Kinetic Study of the Antiport Mechanism of an Escherichia coli Zinc Transporter, ZitB*

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Yang Chao and Dax Fu‡

From the Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

ZitB is a member of the cation diffusion facilitator (CDF) family that mediates efflux of zinc across the plasma membrane of Escherichia coli. We describe the first kinetic study of the purified and reconstituted ZitB by stopped-flow measurements of transmembrane fluxes of metal ions using a metal-sensitive fluorescent indicator encapsulated in proteoliposomes. Metal ion filling experiments showed that the initial rate of Zn$^{2+}$ influx was a linear function of the molar ratio of ZitB to lipid and was related to the concentration of Zn$^{2+}$ or Cd$^{2+}$ by a hyperbola with a Michaelis-Menten constant ($K_m$) of 104.9 ± 5.4 μM and 90.1 ± 3.7 μM, respectively. Depletion of proton stalled Cd$^{2+}$ transport down its diffusion gradient, whereas tetraethylammonium ion substitution for K$^+$ resulted in Cd$^{2+}$ efflux, demonstrating the electrogenic effect of ZitB transport. Taken together, these results indicate that ZitB is an antiporter catalyzing the obligatory exchange of Zn$^{2+}$ or Cd$^{2+}$ for H$^+$. The exchange stoichiometry of metal ion for proton is likely to be 1:1.

Zinc is a micronutrient essential for the growth, development, and differentiation of cells by contributing to a number of important biological processes, including gene expression, DNA synthesis, enzymatic catalysis, hormone storage and release, neurotransmission, memory, and apoptosis (1). The function of zinc in metalloenzymes attributes to its inherent chemical properties as an acid catalyst, a structural ion, and a regulatory co-factor (2–4). Consequently, cellular processes are critically dependent on the maintenance of zinc at optimal physiological levels, ranging from about 10⁻¹¹ M in the cytoplasm of many cells to 10⁻³ M in some vesicles (5). In bacteria and eukaryotic cells, zinc homeostatic control mechanisms have evolved based on a complex network of transport, chelation, and sequestration processes to maintain zinc within narrow physiological ranges and sustain zinc concentration gradients across membranes of different cellular compartments (6–8). Several families of membrane proteins have been identified as zinc uptake or efflux transporters, responsible for zinc homeostasis by moving zinc into and out of cells or intracellular vesicles (9). Of all zinc transporters identified so far, mechanisms by which zinc transporters bind and transport metal ions have not yet been defined in molecular detail.

In Escherichia coli, zinc homeostasis is accomplished largely through the transcriptional control of four zinc transporter systems, including two uptake transporters, the high affinity ABC transporter ZnuABC (10), and the ZIP transporter ZupT (11). The efflux of zinc is mediated by two efflux transporters, the P-type ATPase ZntA (12, 13) and the CDF$^*$ transporter ZitB (14). CDF$^*$ are ubiquitous family of metal transporters found in prokaryotes and eukaryotes (15). Sequence analysis suggests that CDF proteins contain a hydrophilic N-terminal domain followed by a hydrophilic C-terminal domain with large variations both in size and sequence. The number of transmembrane domains and the oligomeric structure of CDF proteins have not yet been established experimentally, but hydropathy profiles of CDF sequences suggest that this family of transporters may contain five to six membrane-spanning domains. Mammalian and yeast CDF transporters have been localized to the plasma and vesicular membranes. They are involved in the removal of cytoplasmic zinc through efflux out of cells or sequestration into intracellular vesicular compartments (16–19).

Like many homologous eukaryotic CDF transporters, the expression of E. coli ZitB was found to be inducible by zinc (14). ZitB was implicated in a role of an efflux pump by the observations that overexpression of ZitB increased zinc tolerance and reduced zinc uptake, whereas double ΔzitB and ΔzntA deletion resulted in zinc hypersensitivity (14). A Zn$^{2+}$/K$^+$ antiporter mechanism was proposed for CDF based on genetic complementation experiments in which ZitB and CzcD, a Bacillus subtilis CDF transporter, were shown to complement K$^+$ uptake deficiency of a mutant E. coli strain in the presence of zinc (20, 21). However, evidence for an antiport mechanism is indirect, because the observed Zn$^{2+}$ efflux could simply reflect a mixed contribution of different transport systems residing in the plasma membrane. The apparent K$^+$ and H$^+$ dependence of CzcD and ZitB functions could result from the interplay of many H$^+$- and K$^+$-linked pumps in cells. Therefore, the transport mechanism of CDF remains obscure. An explicit mechanistic description can only come from a direct kinetic study using a purified reconstitution system.

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† To whom correspondence should be addressed: Biology Dept., Bldg. 463, Brookhaven National Laboratory, Upton, NY 11973. Tel.: 631-344-4208; Fax: 631-344-4207; E-mail: dxf@bnl.gov.

‡ The abbreviations used are: CDF, cation diffusion facilitator; TRA, tetraethylammonium; DDM, n-dodecyl-β-D-maltopyranoside; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; β-ME, β-mercaptoethanol; HPLC, high pressure liquid chromatography.

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Here we describe the kinetic analysis of the purified and reconstituted ZitB using Zn\(^{2+}\)-sensitive fluorescent indicator fluozin-1 to monitor the Zn\(^{2+}\) transport in response to transmembrane chemical gradients and electrical potentials. Fluozin-1 is a single wavelength fluorophore that exhibits more than 200-fold fluorescence enhancement upon Zn\(^{2+}\) or Cd\(^{2+}\) binding with essentially no change in absorption or emission wavelengths (12). Aliquots of protein in a series of 1:2 dilutions were mixed with matrix solution and then spotted onto the sample plate using the sandwich method (23). MALDI-TOF spectrometric data were collected using a Voyager Biospectrometry work station, operated in linear mode. The spectra were externally calibrated with a calibrant mixture containing cytochrome c and bovine serum albumin.

Experiments were prepared from an E. coli polar lipid extract (Avanti Polar Lipids Inc., Alabaster, AL) hydrated to a final concentration of 50 mg/ml in a BTM buffer (2 mM Bis-Tris-MES (BTM), 2 mM β-ME, pH 6.8) by vigorous mixing on a vortex mixer. The suspension of multilamellar liposomes was sonicated under argon in an ice-chilled bath sonicator (Misonix Inc., Farmingdale, NY) until a clear lipid suspension was obtained. Depending on experimental design, the purified ZitB, typically at a concentration of 10 mg/ml, was diluted to a final concentration ranging from 0.01 to 0.25 mg/ml, using an n-octyl-β-D-glucoside (β-OG) buffer (20 mM BTM, pH 6.8, 1% β-OG, 2 mM β-ME, and 50 mM either K\(_2\)SO\(_4\) or TEA\(_2\)SO\(_4\) when indicated). Using the freshly prepared sonicated lipid and ZitB dilution, reconstitution of ZitB proteoliposomes was achieved with 100 µl of a 10% liposome suspension of phosphatidylcholine followed by the addition of 770 µl of ZitB. At this ratio, lipids were completely solubilized by β-OG, as indicated by the disappearance of turbidity. The resulting ZitB-lipid-detergent mixture was incubated at room temperature for 20 min and then applied to an Econo-Pac 10 DG desalting column (Bio-Rad), preequilibrated with an assay buffer (20 mM BTM or 20 mM Tris\(_2\)SO\(_4\) with 50 mM K\(_2\)SO\(_4\) or 50 mM TEA\(_2\)SO\(_4\) when indicated). The cloudy void fraction was collected and subjected to ultracentrifugation at 140,000 g for 45 min to pellet proteoliposomes. The control liposomes were prepared following the same procedure without adding ZitB.

**Encapsulation of Membrane-impermeable Fluorescent Indicators**—Proteoliposome pellets were resuspended in 200 µl of assay buffer. An aliquot of 2 mM Zn\(^{2+}\)-sensitive indicator fluozin-1 (Molecular Probes, Inc., Eugene, OR) or pH-sensitive indicator pyranine (Molecular Probes) was added to the proteoliposome resuspension to a final concentration of 200 µM. The samples were sonicated for 10 s and subjected to one cycle of freeze-thaw (liquid nitrogen, room temperature), followed by an additional 10-s sonication. The untrapped indicator exterior to proteoliposomes was removed using an Econo-Pac 10 DG desalting column, preequilibrated with assay buffer. The indicator-loaded proteoliposomes were eluted in the void fraction.

**Stopped-flow Fluorescence Measurement**—Kinetic experiments were performed in fluorescence mode on a stopped-flow apparatus (KinTek, Round Rock, TX) with a voltage-sensitive indicator fluozin-1 (Molecular Probes). A dead time of 10 ms was used with the instrument time constant set to 500 µs. The reaction temperature was set at 8 °C using a circulating water bath to maintain a constant temperature through jackets surrounding mixing syringes and the mixing cell. Proteoliposome samples and an assay buffer containing varying concentrations of Zn\(_2\)SO\(_4\) or Cd\(_2\)SO\(_4\) were loaded into two separate mixing syringes of equal volume, and transport reactions were initiated by pushing 60 µl of fresh reactants at a 1:1 ratio through the 12-µl mixing cell at a flow rate of 20 µl/s. Fluorescence was monitored at 90° to the incident excitation beam. For measurements of fluo-1 fluorescence changes, samples were excited at 490 nm, and emissions were monitored at 525 nm using a 10-nm band pass cut-off filter. For measurements of intravesicular pH changes by the voltage-sensitive indicator fluozin-1, the flowthrough probe and the instrument time constant were set to 500 µs. The samples were excited alternately by a pair of wavelengths at 450 and 410 nm, and emissions were recorded at 525 nm. The ratio of emission intensities (F\(_{450\text{nm}}\)/F\(_{410\text{nm}}\)) at two excitation wavelengths was calculated. For measurements of transmembrane potential changes, the voltage-sensitive lipophilic indicator oxonol VI (Molecular Probes) was added directly to the proteoliposome resuspension to a final concentration of 2 nM. Fluorescence was excited at 615 nm, and the emission was recorded at 646 nm. The base-line fluorescence for each indicator was set by flushing reactants through the mixing cell and adjusting the photomultiplier tube voltage to a level that would give a 5-V signal in the middle of the 0–10–V detectable range. Data were collected by an IBM work station and kept constant for the duration of each set of experiments. Kinetic traces were recorded using two equal time partitions, with the first half allocated for the fast kinetic component (0–500 ms) and the second half for the slow kinetic component (0.5–500 s). All traces were the cumulative average of 5–10 successive recordings.
Data Analysis—A concentration of fluozin-1 at 200 μM was chosen to achieve a linear fluorescent response (∆F) to the total intravesicular metal concentration (∆[M]). This linear relationship is derived from the following equations. The concentration of the entrapped metal indicator complex ([I-M]) (24) is given by Equation 1,

$$[I-M] = \frac{([I] + [M] - K_j) - \sqrt{([I] + [M] - K_j)^2 - 4[I][M]}}{2} \times \frac{[M]}{2} \cdot \Delta [M]$$ (Eq. 1)

where [I] represents the total indicator concentration, [M] is the total metal ion concentration, and K_j is the binding affinity of fluozin-1 for the metal ion.

The differential of Equation 1 produces Equation 2.

$$\Delta [M] = (1 - \frac{[I] - [M] - K_j}{\sqrt{([I] + [M] - K_j)^2 - 4[I][M]}}) \times \frac{[M]}{2} \times \Delta [M]$$ (Eq. 2)

If it is assumed that [I] > K_j, Equation 2 can be simplified to the following.

$$\Delta [M] = (1 - \frac{[I] - [M] - K_j}{([I] - [M])}) \times \frac{[M]}{2} \times \Delta [M]$$ (Eq. 3)

When [M] < [I], \( \Delta [M] = \Delta [M] \) (Eq. 4)

When [M] > [I], \( \Delta [M] = 0 \) (Eq. 5)

K_j of M binding to fluozin-1 is about 8 μM according to the Molecular Probes handbook. At 200 μM, more than 3% of the K_j of fluozin-1. Therefore, the approximation \( \Delta [M] = \Delta [M] \) holds. The observed \( \Delta F \) is predominantly determined by \( \Delta [I-M] \), which approximately equals \( \Delta [M] \) when \([I] > [M]\). Hence, \( \Delta [M] \) is given by Equation 6,

$$\Delta [M] = k\Delta F/\Delta F_{max}$$ (Eq. 6)

where k is a converting factor. \( \Delta F \) is normalized to the maximum fluorescence change (\( \Delta F_{max} \)) when the entrapped indicator is fully exposed to 2 μM external metal ion by detergent solubilization of proteoliposomes. In principal, the useful metal ion concentration range for this linear relationship, as suggested by Equation 5, is up to the concentration of the encapsulated fluozin-1 (200 μM). Calibration of Equation 6 was performed by exposing fluozin-1-loaded proteoliposomes to a series of controlled Zn^2+ concentrations in the presence of 20 μM zinc ionophore pyridoxal. Linear regression of the calibration curve yielded the converting factor k.

All stopped-flow traces were normalized either to a maximum response elicited by detergent solubilization or to a quasistationary response when indicated. Background traces collected from liposome samples were subtracted, yielding net fluorescence responses that were fit to a biexponential function, \( A_1 \left(1 - \exp(-k_1t)\right) + A_2 \left(1 - \exp(-k_2t)\right) \), where \( A_1, A_2, k_1, \) and \( k_2 \) are amplitudes and rate constants, respectively. The initial rate of transport, \( V_I = \left(\Delta F/\Delta F_{max}\right)/\Delta t_{[M]} \), was calculated as \( A_1/k_1 + A_2/k_2 \). Concentration dependence data were analyzed by least squares fits of the normalized initial transport rate, \( V_{I}/\Delta t_{[M]} \), to a hyperbola defined by Equation 7.

$$V_I = \frac{[M]}{V_{max} + [M]} (Eq. 7)$$

where [M] represents the metal ion concentration, \( V_{max} \) is the maximum initial transport rate when the rate of transport approaches a quasistationary state, and \( K_m \) is the Michaelis-Menten constant. Fits of experimental data were preformed using the data analysis software SIGMAPLOT 4.0 (SPSS Inc., Chicago, IL).

RESULTS

Overexpression and Purification—The overexpression of ZitB was driven by a T7 promoter in E. coli BL21 (DE3) pLysS cells. The optimal A_600 for isopropyl-β-D-thiogalactoside induction was found to be 0.17, and the log phase of growth was arrested 4 h after isopropyl-β-D-thiogalactoside induction to reach an A_600 of 1.8. Cells were harvested, and the membrane fraction was isolated and then solubilized using DDM at a detergent-to-cell ratio of 1:10 (w/w). His-tagged ZitB could be purified to homogeneity by a single step nickel chelate affinity chromatography (Fig. 1A). Overnight incubation of the purified His-ZitB with thrombin at 20 °C resulted in a complete removal of the His tag, as confirmed by Western blot analysis using a His tag-specific antibody (data not shown). The molecular identity of the purified ZitB was confirmed by MALDI-TOF mass spectrometric measurements. Molecular masses of 37,067 ± 7 and 35,179 ± 9 Da were obtained for the purified His-ZitB before and after thrombin digestion. The expected and experimentally measured molecular masses agreed within 23 Da (expected mass: 37,084 and 35,202 Da, respectively). The thrombin-digested ZitB was concentrated to about 10–20 mg/ml, and further purification was achieved by preparative size exclusion HPLC. Analytical size exclusion HPLC analysis indicated that the purified ZitB was free of high molecular weight aggregates, showing a major monodisperse species followed by a minor species with retention times corresponding to apparent molecular masses of 140 and 45 kDa, respectively (Fig. 1B). MALDI-TOF mass spectrometric analysis of these two peak fractions revealed a single mass peak at 35179 Da for the major species and a cluster of mass peaks between 700–800 Da for the minor species, indicating the lipidic nature of the minor species. The monodispersity of the 140-kDa fraction was confirmed by dynamic light scattering analysis. These results indicated that ZitB was purified as a single oligomeric species; however, the order of ZitB oligomerization is yet to be determined.

Functional Reconstitution—The detergent-mediated reconstitution of ZitB was achieved using a detergent removal method as described under “Experimental Procedures.” DDM in the purified ZitB sample was diluted 40–1000-fold with 1% β-OG prior to reconstitution. Encapsulation of fluozin-1 in re-
constituted vesicles was found to be stable over the period of a few days. Exposing ZitB proteoliposomes to 2 mM Zn$^{2+}$ exterior to proteoliposomes caused a rapid rise of fluozin-1 fluorescence. The background Zn$^{2+}$ leakage across the membrane was measured using control liposomes and was subtracted to yield a net Zn$^{2+}$ response attributable to the ZitB-catalyzed Zn$^{2+}$ influx (Fig. 2A). The initial rate of fluozin-1 response increased linearly with the ZitB-to-lipid ratio (Fig. 2B), demonstrating a linear relationship between the amount of the reconstituted ZitB and the initial rate of the fluorescence response. At a ZitB-to-lipid molar ratio of 8:1,1517 (equal to 1:1440), the background Zn$^{2+}$ leakage is negligible. Hence, this ZitB-to-lipid ratio was used thereafter for all stopped-flow measurements unless otherwise indicated. Furthermore, the quasistationary fluorescence responses also increased with ZitB-to-lipid ratios (Fig. 2A), indicative of the presence of a mixed population of proteoliposomes and liposomes in the reconstituted suspension. Increasing the ZitB-to-lipid ratio resulted in an increase of the proteoliposome population and a consequent increase of the quasistationary response. The quasistationary fluorescence was normalized to the maximum fluorescence, measured by adding β-OG to 1% at the end of each experiment to solubilize both proteoliposomes and liposomes. The resulting relative fluorescence varied from 0.05 to 0.5 depending on ZitB-to-lipid ratios (Fig. 2A).

To determine the specific activity of ZitB, the relationship between the intravesicular Zn$^{2+}$ concentration and the fluorescence response was calibrated based on Equation 6 as described under “Experimental Procedures.” Proteoliposomes were prepared at three ZitB-to-lipid ratios: 4:1,1517, 8:1,1517, and 16:11,517. Correspondingly, relative quasistationary responses were 0.43, 0.61, and 0.52. The maximum quasistationary response occurred at a ZitB-to-lipid ratio of 8:1,1517. The reduced response at a lower ratio was due to the presence of empty liposomes, whereas the decline at a higher ratio might be caused by the presence of multiple copies of ZitB in one proteoliposome. A calibration curve was obtained using the optimal ZitB-to-lipid ratio of 8:1,1517 (Fig. 2C). Under this condition, ΔF/ΔF$_{max}$ was linearly related to [Zn$^{2+}$] up to a value of 0.28, corresponding to a zinc concentration of 50 μM. Although this experimentally determined linear range was narrower compared with the theoretical prediction, it was sufficient to quantify initial zinc responses that fell within the range of 0–20% of the ΔF$_{max}$. The specific activity was calculated by converting the normalized initial Zn$^{2+}$ response (ΔF/ΔF$_{max}$) at Δt to Δ[Zn$^{2+}$]/Δ(t) (μmol/s), multiplying by the total proteoliposome internal volume (μl) and then normalizing to the total ZitB (μmol) in the reconstitution system. Using the calibration curve obtained from the same sample, the specific activity was determined to be 2.36 ± 0.42 (n = 3) μmol of Zn$^{2+}$/μmol of ZitB, giving an average turnover number of 2.36 s$^{-1}$ at 8 °C.

**Substrate Concentration Dependence**—The initial rate of Zn$^{2+}$ influx was measured in a zinc concentration range between 0 and 4 mM exterior to proteoliposomes. Proteoliposomes preloaded with fluozin-1 were rapidly mixed with an assay buffer containing various concentrations of Zn$^{2+}$, resulting in an inward Zn$^{2+}$ influx that drove Zn$^{2+}$ diffusion into the proteoliposome population and a consequent increase of the fluorescence response. The relative initial rate of the fluorescence responses increased in a hyperbolic manner with the Zn$^{2+}$ concentration and a fit of the relative rate data to a single hyperbolic function yielded a K$_m$ of 104.9 ± 5.4 μM (Fig. 3A). This kinetic behavior is consistent with a two-step process shown in Scheme 1,

\[ M + T_1 \xrightarrow{k_1} MT \xrightarrow{k_2} T_2 + M \]

**Scheme 1**

where T$_1$ and T$_2$ are different conformational states of ZitB, and M is the substrate. The first step is a rapid equilibrium with a binding constant k$^{-1}$, followed by a rate-limiting conformational transition from T1 to T2 with a rate constant k$^2$. The relationship among k$_1$, k$^{-1}$, and k$^2$ is defined as K$_m$ = (k$^{-2}$ + k$^{-1}$)k$_1$. This kinetic scheme suggests that ZitB catalyzes Zn$^{2+}$ transport with a stoichiometry of one ZitB for one Zn$^{2+}$.

In a parallel set of experiments, the Cd$^{2+}$ concentration dependence of ZitB transport was investigated. The ZitB-me-
diated Cd\textsuperscript{2+} influx showed a rapid rise of fluozin-1 fluorescence upon the mixing of proteoliposomes with an assay buffer containing varying concentrations of Cd\textsuperscript{2+} ranging from 0 up to 4 mM. The initial rate of the fluorescence response increased in a hyperbolic manner with the Cd\textsuperscript{2+} concentration. The concentration dependence of the relative initial rate, when fitted to a two-step process as described above for the Zn\textsuperscript{2+} transport. This result indicated that both Zn\textsuperscript{2+} and Cd\textsuperscript{2+} are effective substrates of ZitB. In comparison with Zn\textsuperscript{2+}, transport of Cd\textsuperscript{2+} appeared to have a relatively faster kinetics with a slightly lower \( K_m \). Because Cd\textsuperscript{2+} background response was found significantly lower than that of Zn\textsuperscript{2+}, Cd\textsuperscript{2+} was used for all kinetic experiments thereafter.

**Coupling Ion**—Evidence from genetic complementation and whole cell zinc uptake experiments suggested that ZitB might utilize an antiporter mechanism for extrusion of cytoplasmic zinc in exchange for extracellular potassium (20, 21). Protons were also shown as a possible coupling ion either alone or in concert with K\textsuperscript{+}. To directly test the antiporter mechanism, we used a purified reconstituted system to examine K\textsuperscript{+} and H\textsuperscript{+} dependencies of ZitB transport for Cd\textsuperscript{2+}. It is expected that the translocation of Cd\textsuperscript{2+} in antiport would be obligatory upon the transport of a coupling ion in the opposite direction. An inward Cd\textsuperscript{2+} diffusion gradient was rapidly applied by exposing proteoliposomes to an assay buffer containing 2 mM Cd\textsuperscript{2+}. Cd\textsuperscript{2+} influxes driven by this imposed Cd\textsuperscript{2+} diffusion gradient were measured under otherwise identical conditions except that either K\textsuperscript{+} or H\textsuperscript{+} was used as the major coupling ion species. The effects of any pH and ionic conditions on the fluozin-1 response were corrected by normalizing the fluorescence change to the quasistationary fluorescence change. In the first set of experiments, TEA\textsuperscript{+} was used as a control osmolyte to completely substitute for K\textsuperscript{+} both inside and outside of vesicles. The kinetics of the Cd\textsuperscript{2+} influx in the TEA\textsuperscript{+} medium (Fig. 4A, upper panel) was identical to that observed in the regular K\textsuperscript{+} assay buffer. (Fig. 4A, lower panel). This result precluded K\textsuperscript{+} being a coupling ion for Zn\textsuperscript{2+} transport. In the second set of experiments, pH of the assay buffer was set to 6.8 or 8.8 using Tris buffer instead of the standard BTM buffer to extend the pH buffering range. The Cd\textsuperscript{2+} response at pH 6.8 rose with a rapid rise time course that reached a quasistationary state in about 2 s (Fig. 4B, upper panel). In contrast, the Cd\textsuperscript{2+} response virtually stalled at pH 8.8, showing a significantly slower kinetics, taking more than 200 s to reach a quasistationary state (Fig. 4B, lower panel). These results demonstrated that a Cd\textsuperscript{2+} diffusion gradient alone was not sufficient to support a rapid Cd\textsuperscript{2+} influx. The increased proton concentration at pH 6.8 resumed the rate of Cd\textsuperscript{2+} influx to a level equal to that observed with the standard BTM assay buffer (Fig. 4A), indicating that Tris buffer itself did not influence Cd\textsuperscript{2+} transport. Therefore, the pH dependence of Cd\textsuperscript{2+} transport is consistent with the role of a proton as an obligatory coupling ion that was required for completing the transport cycle of Cd\textsuperscript{2+} down its diffusion gradient.

The Effect of [H\textsuperscript{+}] on ZitB-catalyzed Cd\textsuperscript{2+} Transport—To quantitatively examine the coupling between Cd\textsuperscript{2+} and H\textsuperscript{+}, the proton dependence of Cd\textsuperscript{2+} transport was exploited to study the H\textsuperscript{+} transport process. Aliquots of proteoliposome were diluted 100-fold to a series of assay buffers with varying pH values ranging from 6.8 to 9.1. The natural high permeability of lipid membrane to H\textsuperscript{+} allowed free equilibration of the intravesicular pH to the value set by the external Tris-based assay buffer (25). Initial rates of Cd\textsuperscript{2+} influxes in response to 2 mM external Cd\textsuperscript{2+} were measured at various pH values. The relative initial rates were found to increase progressively with H\textsuperscript{+} concentrations, showing a [H\textsuperscript{+}]-dependent profile that could be fit to a

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**Fig. 3. Kinetics of ZitB-mediated metal ion transport.** Fluozin-1-loaded proteoliposomes were mixed 1:1 with BTM assay buffer containing the indicated concentration of ZnSO\textsubscript{4} (A) or CdSO\textsubscript{4} (B). \( V/V_{\text{max}} \) is the relative rate normalized to the rate measured at 4 mM Zn\textsuperscript{2+} or 2 mM Cd\textsuperscript{2+}, respectively. The data were fitted to a hyperbolic equation by the least-squares method.

**Fig. 4. H\textsuperscript{+}-coupled Cd\textsuperscript{2+} influx.** Responses of fluozin-1-loaded proteoliposomes to the addition of 2 mM extravesicular CdSO\textsubscript{4} were normalized to the respective quasistationary fluorescence changes. A, the effect of K\textsuperscript{+} on Cd\textsuperscript{2+} influx. Stopped-flow traces were recorded at pH 6.8 in the presence of 50 mM TEA\textsubscript{SO\textsubscript{4}} (upper panel) or 50 mM K\textsubscript{2}SO\textsubscript{4} (lower panel), both inside and outside of vesicles. TEA\textsuperscript{+} was used as an osmolyte substitute for K\textsuperscript{+}. B, the effect of H\textsuperscript{+} on Cd\textsuperscript{2+} influx. Stopped-flow traces were recorded at pH 6.8 (upper panel) and 8.8 (lower panel). The pH was buffered using 20 mM Tris and titrated to the indicated pH value using H\textsubscript{2}SO\textsubscript{4}.
single hyperbolic function with a $K_m$ of 19.9 ± 1.6 nM (Fig. 5). Likewise, a fit to the Hill equation, $S^n/K_m^n + K_{n,5}$, yielded a $K_{n,5}$ of 19.9 ± 1.7 nM and a stoichiometry $n = 0.94 ± 0.07$. The near unity $n$ value indicates that the H$^+$ dependence is a noncooperative process. The lack of H$^+$ cooperativity is consistent with a 1:1 stoichiometry for Cd$^{2+}$/H$^+$ exchange.

**Cd$^{2+}$ Exchange against H$^+$ Diffusion Gradient** — The coupled exchange of Cd$^{2+}$ for H$^+$ suggests that a transmembrane proton gradient could be as effective an energetic driving force as a Cd$^{2+}$ gradient to move Cd$^{2+}$ across the membrane. To directly examine the Cd$^{2+}$ flux in response to the imposition of an artificial transmembrane proton diffusion gradient, fluorescent indicators fluozin-1 and pyranine (26) were also loaded into proteoliposomes to monitor the intravesicular Cd$^{2+}$ and H$^+$ concentration changes, respectively. A calibration of pyranine ratio-metric fluorescence responses to a series of pH calibrants revealed a linear relationship with pH changes within 1.2 pH units centered at pH 7.5 (Fig. 6A). This linear range was exploited for all Cd$^{2+}$ exchange experiments that were performed at pH 7.5 using proteoliposomes preloaded with 100 μM Cd$^{2+}$ in equilibrium with the same Cd$^{2+}$ concentration in the external assay buffer. Transmembrane H$^+$ gradients were established by the imposition of K$^+$ gradients in the presence of an electroneutral H$^+$/K$^+$ exchanger nigericin that can utilize the imposed K$^+$ gradients to drive H$^+$ into or out of vesicles (27). An inward K$^+$ gradient, corresponding to inside 0 mM K$^+$ + 100 mM TEA$^-$ and outside 50 mM K$^+$ + 50 mM TEA$^-$, caused an increase in pyranine fluorescence (Fig. 6B, upper panel), indicating a decrease in the invesicular H$^+$ concentration and consequently the establishment of an inward H$^+$ diffusion gradient. Under the same experimental condition, a decrease of Fluozin-1 fluorescence (Fig. 6B, lower panel) was observed, indicating a Cd$^{2+}$ efflux concurrent with the imposition of the inward H$^+$ gradient. In a mirror experiment, an outward H$^+$ diffusion gradient, corresponding to inside 100 mM K$^+$ + 0 mM TEA$^-$ and outside 50 mM K$^+$ + 50 mM TEA$^-$, caused a decrease in pyranine response (Fig. 6C, upper panel) and a concurrent increase of fluozin-1 fluorescence (Fig. 6C, lower panel). Taken together, these results demonstrated that the Cd$^{2+}$ transmembrane flux, either inward or outward, was driven by an oppositely oriented H$^+$ diffusion gradient. A quantitative comparison between H$^+$-driven and Cd$^{2+}$-driven fluxes was difficult because of the nonlinearity of fluozin-1 response at a Cd$^{2+}$ concentration above 50 μM (Fig. 2C). Nevertheless, relative quasistationary amplitudes of 1.2 for Cd$^{2+}$ influx and 1.9 for Cd$^{2+}$ efflux were obtained by normalizing the H$^+$-driven Cd$^{2+}$ response to a Cd$^{2+}$ response driven by a one-fold Cd$^{2+}$ concentration difference, corresponding to inside 100 μM outside 200 μM and inside 100 μM outside 50 μM for Cd$^{2+}$ influx and efflux, respectively (Fig. 6, C (lower panel) and B (lower panel)). These results qualitatively demonstrated that ZitB mediates an efficient energetic propagation from K$^+$ diffusion gradients through proton gradients to Cd$^{2+}$ fluxes.

**Membrane Potential Dependence** — The possible 1:1 exchange of Cd$^{2+}$ for H$^+$ suggests that ZitB transport is an electrogenic process. If this is the case, the transmembrane potential could be as effective an energetic driving force as a Cd$^{2+}$ or H$^+$ gradient. Cd$^{2+}$ fluxes in response to the imposition of transmembrane potentials were examined using proteoliposomes in the presence of 100 μM Cd$^{2+}$ in equilibrium between the internal and the external medium. Membrane potentials were generated by applying K$^+$ transmembrane gradients in the presence of 2 μM potassium ionophore valinomycin (28). The valinomycin-mediated K$^+$ flux down its diffusion gradient would create a transmembrane electrical potential according to the Nernst equilibrium. This membrane potential was detected by the voltage-sensitive fluorescence of a lipophilic anion indicator, oxonol VI (29). Upon exposure of proteoliposomes preloaded with no mM K$^+$ + 100 mM TEA$^-$ to an assay buffer containing 50 mM K$^+$ + 50 mM TEA$^-$, an inside-positive potential was established, as indicated by a rise of oxonol VI fluorescence (Fig. 7A, upper panel). Under the same experimental condition, a concurrent Cd$^{2+}$ efflux was observed, as indicated by a decrease in fluozin-1 fluorescence (Fig. 7A, lower panel). Taken together, these results demonstrated that an outwardly directed membrane potential drove a Cd$^{2+}$ efflux down its electrical gradient. The quasistationary response to this imposed inside-positive potential was 1.2-fold relative to that...
higher oxonol VI concentration, a decrease in oxonol VI response potential and by a Cd²⁺ to proteoliposomes loaded with a TEA⁺/H⁺ was increased from 1 to 20. Fluorescence was observed when the concentration of oxonol VI was normalized to a quasistationary response generated by ex- conditions, normalized to a quasistationary response generated by ex- posing proteoliposomes to a TEA⁺ medium minus 50 μM CdSO₄. The direction of the membrane potential and the resulting Cd²⁺ flux are indicated by a triangle and arrow, respectively, in the inset, B, mirror experiments to A. Note that the ΔF/F₀ for 1 μM oxonol VI is negligible, as shown in the upper panel (solid line), whereas the ΔF/F₀ for 20 μM oxonol VI shows a significant decrease (dotted line).

driven by an outward Cd²⁺ gradient corresponding to inside 100 mM outside 50 μM Cd²⁺ (Fig. 7A, lower panel). This qualitative comparison of Cd²⁺ effluxes driven by an electrical potential and by a Cd²⁺ gradient indicates that the membrane potential is in effect comparable with the chemical gradient as a driving force for ZitB transport. However, neither the expectant inside-negative membrane potential nor the Cd²⁺ influx was detected in a mirror experiment in which an outward K⁺ gradient (inside 100 mM K⁺ and 0 mM TEA⁺/outside 50 mM K⁺ + 50 mM TEA⁺) was imposed (Fig. 7B). It is noted that the efflux of a limited supply of K⁺ is insufficient to generate a Nernst potential because of the low intravesicular volume and the relatively high membrane capacitance. Therefore, the electrogenic effect of an outward K⁺ gradient is not symmetrical to that of an inward K⁺ gradient. In the latter case, the external K⁺ supply is unlimited. Nevertheless, a decrease in oxonol VI fluorescence was observed when the concentration of oxonol VI was increased from 1 to 20 μM (Fig. 7B, upper panel). At this higher oxonol VI concentration, a decrease in oxonol VI response was probably caused either by a small inside-negative membrane potential not detectable to 1 μM oxonol VI or by the self-quenching of oxonol VI fluorescence at this high concentration.

**DISCUSSION**

The experiments described herein provide the first kinetic analysis of the transport mechanism of ZitB. The kinetics of ZitB exhibits a hyperbolic relationship between the initial transport rate and the metal ion concentration, indicative of a substrate-saturable transport process that can be described by a two-step kinetic process involving a rapid binding followed by a rate-limiting step of conformational transition for moving the metal ion across the membrane. The $K_m$ is 104.9 ± 5.4 μM for Zn²⁺ and 90.1 ± 3.7 μM for Cd²⁺. The turnover number is 2.36 s⁻¹ at 8 °C for the physiological substrate Zn²⁺ when it is assumed that all ZitB is reconstituted functionally. The low overexpression level of ZitB and the high background zinc permeability in E. coli cells prevent a reliable measurement of the ZitB turnover number in the native membrane environment. It is not known to what extent the reconstituted ZitB is active. To prevent possible denaturation of ZitB in the purification and reconstitution processes, we have removed protein aggregates by size exclusion HPLC and reconstituted the monodisperse faction of the purified ZitB into the native E. coli lipids. The downhill flux of Cd²⁺ through ZitB stalled at pH 8.8 but could be restored at pH 6.8, indicating that the transport of metal ion is coupled to H⁺. Measurements of Cd²⁺ fluxes in response to various pH gradients provided internally consistent results, demonstrating that the transport of Cd²⁺ can be driven by a proton potential artificially imposed in an opposite direction with the aid of nigericin. Since proteoliposomes may contain a mix of outward facing and inward facing ZitB, the proton-driven Cd²⁺ influx or efflux may reflect the activity of two different populations of ZitB, with the activity of each population being dependent on the orientation of the proton gradient. From an energetic point of view, the accumulation of Cd²⁺ is achieved at the expense of the proton diffusion potential. ZitB captures the free energy of the downhill H⁺ flux and uses it to drive the uphill Cd²⁺ flux. The H⁺ flux, although not demonstrated directly, conceivably occurs simultaneously with the flux of Cd²⁺. This obligatory coupling between two fluxes ensures that the proton electrochemical driving force is effectively utilized for the concurrent accumulation of Cd²⁺.

The Cd²⁺ flux against a H⁺ gradient represents a heterolo- gous Cd²⁺/H⁺ exchange likely with a stoichiometry of one Cd²⁺ for one H⁺, as inferred by the hyperbolic H⁺ dependence of the Cd²⁺ transport. It is noted that a reliable transport stoichiometry for the coupling ion should be measured directly rather than inferred from the effect of the coupling ion on transport. The high natural membrane permeability to H⁺ precludes a direct measurement. Alternatively, the electrogenic effect of the ZitB transport was examined to deduce the transport stoichiometry, because a 1:1 exchange of Cd²⁺ for H⁺ would result in a vectorial accumulation of positive charge along the direction of the Cd²⁺ flux, whereas a 1:2 exchange would favor an electroneutral process. In experiments using ionophorous valinomycin to generate artificial membrane potentials, only a sustained inside-positive potassium Nernst potential was established, and such a membrane potential supported the efflux of Cd²⁺ across a membrane equilibrated with Cd²⁺ on both sides. This result demonstrates that imposition of an artificial membrane potential drives a Cd²⁺ flux along the direction of the membrane potential, therefore favoring a 1:1 electrogenic exchange of Cd²⁺ for H⁺. The Cd²⁺ efflux driven by the membrane electrical potential is on the same order of magnitude with those driven either by proton potential or by Cd²⁺ chemical potential. These results suggest that the membrane electrical potential and the chemical potentials of the substrate and the coupling ion are interconvertible components of an integrated transmembrane electrochemical potential that acts as a whole to power the ZitB pumping activity.

ZitB is a member of an extensive family of CDF transporters, found in both prokaryotes and eukaryotes including seven human isoforms, ZnT1–ZnT7 (30). Homologies among the sequenced CDF members suggest a common genetic origin and therefore a common mechanism that has not yet been described with certainty. Cation diffusion facilitator, implied by its name, favors a porelike mechanism by which metal ions move independent of other coupling elements. Our results obtained from the purified and reconstituted ZitB indicate that the Cd²⁺ transport cycle can only be completed in the presence of H⁺ as a coupling ion, in direct contrast to the implied uniport or channel mechanism. The H⁺-linked antiport mechanism of
ZitB also partly contradicts earlier studies suggesting that CDF transporters catalyze the active efflux of Zn\(^{2+}\) in exchange for K\(^+\) (20). ZitB transport kinetics is virtually unchanged under experimental conditions completely devoid of K\(^+\), clearly indicating that K\(^+\) is not a coupling ion for ZitB transport. It is noted that those earlier studies were based on growth experiments and whole cell transport assays under the conditions of overexpression of ZitB or CzcD. Interpretations of these experimental results could be obscured by many overlapping transport activities in a whole-cell overexpression system.

The Zn\(^{2+}/H^+\) antiporter mechanism of ZitB is consistent with its functional role as a zinc efflux pump. In most common bacteria including *E. coli*, the chief link between metabolism and secondary transport activities is the global circulation of protons across the plasma membrane. Vectorial electrogenic H\(^+\) extrusion through the respiratory chain generates a proton potential, making the cytoplasm electronegative and alkaline. ZitB may provide a pathway that allows the proton at the external surface to be pulled back across the membrane down the electrochemical gradient established by respiration. The free energy of this downhill H\(^+\) influx is coupled to the uphill pumping of Zn\(^{2+}\) out of the cell. 

The Zn\(^{2+}\) transport system is dependent on a pH range of 7.6 to 7.8 found in the cytoplasm of a functioning *E. coli* cell (31, 32).

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