{2+} transport site(s).

Our results can be explained as follows. (i) In the presence of Cd^{2+} , the SH groups of the CPC motif cysteines bind Cd^{2+} , and this prevents NEM from reacting with them. (ii) In the presence of EGTA, however, any bound metal is removed from the CPC cysteine SH groups, and NEM is free to react with them. In Scheme 1, NEM would only react with *E*, driving the alkylated proteins into a dead-end conformation, unable to be phosphorylated either by P_i or by ATP, a reaction that takes place in the large cytoplasmic loop, far from the membranous CPC motif. In terms of the mechanism of P-type ATPases, this probably means that the alkylated protein cannot undergo the conformational changes induced by the presence or absence of Cd^{2+} at the transport site(s) that allow phosphorylation to take place at the catalytic site. Namely, the alkylated protein is neither in conformation *E*, which allows phosphorylation by P_i (Scheme 1, step 4), nor in conformation Cd_nE , which allows phosphorylation by ATP (Scheme 1, step 2). Therefore we propose that the CPC cysteines are present in the Cd^{2+} transport site(s). Similar conclusions were reached by Pick and Racker [29] in the first report showing that the Ca^{2+} transport sites of Serca1a are in the membrane. In that study, the protein was labelled with dicyclohexylcarbodi-imide – a carboxyl group reagent – which inhibited Serca1a provided that the reaction was performed in the presence of EGTA. Since then, site-directed mutagenesis has confirmed the location of the Ca^{2+} transport sites and the presence of acidic residues at these sites [30].

Our results emphasize the functional role of the CPC motif cysteines, in agreement with complementation assays showing (i) the failure of CPC → CPA-mutated Cu^+ -ATPase from *Caenorhabditis elegans* [23] and of CPC → SPS-mutated ATP7B [21] to replace Ccc2p functionally in *S. cerevisiae*, and (ii) the inability of the CPH → SPH mutant of CopB from *E. hirae* to restore a wild-type phenotype [31]. Our results are also in agreement with the identification of the C1000R and C985Y mutations in the CPC motif of Cu^+ -ATPases from patients with Menkes and Wilson diseases respectively [32,33]. Interestingly, analysis of around 100 prokaryotic or eukaryotic sequences reveals that the two cysteines in the CPC motif are not always conserved in the putative sixth transmembrane segment of P1-type ATPases. A CPH motif is found in histidine-rich P1-type ATPases; furthermore SPC, and more rarely APC, TPC or CPS, can replace the consensus CPC motif. In all of these variants one cysteine is always preserved, the second being replaced by an amino acid with similar steric hindrance and polarity properties (except for Ala).

Role of the cysteines in the MBD

Although the effect of NEM was observed with ΔMBD, and therefore is likely to occur on the CPC cysteines, the possibility of NEM also reacting with the cysteines in the CTNC motif of CadA cannot be ruled out. Such information is not yet available, since it would require radioactive NEM labelling of CadA and ΔMBD.

A comparison between metal-depleted CadA and ΔMBD shows that the reactivity towards NEM varies depending on

whether the MBD is present or not (Figure 4). This suggests that, in the absence of Cd^{2+} , MBD lowers the reactivity to NEM of the SH groups in the CPC cysteines. Since NEM is at least 1000-fold more concentrated than the CadA cysteines, and given the high probability that the CPC cysteine SH groups are present in the Cd^{2+} transport site(s), this suggests that the MBD interacts with this site in native CadA. This is of importance: two functions have been proposed to date for the MBD, namely a role in the Cu^+ -ATPase trafficking signal [34] and representing the Cu^+ -metallochaperone target [15]; however, neither of these have been observed for bacterial non- Cu^+ -ATPases, which represent a significant proportion of P1-type ATPases.

It is not yet possible to explain how the MBD acts on the metal transport site. However, such an interaction between the MBD and the rest of the protein in the absence of Cd^{2+} has already been suggested by comparing the Cd^{2+} -dependence of CadA and ΔMBD ATPase activities [20]. An interaction between MBD and a part of the large cytoplasmic loop has also been demonstrated in ATP7B, and this interaction required the absence of Cu^+ [35].

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