Energetics and Topology of CzcA, a Cation/Proton Antiporter of the Resistance-Nodulation-Cell Division Protein Family*

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The membrane-bound CzcA protein, a member of the resistance-nodulation-cell division (RND) permease superfamily, is part of the CzcCB2A complex that mediates heavy metal resistance in Ralstonia sp. CH34 by an active cation efflux mechanism driven by cation/proton antiport. CzcA was purified to homogeneity after expression in Escherichia coli, reconstituted into proteoliposomes, and the kinetics of heavy metal transport by CzcA was determined. CzcA is composed of 12 transmembrane $\alpha$-helices and two large periplasmic domains. Two conserved aspartate and a glutamate residue in one of these transmembrane spans are essential for heavy metal resistance and proton/cation antiport but not for facilitated diffusion of cations. Generalization of the resulting model for the function of CzcA as a two-channel pump might help to explain the functions of other RND proteins in bacteria and eukaryotes.

Multiple drug resistant bacteria poses a threat to man’s fight against infectious diseases. Some multiple drug resistance systems may detoxify their substrates by transport across the complete cell wall of Gram-negative bacteria, across cytoplasmic membrane, periplasm, and outer membrane. These assumed transenvelope transporters are composed of a pump protein that energizes the transport, in addition to a membrane fusion and an outer membrane-associated protein (1, 2). The pump protein may be an ATP-binding cassette transporter (3, 4), a transporter of the major facilitator superfamily (5), or a resistance-nodulation-cell division (RND) protein (4, 6, 7). The archetype of the RND permease superfamily family is CzcA from the Gram-negative bacterium Ralstonia sp. CH34 (formerly Alcaligenes eutrophus strain CH34) (8–12).

This bacterium contains at least seven heavy metal resistance determinants, located either on the bacterial chromosome or on one of the two indigenous plasmids pMOL28 (163 kilobase pairs) and pMOL30 (238 kb) (8, 13–16). One of them, the $czc$-determinant of plasmid pMOL30, mediates inducible resistance to millimolar concentrations of $Co^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ in strain CH34 (8, 17). The products of the genes $czcA$, $czcB$, and $czcC$ form a membrane-bound protein complex catalyzing an energy-dependent flux of these three metal cations (9, 11), probably across the complete envelope. The mechanism of action of CzcCB2A is that of a proton/cation antiporter, and the $K_m$ values of the efflux system for the substrate heavy metal cations are also in the millimolar range (10).

Although indirect evidence led to the assumption that CzcA is the central cation/proton antiporter of the CzcCB2A complex (10), this has not been shown directly. This paper demonstrates that CzcA is a cation/proton antiporter, and develops the model of CzcA as a two-channel pump based on topology studies and the function of CzcA mutant proteins. This model sheds some light on other RND proteins involved in multiple drug resistance of bacteria or with previously unknown functions in mammals.2

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—Ralstonia sp. strain AE104 (14) is a metal-sensitive, plasmid-free derivative of strain CH34. Escherichia coli K38(pGPI–2) (19) was used for expression of $czc$BAD derivatives under control of the phage T7 promoter as described (20). Tris-buffered mineral salts medium (14) containing 2 g/liter sodium glucose was used for growth.**

**Genetic Techniques—Standard molecular genetic techniques were used (8, 21). Transformation of E. coli strains was conducted as described previously (8). For expression of $czc$BAD derivatives under control of the lac promoter in Ralstonia strain AE104, the plasmid pT7-5-derivatives containing the various $czc$ constructs were cut with EcoRI and XbaI and cloned into the broad host range plasmid pVDZ2-2 (23). The pVDZ2-derivatives were transformed into E. coli S17–1 (24) and transferred into Ralstonia AE104 by conjugation as described (8). For reporter gene fusions, fusion vector pECD500 (11) and E. coli CC118 were used (25). All fusions were done immediately downstream of an arginine or lysine residue of CzcA. Activity of alkaline phosphatase (25) was determined in triplicate as published previously.

**Mutations in the $czc$ gene were constructed using PCR by an overlap extension method (26) as published previously (11). The 5’ part of the internal 1,034-base pair $NheI-MunI$ internal fragment of $czcA$ (position 3,489 to 4,523) (9) was amplified from plasmid $pECD110$ (9) using a primer corresponding to the sequence at the $NheI$ site on the $czcA$ gene and a primer corresponding to the mutated region. These (mutations underlined) TC-GAAAACAGTGTGAGGCGA for C417S, TGGCGCGTGCGCAGGAACA for H423R, AGGAACGCCATGGCCGGC for H427R, GTGGTGATTGTCCAACTG for H428R, TCCGAGCGGTTCCGTGAGGT for H439R, GGCCGGC for H427R, and ATCGATATCATGGCCGGC for D402N. The 3’ part of the 1,034 fragment from the region of the mutation in the $MunI$ site. These fragments were amplified from pECD110 (9) with primers inverse to the primers listed above and a MunI region primer (AAAGGGCCTCCAG-GAACATTTGACCC, MunI-primer). After purification (Wizard PCR Clean-Up System, Promega, Madison, WI), they were cloned into the EcoRI site of pECD500 (11) and transferred into Ralstonia CH34 by conjugation as described (8).**

**Materials—Plasmids were isolated by alkaline lysis (22), unless otherwise stated.**

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Function of the CzcA Subunit

RESULTS

Purification of CzcA and Its Active Reconstitution into Proteoliposomes—CzcA was purified to homogeneity (Fig. 1). The first amino acids of CzcA were determined as MFE as expected from DNA sequence analysis (9). CzcA was reconstituted into proteoliposomes, which were incubated in 100 mM Tris-Cl, pH 5.0. At 1 mM Zn2+, CzcA proteoliposomes accumulated about 70 mol of Zn2+/mol of CzcA (c0 = 0.7 mM) more within the first 15 s than control liposomes (Fig. 2A). In the following 2 min, another 100 mol of Zn2+/CzcA (final c0 = 1.7 mM) were accumulated, whereas binding of zinc by control liposomes did not increase with time. The rapid transport of zinc by CzcA proteoliposome was measured, could be inhibited by 100 μM of the protophosphate FCCP (Fig. 2A) or using Tris buffer, pH 5.0, instead of pH 7.0 for dilution (data not shown). Thus, this rapid transport was probably driven by the zinc concentration gradient across the proteoliposome membrane. Increased amounts of CzcA proteoliposome led to increased metal transport, but increased amounts of control liposomes did not (Fig. 2B). Thus, CzcA was
presence of 100 m M Tris system was compared with CzcA-containing proteoliposomes in the parallel experiment, which was divided by the CzcA content of the proteoliposomes. A volume of 3 l of proteoliposomes (3 g of CzcA/liter) in 100 mM Tris-HCl, pH 5.0, was added into 100 mM Tris-HCl buffer, pH 7.0 (panels A and B), or proteoliposomes charged with 0.5 m M NH4Cl were diluted into choline buffer, pH 9.0 (panels C and D). After 1 or 5 min, 65Zn2+ was added. 5-l samples (containing 0.5 l of CzcA each) were filtrated, washed, and used to calculate the mols of zinc transported into the proteoliposomes per mol of CzcA (molecular mass of CzcA-strep-tag, 116,611 Da). Concentration higher than 10 mM gave no significant uptake, probably due to the toxic effect of zinc on the integrity of the proteoliposomes (data not shown).

Cobalt transport by CzcA was much slower than zinc transport. To measure any significant transport, 1.5 l of proteoliposomes instead of 0.3 l had to be used, and the cobalt concentration had to be raised to 10 m M (Fig. 4A). The substrate saturation of cobalt transport by CzcA was again sigmoidal (Fig. 3A), and, due to a toxic effect, no uptake was detectable at concentrations higher than 50 mM. Using the velocity measured at 50 mM, a Hill plot was performed (Fig. 3B) yielding n = 2 and a K1/2 of 18.5 mM (Table I). Cobalt transport by CzcA was even slower than cobalt uptake (Fig. 4B). No uptake could be detected at concentrations lower than 1 mM or higher than 5 mM. In this narrow range of substrate concentration (Fig. 3A), a Lineweaver-Burk plot was linear and yielded a Vmax of 28 s−1 and a K1/2 of 7.7 mM (Table I).

Topolgy of CzcA and Aminoacyl Residues Essential for Cation/Proton Antiport—Computer predictions (data not shown) indicated 12 hydrophobic peaks in the amino acid sequence of CzcA, which might resemble membrane-spanning a-helices (TMHs), and two large hydrophilic regions between TMH I/II and TMH VII/VIII, respectively. The specific activities of CzcA::PhoA translational fusions (Table II) gave evidence for (i) a cytoplasmic location of both termini of CzcA, (ii) a periplasmic location of both large hydrophilic domains, (iii) the presence of TMHs I (between N terminus and large hydrophobic domain I), II, III, IV, VII, VIII, IX, XI, and XII. No fusion could be isolated between TMHs IX and X, and the low specific activity of the fusion between TMHs V and VI (position 475) gave no evidence for a periplasmic location of this region (Table II).

The highly conserved (Fig. 5) aminoacyl residues Asp-402, Asp-408, and Glu-415 of CzcA were mutated to D402N, D408N, E415Q, and E415D. The predicted structure of CzcA leaves as possible metal-binding residues 3 histidine residues and 1 cysteine residue between TMH IV/V, these were mutated to C417S, H423R, H427R, and H439R. None of the mutations in the possible metal binding site yielded any decrease in metal resistance, neither on solid medium (Table III) nor in liquid culture (data not shown). Thus, the residues Cys-417, His-423, His-427, and His-439 alone are not essential for transport of any of the three CzcA substrate cations Co2+, Zn2+, or Cd2+. In contrast, mutation to Asn or Gln of the negatively charged aminoacyl residues in the middle of the transmembrane a-helix

**Fig. 2. Activity of CzcA-containing proteoliposomes.** A volume of 3 l of proteoliposomes (3 g of CzcA/liter) in 100 mM Tris-HCl, pH 5.0, was added into 100 mM Tris-HCl buffer, pH 7.0 (panels A and B), or proteoliposomes charged with 0.5 m M NH4Cl were diluted into choline buffer, pH 9.0 (panels C and D). After 1 or 5 min, 65Zn2+ was added. 5-l samples (containing 0.5 l of CzcA each) were filtrated, washed, and used to calculate the mols of zinc transported into the proteoliposomes per mol of CzcA (molecular mass of CzcA-strep-tag, 116,611 Da). Concentration higher than 10 mM gave no significant uptake, probably due to the toxic effect of zinc on the integrity of the proteoliposomes (data not shown).

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**Fig. 3. Kinetics of cation transport by CzcA.** The initial velocity of zinc (●), cobalt (○), or cadmium (△, only in panel A) uptake into CzcA proteoliposomes was determined and plotted against the substrate concentrations used (panel A) or in a Hill plot (panel B).
The uptake of 10 mM $^{57}$Co²⁺ for cobalt, it was the velocity at 50 mM. Higher concentrations of either metal inactivated the proteoliposomes. With this $V_{max}$ value, a Hill plot using the equation $\ln(V/V_{max} - V) = n \cdot \ln S - n \cdot \ln K_m$ was performed yielding the resulting values. For cadmium, concentration higher than 5 mM inactivated the proteoliposomes, and no significant transport could be measured with concentrations lower than 1 mM. Within this range of concentrations, the Lineweaver-Burk plot was linear and yielded the listed $K_m$ and $V_{max}$ values.

| Metal ion | $n$ | $K_m$/mM | $V_{max}$/μmol/min/mg |
|-----------|-----|-----------|-----------------------|
| $\text{Zn}^{2+}$ | 1.96 | 6.6 | 385 |
| $\text{Co}^{2+}$ | 1.91 | 18.5 | 100 |
| $\text{Cd}^{2+}$ | (1) | 7.7 | 28 |

**TABLE II**  Specific activity and proposed localization of CzcA::PhoA translational fusions

| Amino acid residue of CzcA | Predicted topology of CzcA | Specific activity of PhoA fusions, d.w. |
|---------------------------|---------------------------|---------------------------------------|
| 13 | N terminus | 0.15 ± 0.02 |
| 33 | Large hydrophilic domain 1 | 2.14 ± 0.10 |
| 64 | Large hydrophilic domain 1 | 2.60 ± 0.03 |
| 192 | Large hydrophilic domain 1 | 4.31 ± 0.14 |
| 334 | Large hydrophilic domain 1 | 3.80 ± 0.01 |
| 345 | Large hydrophilic domain 1 | 3.97 ± 0.07 |
| 366 | TMH II/III | 0.50 ± 0.00 |
| 390 | TMH III/IV | 2.89 ± 0.03 |
| 419 | TMH IV | 2.38 ± 0.17 |
| 449 | TMH IV/V | 0.37 ± 0.01 |
| 475 | TMH V/V | 0.28 ± 0.03 |
| 510 | TMH VI/VII | 0.35 ± 0.02 |
| 532 | TMH VI/VII | 0.32 ± 0.01 |
| 557 | TMH VII | 0.46 ± 0.02 |
| 880 | Large hydrophilic domain 2 | 0.99 ± 0.04 |
| 927 | TMH VIII/IV | 0.20 ± 0.02 |
| 980 | TMH XXI | 0.16 ± 0.04 |
| 1008 | TMH XI/XII | 2.13 ± 0.02 |
| 1038 | C terminus | 0.16 ± 0.03 |

*Mean ± S.D. of triplicate determinations are shown.

The position of the carboxyl group of aminoacyl residue 415 of the CzcA protein diminished the efficiency of heavy metal transport in the respective mutant strain.

**FIG. 4.** Transport of cobalt and cadmium by CzcA. Panel A gives the uptake of 10 mM $^{57}$Co²⁺ into CzcA proteoliposomes (3 μg CzcA/μl) (●) and into control liposomes (○); panel B gives the uptake of 1 mM $^{109}$Cd²⁺ (185 mCi/mM).

**TABLE III**  Metal resistance of mutant strains carrying a czcA mutation

| Genotype* | Co²⁺ (mM) | Zn²⁺ (mM) | Cd²⁺ (mM) |
|-----------|-----------|-----------|-----------|
| No czcCBA | 0.2 | 0.3 | 0.6 |
| Positive control | 20 | 15 | 5 |
| D402N | 0.2 | 0.3 | 0.6 |
| D408N | 0.2 | 0.3 | 0.6 |
| E415D | 20 | 15 | 5 |
| E418Q | 0.2 | 0.3 | 0.6 |
| C417S | 20 | 15 | 5 |
| H423R | 20 | 15 | 5 |
| H427R | 20 | 15 | 5 |
| H439R | 20 | 15 | 5 |

*Done with Ralstonia sp. AE104 strains harboring plasmid pVDZ2 derivatives with mutant czcCBA operons cloned under control of the lac promoter, which is constitutive in this bacterium.

† Determined after 3 days at 30 °C.

‡ As the negative control, Ralstonia sp. strain AE104(pVDZ2) was used.

§ As positive control, strain AE104(pDNA130) was used.

IV led to a complete loss of metal resistance to each of the three heavy metals (Table III), and not the slightest residual resistance could be observed in liquid culture (data not shown).

Although the E415Q mutant CzcA protein was not functional, the E415D protein gave full resistance on solid media (Table III). However, in liquid culture in the presence of 2.5 mM Co²⁺, 2.5 mM Zn²⁺, or 1 mM Cd²⁺, an AE104 derivative with the E415D mutation in czcA grew slower than the respective wild type strain (data not shown). Thus, the small change in the position of the carboxyl group of aminoacyl residue 415 of the CzcA protein diminished the efficiency of heavy metal transport in the respective mutant strain.

CzcA as a Two-Channel Pump—The mutant proteins with defects in the aspartate residues, CzcA-D402N (pECD557) and CzcA-D408N (pECD558), were purified like the CzcA wild type protein and reconstituted into proteoliposomes. When they were compared with the wild type protein, all three proteins displayed the rapid facilitated diffusion of zinc (Fig. 6A), but the mutant proteins were no longer able to catalyze the slower proton/zinc antiport. To analyze, if the zinc bound in the rapid reaction was transported into the inside of the proteoliposomes or were bound at the outside, proteoliposomes with the wild type (data not shown) and the mutant proteins (Fig. 6B) were incubated for 2 min with 1 mM [65Zn]Zn²⁺, and then an additional 10 mM of nonradioactive zinc was added. In all cases, CzcA-containing proteoliposomes accumulated more zinc in this isotope competition experiment, although the amount of zinc bound by the control liposomes decreased. Transport of Co²⁺ and Cd²⁺ by wild type and both mutant proteins was not...
were incubated with 1 mM \( ^{65}\text{Zn}^2 \) cation, and this has been demonstrated for YbdE from motif like SilA may be involved in transport of a monovalent which detoxifies Ag.

mutant proteins D402N (\( \text{Panel A} \)) and D408N (\( \text{Panel B} \)) or D408N (\( \text{Panel C} \)) were added. After 2 min (arrows), 10 mM of nonradioactive \( ^{65}\text{Zn}^2 \) was added.

different (data not shown). Thus, the mutant proteins were still able to transport metal cations into proteoliposomes but were unable to catalyze a proton/cation antiport.

**DISCUSSION**

The roughly determined structure of CzaA as N terminus/ TMH I/periplasmic domain 1/TMHs II-VII/periplasmic domain 2/TMHs VIII-XII/cyttoplasmic C terminus fits into the model of a “6–6 spanner transporter” (37). If two independent channels exist in CzaA, they may have different functions. The highly conserved “DDE” motif in one of these channel was essential for CzaA function in vivo and for proton/cation antiport in vitro but not for facilitated diffusion of cations. Thus, the DDE channel could form a charge-relays system (38) with the DDE residues required for proton transport, whereas the second channel may be a cation channel. Therefore, the best model to describe the function of CzaA, so far, is that of a two-channel pump.

In a working model, binding of \( ^{65}\text{Zn}^2 \) to a cytoplasmic metal-binding site of CzaA may trigger proton transport across the proton channel into the cytoplasm. This could create negative charges in the large periplasmic domains of CzaA. According to calculations using Fick’s first law, diffusion of \( ^{65}\text{Zn}^2 \) through CzaA should be far too slow to explain the observed turnover number of CzaA, thus, these negative charges plus the bound cation could generate an electrical field that drives the cation through the cation channel to the periplasm. Finally, the cation could be exchanged for protons from the periplasm.

**Other RND Proteins**—Asp-402 is not conserved (Fig. 5) in the SiaA protein (39) involved in efflux of Ag+, but Asp-408 and Glu-415 are conserved, which may be explained by a 1H+/1Ag+ ratio of transport by this system in contrast with a ratio of 2 H+/1 \( ^{65}\text{Zn}^2 \) for CzaA (10). Therefore, RND proteins with a DE motif like SiaA may be involved in transport of a monovalent cation, and this has been demonstrated for YbdE from \( E. \) coli, which detoxifies Ag. 3 Surprisingly, the Acr-like RND proteins (HAE1 family) (7) contain another conserved DDE motif in the respective region after TMH IV (Table II, Fig. 5); however, in contrast to CzaA, Asp-402 is not conserved, but another Asp at the (CzaA-) position Asp-409.

In Acr proteins, the substrates do not have to carry any charge, increased hydrophobicity even enhances transport (40). Thus, although the conserved proton channel suggests that Acr-like RND proteins are driven by the proton gradient, an electrical field is unlikely to drive export of substances like toluene, hexadecan, or \( \beta \)-lactams with highly hydrophobic side chains (41–43). In Acr-like proteins, protonation/deprotonation may switch a periplasmic substrate-binding site between a hydrophilic and a hydrophobic character. If a hydrophobic substrate is bound to this site, it may be driven off into the periplasm when the site becomes hydrophilic as a consequence of protonation.

Some other bacterial proteins are related to RND proteins or even members of this family. 2 SecD is involved in protein export and seems to catalyze the release of the transported preprotein portion from the Sec pore. This reaction is also proton-dependent (44, 45). Because SecD is related to the proposed proton channel of RND proteins (7), the SecD result supports the reaction mechanism proposed above. Moreover, some proteins in eukaryotes may also be RND members, 2 therefore, they may catalyze comparable reactions. The Niemann-Pick disease C1-protein NPC1_MAN seems to be a lysosomal cholesterol transporter (46–48), and should be proton-driven. Members of the patched gene regulator family are involved in a signal transduction chain of the hedgehog developmental signal (18, 49–51); these proteins should be proton-driven transporters too. The homology to NPC1_MAN suggests a function as a cholesterol transporter. Because covalently bound cholesterol is essential for the activity of the hedgehog protein, release and uptake of the hedgehog-bound cholesterol might be required to set back the hedgehog signal.

Thus, the data obtained with the purified and reconstituted CzaA protein might be of help to understand the function of other RND-related proteins involved in multiple drug resistance and protein export in bacteria, and of proteins involved in cholesterol metabolism, development and cancer generation in mammals.

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