AztD, a Periplasmic Zinc Metallochaperone to an ATP-binding Cassette (ABC) Transporter System in Paracoccus denitrificans*

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Bacterial ATP-binding cassette (ABC) transporters of transition metals are essential for acquisition of necessary elements from the environment. A large number of Gram-negative bacteria, including human pathogens, have a fourth conserved gene of unknown function adjacent to the canonical permease, ATPase, and solute-binding protein (SBP) genes of the AztABC zinc transporter system. To assess the function of this putative accessory factor (AztD) from Paracoccus denitrificans, we have analyzed its transcriptional regulation, metal binding properties, and interaction with the SBP (AztC). Transcription of the aztD gene is significantly up-regulated under conditions of zinc starvation. Recombinantly expressed AztD purifies with slightly substoichiometric zinc from the periplasm of Escherichia coli and is capable of binding up to three zinc ions with high affinity. Size exclusion chromatography and a simple intrinsic fluorescence assay were used to determine that AztD as isolated is able to transfer bound zinc nearly quantitatively to apo-AztC. Transfer occurs through a direct, associative mechanism that prevents loss of metal to the solvent. These results indicate that AztD is a zinc chaperone to AztC and likely functions to maintain zinc homeostasis through interaction with the AztABC system. This work extends our understanding of periplasmic zinc trafficking and the function of chaperones in this process.

Zinc is an essential element required for a large number of biological processes with roles as a cofactor in enzyme catalysis and in the maintenance of protein structures (1). Therefore, its acquisition from the environment is essential for cell survival. However, zinc levels must also be tightly regulated to prevent metal ion toxicity and misincorporation into off-target proteins. Zinc homeostasis is achieved in part by the transcriptional regulation of import (2) and efflux systems (3) in response to changing intracellular zinc concentrations. Proteins involved in both processes have been identified as virulence factors in human pathogens as they enable bacteria to survive conditions of zinc starvation and intoxication, either of which may be employed by the innate host immune system (4–6). Consequently, there is significant interest in targeting prokaryotic mechanisms of zinc homeostasis for the development of novel antibiotics against resistant bacteria.

The intracellular concentration of “free” zinc in Escherichia coli is estimated to be maintained in the femtomolar to nanomolar range, whereas total intracellular zinc approaches millimolar concentrations (7, 8). The vast majority of zinc is bound to high affinity sites on proteins and nucleic acids that buffer the available zinc concentration to virtually zero. As such, there is a strong implication for the presence of cytosolic zinc chaperones that can sequester metal during excess metal stress and deliver it to appropriate targets under metal starvation. Although none have been conclusively identified to date, there is evidence that members of the COG0523 (9, 10) (NiFe hydrogenase and urease maturation factor) and Atx1 (11, 12) (copper metallochaperone) families may act in this capacity. In eukaryotes, metallothioneine (13) and histidine-proline-rich glycoprotein (14) have been implicated as possible zinc metallochaperones.

The situation in the periplasm of Gram-negative bacteria is markedly different as metal ions are allowed to diffuse more or less freely across the outer membrane. However, even here there is evidence that the concentration of zinc is managed by protein binding. Under severe zinc limitation, high affinity solute-binding proteins (SBPs)3 such as ZnuA compete effectively for zinc (15), delivering it to the cytoplasm via the ATP-binding

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3 The abbreviations used are: SBP, solute-binding protein; ABC, ATP-binding cassette; ICP-OES, inductively coupled plasma-optical emission spectroscopy; MF-2, mag fura-2; SEC, size exclusion chromatography; TPEN, N,N,N,N’-tetrakis(2-pyridylmethyl)ethane-1,2-diamine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CV, column volume; FT-ICR, Fourier transform-ion cyclotron resonance.

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AztD homologues, this protein likely plays an important role in the management of periplasmic zinc by many Gram-negative species.

Experimental Procedures

Bacterial Growth Conditions and Harvesting of Cells—A minimal media composition using an organic phosphate source and succinate as a carbon source was developed based on Graham et al. (18) and Wang et al. (24). The details of zinc-replete (50 μM zinc), -depleted (0 μM zinc), and -chelated (0 μM zinc, 50 μM N,N′,N′′,N′′-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN)) media compositions are given in a previous publication (21).

An overnight culture of P. denitrificans PD1222 cells grown in replete media was washed once with zinc-depleted media and used to inoculate three replicates of each of the different media types described above. The growth of cells in all three conditions was monitored at 600 nm using an Agilent Cary 60 UV-visible spectrophotometer, and 5 ml of cells at midexponential phase (A600nm 0.4–0.6) were harvested from each condition. To preserve RNA, 2 ml of ice-cold 5% v/v phenol in ethanol were added and incubated on ice for 30 min prior to centrifugation and storage at −80 °C until RNA was isolated.

RNA Extraction and Quantitative RT-PCR—RNA was extracted and purified from the replicate samples described above using a PureLink® RNA mini kit (Ambion) according to the manufacturer’s instruction. Contaminating DNA was removed by an on-column DNase digestion protocol (Invitrogen). RNA concentration and purity were determined spectrophotometrically using a Nanodrop Spectrophotometer (ND 1000).

Purified RNA (1 μg) was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA™ kit (Applied Biosystems) according to the manufacturer’s instructions. cDNA was diluted 5-fold in H2O before use in quantitative PCR experiments. Primers were designed to amplify 100–150-bp regions of each gene with a Tm of ~60 °C and used at a final concentration of 0.3 μM. Quantitative real time PCR was done using Power SYBR® Green PCR Master Mix (Applied Biosystems) CFX96 RT-PCR real time system combined with a C1000 thermal cycler (Bio-Rad). Relative transcript abundance was normalized to dnaN, encoding the β-subunit of DNA-polymerase III (Pden_0970), a housekeeping gene previously used for P. denitrificans RT-PCR experiments (25). Standard PCR was performed using the forward RT-PCR primer for aztC and the reverse primer for aztD to amplify a 1157-bp product spanning the intergenic region. Genomic DNA (gDNA) or complementary DNA (cDNA) from RT-PCR experiments were used as templates.

Cloning, Heterologous Expression, and Purification—AztC was expressed and purified as described previously (21). The entire pden1598 gene encoding AztD was amplified by PCR from P. denitrificans genomic DNA using the following primers: 5′-ACTATCATATGATGCTACGACATCCTGCG-3′ forward and 5′-ACTATGGTACCTCAGTGCGTCACGCCGCT-3′ reverse. The PCR product was cloned into a pCDFDuet™-TM plasmid using the EcoRV restriction site and transformed into E. coli DH5α. Plasmids were purified (MoBio Nucleic Acid Purification Kit) and sequenced to confirm the correct sequence. Plasmids were transformed into E. coli PD1222 and growth in zinc-depleted minimal media was confirmed.

P. denitrificans was lysed using French pressure and the cytoplasmic fraction was subjected to ammonium sulfate precipitation and dialysis before being loaded onto a Ni-nitrilotriacetic acid column. Purified RNA (1 μg) was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA™ kit (Applied Biosystems) according to the manufacturer’s instructions. cDNA was diluted 5-fold in H2O before use in quantitative PCR experiments. Primers were designed to amplify 100–150-bp regions of each gene with a Tm of ~60 °C and used at a final concentration of 0.3 μM. Quantitative real time PCR was done using Power SYBR® Green PCR Master Mix (Applied Biosystems) CFX96 RT-PCR real time system combined with a C1000 thermal cycler (Bio-Rad). Relative transcript abundance was normalized to dnaN, encoding the β-subunit of DNA-polymerase III (Pden_0970), a housekeeping gene previously used for P. denitrificans RT-PCR experiments (25). Standard PCR was performed using the forward RT-PCR primer for aztC and the reverse primer for aztD to amplify a 1157-bp product spanning the intergenic region. Genomic DNA (gDNA) or complementary DNA (cDNA) from RT-PCR experiments were used as templates.

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1 vector (Novagen) using NdeI and Acc65I restriction sites, resulting in a translated protein lacking any affinity tags and containing the N-terminal periplasmic targeting sequence. Plasmid was transformed into BL21 DE3 E. coli cells, which were grown in LB medium containing 50 µg/ml streptomycin at 37 °C and 250 rpm to an A600 = 0.8–1.0. Overexpression was then induced by addition of IPTG to 1.0 mM, the temperature decreased to 20 °C, and cells were grown with shaking overnight. Cells were harvested by centrifugation at 3000 × g for 30 min at 4 °C.

The periplasmic fraction was obtained using an osmotic shock protocol adapted from Wang et al. (24). Briefly, the cell pellet was resuspended at 5 ml/g of wet weight cells in 50 mM phosphate, pH 8.0, 0.5 M sucrose, 0.67 mM EDTA, and 7.5 mg/ml lysozyme and incubated at 30 °C for 15 min. An equal volume of deionized water was added and incubated a further 45 min at 30 °C. Cell debris was removed by centrifugation at 25,000 × g for 30 min, leaving the periplasmic fraction in the supernatant. Polyethyleneimine was added to 0.5% by volume to precipitate nucleic acids, which were removed by centrifugation at 25,000 × g for 30 min. Finally, protein was precipitated by addition of ammonium sulfate to 70% and centrifuged as above.

The ammonium sulfate pellet was dissolved in 20 mM Tris, pH 8.0, and centrifuged at 20,000 × g for 30 min to remove any remaining particulates. The supernatant was loaded onto a HiTrap Q HP column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0. The buffer was gradually changed to 20 mM bis-tris, pH 6.5, over 3 column volumes. NaCl was stepped to 100 mM followed by elution of the protein on a linear gradient of NaCl. The peak containing AztD eluted at ~200 mM NaCl. Fractions containing AztD were combined and concentrated and applied to a HiPrep Sephacryl S-100 HR column (GE Healthcare) equilibrated with 50 mM Tris, pH 8.0, 150 mM, 0.1 mM DTT. SDS-PAGE was used to quantitatively assess protein purity. Protein concentration was determined using an extinction coefficient at 280 nm of 33,666 M⁻¹ cm⁻¹ calculated as described previously (26).

Trypsin Digests and Mass Spectrometry—Purified AztD was reduced and denatured in 3 M urea, 10 mM DTT for 30 min at 37 °C. Iodoacetamide was added to 20 mM and incubated for 30 min at room temperature in the dark. A further 16 mM DTT was added to quench the reaction. Urea was diluted to <1.2 mM in trypsin digest buffer (final concentration 50 mM Tris, pH 8.0, 20 mM CaCl₂) prior to addition of proteomics grade trypsin (New England Biolabs) to a ratio of 1:20 by mass. After incubation at 37 °C overnight, peptides were desalted/exchanged into 75:25 (v/v) acetonitrile/water with 0.1% formic acid using C18 resin ZipTip® pipette tips (Millipore) prior to introduction into the mass spectrometer. Peptide solutions were analyzed by direct-infusion electrospray ionization FT-ICR mass spectrometry (Thermo LTQ FT) in positive ion mode. Nanoelectrospray was performed with an Advion NanoMate, and resultant peptide ions were measured in a parent-ion FT-ICR scan at a resolving power of m/Δm50% = 100,000 at m/z 400. Fragment ion spectra were generated in the linear ion trap. Parent ions were picked from the FT-ICR spectrum with dynamic exclusion enabled. Parent ion and MS/MS data were manually inspected using Xcalibur software version 2.1.0 to identify and confirm peptides of interest.

Metal Content Analysis—AztD at 200 µM was incubated with 0–4 molar eq of ZnSO₄ for 15 min at room temperature. A control sample at the highest zinc concentration but lacking protein was used to confirm exogenous zinc removal. Samples were then desalted using Zeba™ desalting spin columns (Thermo Scientific) equilibrated with binding buffer (20 mM HEPES, pH 7.2, 200 mM NaCl, and 5% glycerol treated with Chelex 100 resin (Bio-Rad) to remove trace metal contaminants). Protein concentrations were determined by absorbance at 280 nm, and samples were diluted to 10 µM in 4 M HNO₃ for overnight digestion at 70 °C. Prior to metal analysis, samples were diluted 2.5-fold with MilliQ water. Samples were analyzed on a PerkinElmer Life Sciences 2100 DV inductively coupled plasma-optical emission spectrometer (ICP-OES), calibrated with a multielement standard (Ricca Chemical). The wavelengths for measuring manganese and zinc were 257.610 and 206.200 nm, respectively, and samples were run in triplicate.

Circular Dichroism—Circular dichroism (CD) spectra were recorded at 25 °C using a Jasco-810 spectropolarimeter with a cuvette chamber regulated by a PTC-4235 Peltier device (Jasco). AztD as isolated was diluted to 5 µM in 5 mM HK₃PO₄, pH 8.0, 150 mM NaCl in a 1-mm quartz cuvette. Spectra were acquired from 190 to 260 nm at 1 nm bandwidth, 2 s response time, 0.5-nm data pitch, and 10 nm/min scan speed. Each spectrum is the average of three accumulations and has been converted to mean residue ellipticity. Spectra were fitted through the DICHROWEB interface (27) using the CDSSTR algorithm and associated reference sets (28). For thermal stability experiments, ellipticity at 215 nm was monitored from 25 to 90 °C every 0.2 °C with a constant heating rate of 1.0 °C/min. After thermal denaturation experiments, the spectra from 190 to 260 nm were again collected after returning to 25 °C to determine reversibility.

Generation of Apoproteins—Apo-AztC was generated as described previously (21). Apo-AztD was generated by heating in the presence of Chelex. Circular dichroism (CD) demonstrated no significant loss of secondary structure up to 90 °C. Therefore, 55 mg of Chelex was added to AztD in a microcentrifuge tube, and the suspension was incubated for 15 min at 90 °C with occasional mixing. Resin was subsequently removed by centrifugation, and the metal content was determined as described above.

Metal Binding Affinity—All of the metal binding affinity experiments were performed in Chelex-treated binding buffer using proteins that had been desalted/exchanged into binding buffer. The fluorescent dye mag-fura-2 (MF-2) (Invitrogen) was used to analyze the metal binding affinities of AztD for manganese and zinc as described previously (29). All fluorescence measurements were made using a Varian Cary Eclipse fluorescence spectrophotometer with entrance and exit slits set to 10 nm. Protein concentration was measured before each experiment, and MF-2 concentration was determined using an extinction coefficient at 369 nm of 22,000 M⁻¹ cm⁻¹ (29). In each experiment, 15.0 µM apo-AztD and 0.5 µM MF-2 were titrated with increasing concentrations of MnCl₂ or ZnSO₄, keeping the total volume of titrant added to less than 10%.
excitation spectra were scanned from 250 to 450 nm while monitoring emission at 505 nm. The fluorescence intensities at \( \lambda_{ex} = 330 \) nm for zinc titrations were fit using the program DYNAFIT (30, 31) using scripts adapted from Golynskiy et al. (29).

**Metal Transfer by Intrinsic Fluorescence**—All experiments were performed at room temperature in a fluorescence microcuvette containing 200 \( \mu \)l of binding buffer with or without 1 mM EDTA. All proteins were desalted/exchanged into binding buffer prior to use. Apo- or holo-AztC at 10 \( \mu \)M was titrated with \( \text{ZnSO}_4 \), apo-AztD, or holo-AztD. Fluorescence emission intensity was monitored from 290 to 450 nm with an excitation wavelength of 278 nm using a Varian Cary Eclipse fluorescence spectrophotometer with entrance and exit slits set to 5 nm. In titrations of apo-AztC with apo- and holo-AztD, 15 min of equilibration was allowed between measurements. After addition of apo- or holo-AztD to 12 \( \mu \)M, 20 \( \mu \)M of \( \text{ZnSO}_4 \) was added to determine saturation.

**Metal Transfer by Size Exclusion Chromatography**—A HiPrep Sephacryl S-100 HR column (GE Healthcare) was prepared with washing with 0.5 column volumes (CV) 0.2 M NaOH followed by 2 CV of 50 mM EDTA, pH 8.0, and finally equilibrated with 2 CV of Chelex-treated binding buffer. This same washing protocol was performed between each sample run. In control experiments, 500 \( \mu \)l of apo-AztC or holo-AztD at 100–135 \( \mu \)M in binding buffer was loaded onto the column and eluted at a flow rate of 0.5 ml/min. For zinc transfer experiments, apo-AztC and holo-AztD at 160 \( \mu \)M each were incubated for 30 min at room temperature prior to loading onto the column. The elution profile was fitted with two Gaussian curves representing AztC and AztD using the program MagicPlot (St. Petersburg, Russia). These were then converted to protein concentration using the respective extinction coefficients. SDS-PAGE was used to confirm the identity of protein found in each chromatographic fraction. Fractions were collected and digested in 4 M HNO\(_3\) for zinc content analysis by ICP-OES.

**Results**

**Differential Expression of aztD under Zinc Starvation**—The expressions of \( \text{aztC} \) and \( \text{aztD} \) were analyzed using quantitative RT-PCR of \( P. \text{denitrificans} \) cells grown in zinc-replete (50 \( \mu \)M added zinc), zinc-depleted (0 \( \mu \)M added zinc), and zinc-chelated (0 \( \mu \)M added zinc, 50 \( \mu \)M TPEN) conditions (Fig. 2A). Expression of \( \text{aztC} \) was increased \( \sim 5\)-fold in zinc-depleted and zinc-chelated conditions consistent with previous results (21). The expression of \( \text{aztD} \) increased nearly 20- and 30-fold under zinc-depleted and zinc-chelated conditions, respectively. To determine whether \( \text{aztC} \) and \( \text{aztD} \) are co-cistronic, we attempted to amplify a sequence across the \( \text{aztC-D} \) boundary using cDNA as a template. Despite repeated attempts, no product could be amplified from cDNA, whereas a strong band was observed using gDNA as a template (Fig. 2B), suggesting that \( \text{aztC} \) and \( \text{aztD} \) are not co-cistronic, although both are induced under zinc starvation to varying degrees.

**Expression and Purification of AztD**—A plasmid bearing the full-length \( pde1 \)598 gene encoding AztD, including the N-terminal 22 residues comprising the predicted periplasmic localization signal peptide (32), was transformed into \( E. \text{coli} \), over-expressed, and purified from the periplasm by osmotic shock (Fig. 3A). SDS-PAGE identified a band of the correct size for full-length AztD in the induced whole cell fraction, although a band of slightly smaller apparent molecular weight was enriched in the periplasmic fraction. The processed protein was purified to homogeneity from the periplasmic fraction by ion exchange and size exclusion chromatography (SEC). The identity of the purified protein as AztD was confirmed by trypsin cleavage and mass spectrometry. A non-tryptic cleavage site between Ala-21 and Gln-22 was observed (data not shown), indicating that the signal sequence is recognized by \( E. \text{coli} \) and cleaved upon export into the periplasm.

Analysis of metal content by ICP-OES revealed that AztD was isolated with 0.5–0.7 eq of bound zinc for several independent preparations. Manganese content of AztD was undetectable, consistent with its association with a zinc-specific ABC transporter. Because zinc content was found to be sub-stoichiometric, protein samples were incubated with increasing concentrations of zinc, desalted to remove any adventitiously bound metal, and quantified by ICP-OES. The results show that AztD begins to saturate at \( \sim 2.5 \) eq of zinc (Fig. 3B), suggesting that this protein may have as many as three high affinity zinc-binding sites.

**Metal Binding Affinities**—CD spectroscopy showed a predominantly \( \beta \)-sheet structure for AztD with remarkable thermal stability (Fig. 4), allowing for the generation of apo-AztD as described under “Experimental Procedures.” Apo-AztD was confirmed by ICP-OES to contain no more than 0.05 eq of zinc. The chelating fluorophore Mag-fura-2 (MF-2) forms well characterized 1:1 complexes with transition metals, and competition assays using this molecule have been extensively used to estimate protein metal binding affinities (18, 29, 33–37). Binding of zinc to MF-2 causes a shift in the fluorescence excitation peak from \( \sim 360 \) to \( \sim 330 \) nm (Fig. 5A), whereas manganese binding causes a quenching of fluorescence (Fig. 5C). Comparison of titrations of 0.5 \( \mu \)M MF-2 with zinc in the presence and absence of 15.0 \( \mu \)M apo-AztD (Fig. 5B) shows that the protein competes very effectively against MF-2 for zinc binding. Conversely, the presence of apo-AztD had no significant effect on
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FIGURE 3. A, SDS-polyacrylamide gel. Lanes 1 and 7, molecular weight ladder; lane 2, total cellular protein before IPTG induction; lane 3, total cellular protein after IPTG induction; lane 4, periplasmic fraction; lane 5, combined fractions containing AztD after anion exchange chromatography; lane 6, combined fraction containing AztD after size exclusion chromatography. B, zinc content of AztD after addition of up to 4 molar eq of ZnSO₄. Samples were desalted to remove adventitious metal prior to ICP-OES.

FIGURE 4. A, CD spectrum of AztD at 25 °C fitted with secondary structure composed of 52% β-sheet, 18% turn, 22% unordered, and 5% α-helix as described under ”Experimental Procedures.” B, ellipticity at 215 nm monitored as a function of temperature.

Metal Transfer to AztC—We next set out to determine whether AztD could transfer zinc directly to AztC, consistent with a role as a metallochaperone. For these experiments, AztD was used as isolated with 0.7 eq of bound zinc, which will be referred to as holo-AztD. AztC contains a single Trp residue and 10 Tyr residues, leading to an intrinsic fluorescence emission band centered at 315 nm upon excitation at 278 nm. The intensity of this peak increases more than 2-fold as apo-AztC is saturated with zinc (Fig. 6A). AztD, with 3 Trp and 13 Tyr residues, exhibits fluorescence emission around 345 nm, the intensity of which is essentially insensitive to zinc binding (Fig. 6B).

The dramatic difference in fluorescence behavior between these proteins was exploited to assess zinc transfer from holo-AztD to apo-AztC. Titration of 10 μM apo-AztC with holo-AztD leads to a significant increase in fluorescence around 315–325 nm, consistent with the formation of holo-AztC (Fig. 7A). After the addition of 12 μM holo-AztD, the further addition of 20 μM of ZnSO₄ had no effect on the spectrum, indicating that AztC was saturated with zinc. Saturation consistently occurs slightly earlier than anticipated, even assuming stoichiometric zinc transfer. The reason for this is currently unclear, although it may be due in part to slight errors on the calculated titration curve for MF-2 with manganese (Fig. 5D), indicating that if this protein binds manganese, it does so with much lower affinity than MF-2 (Kₐ = 0.97 μM).

Up to 10 μM of added zinc, there was virtually no change in MF-2 fluorescence, and greater than 30 μM of added zinc was required to saturate MF-2. This indicates the presence of at least two high affinity binding sites. In fact, the data are best fit to a three-site model (Fig. 5) consistent with the observation of nearly 3 eq of zinc in reconstituted AztD. The results of the fitted parameters from four independent experiments are given in Table 1. Binding of the first zinc has an apparent Kₐ value in the sub-nanomolar regime, and the second and third zinc ions bind with mid and high nanomolar affinities, respectively. It should be noted that the fitted value for protein concentration may be due to some protein damage incurred during the zinc removal process, resulting in a population of non-functional protein. In any case, the MF-2 assays demonstrate that AztD binds at least two zinc ions with very high affinity and completely excludes manganese binding.

### Table 1

| Parameter | Value ± S.D. (n = 4) |
|-----------|----------------------|
| Kₐ (nM)  | 0.7 ± 0.3            |
| Kₐ (nM)  | 54 ± 8               |
| Kₐ (nM)  | 340 ± 110            |
| Stoichiometry | 2.2 ± 0.1           |

The dramatic difference in fluorescence behavior between these proteins was exploited to assess zinc transfer from holo-AztD to apo-AztC. Titration of 10 μM apo-AztC with holo-AztD leads to a significant increase in fluorescence around 315–325 nm, consistent with the formation of holo-AztC (Fig. 7A). After the addition of 12 μM holo-AztD, the further addition of 20 μM of ZnSO₄ had no effect on the spectrum, indicating that AztC was saturated with zinc. Saturation consistently occurs slightly earlier than anticipated, even assuming stoichiometric zinc transfer. The reason for this is currently unclear, although it may be due in part to slight errors on the calculated
concentrations of AztD and/or AztC. In contrast, the titration of apo-AztC with apo-AztD showed very different results (Fig. 7B). In this case, only modest increases in fluorescence were observed consistent with the sum of apoprotein fluorescence as the concentration of apo-AztD was increased. Further zinc addition caused a dramatic increase in fluorescence consistent with the formation of holo-AztC. This demonstrates that the fluorescence changes observed upon titration of apo-AztC with holo-AztD are due to zinc transfer, rather than the burial of hydrophobic residues at a protein-protein interface.

Back transfer of zinc from holo-AztC to apo-AztD was assessed by titration of the former with the latter. In this case, zinc transfer would be expected to result in a loss in fluorescence intensity at 315 nm. With the first addition of apo-AztD, a slight decrease in fluorescence at this wavelength is observed (Fig. 7, C and D). However, based on previous AztC titrations, this change accounts for a loss of no more than 5% of the total zinc from holo-AztC. Throughout the remainder of the titration, only modest linear increases in fluorescence intensity were observed due to the increasing concentrations of AztD. Addition of 20 μM ZnSO4 after the titration had no effect on fluorescence intensity, indicating that AztC was essentially saturated with zinc throughout the experiment. We conclude that, although a small amount of metal transfer from holo-AztC to apo-AztD may be possible, it is highly unfavorable.

The above results indicate nearly stoichiometric directional metal transfer from AztD to AztC consistent with a direct associative mechanism. However, we wished to rule out the possibility that zinc dissociates from AztD and is subsequently acquired by AztC from solution. To that end, titrations of apo-AztC with ZnSO4 or holo-AztD were repeated in the presence of 1 mM EDTA. This concentration of EDTA is sufficient to prevent any zinc binding by AztC from solution (Fig. 7E). However, metal transfer is still clearly observed when holo-AztD is used as the zinc source (Fig. 7F). These results demonstrate that AztD is able to transfer zinc to AztC through a direct mechanism that prevents metal loss to the environment.

Size exclusion chromatography was employed to determine whether zinc is transferred through the formation of a stable protein complex. Extensive washing of the column with 50 mM EDTA and the use of Chelex-treated running buffer were necessary to prevent the incorporation of exogenous zinc into the proteins as they are passed through the column. Holo-AztD (41 kDa) and apo-AztC (30 kDa) both run exclusively as monomers. More importantly, ICP-OES of chromatographic fractions demonstrates that holo-AztD retains its native zinc, and apo-AztC does not acquire zinc after SEC (Fig. 8, A and B). The elution profile of apo-AztC incubated with stoichiometric holo-AztD is consistent with elution of the proteins as individual monomers (Fig. 8C), and absolutely no higher molecular weight species were observed. As such, the chromatogram fits well to two Gaussian peaks representing the partially overlapping elution profiles of AztC and AztD. This fitting allows for the application of the individual extinction coefficients for each protein and the estimation of their respective concentrations in each fraction (Fig. 8D). Plotting protein and zinc concentrations on the same graph clearly demonstrates that zinc is associated almost exclusively with AztC, which is confirmed by comparing SDS-PAGE and zinc quantitation of individual fractions (Fig. 8E). These results demonstrate virtually stoichiometric zinc transfer from AztD to AztC without the formation of a stable complex.

Discussion

The results described herein suggest a role in zinc homeostasis for the periplasmic protein AztD from *P. denitrificans*. Like AztC and other genes involved in zinc acquisition, its transcription is significantly enhanced under conditions of zinc starvation (Fig. 2A). Zinc-dependent transcriptional regulation in bacteria is largely undertaken by the transcriptional repressor Zur (zinc uptake regulator), which binds to inverted repeat sequences in the promoter regions of regulated genes (38). The
The apparent zinc chaperone activity of AztD, which was shown to be functional in vitro, 
suggests that it has the potential to serve as a zinc chaperone in vivo. This is 
consistent with the finding that AztD is able to transfer zinc from one protein 
to another in a defined reaction mixture. The mechanism of metal transfer by 
AztD is not yet fully understood, but it is clear that it is not a simple diffusion 
process. Rather, AztD appears to play a role in regulating the availability of 
zinc to other proteins.

The role of AztD as a zinc chaperone is supported by the observation that it 
interacts with AztC, which is the metal-binding protein in the system. The 
interaction between AztD and AztC is strong and specific, and it is not observed 
in the absence of zinc. This suggests that AztD is able to direct zinc to AztC, 
thereby facilitating its incorporation into the membrane protein.

The mechanisms by which AztD regulates the availability of zinc to AztC are 
not yet fully understood. However, it is clear that AztD is able to transfer 
zinc from one protein to another in a defined reaction mixture, and this 
ability is likely to be important in regulating the availability of zinc to the 
membrane protein.

The ability of AztD to transfer zinc from one protein to another is 
important for the efficient and directed transfer of zinc. This is supported by 
the observation that AztD is able to transfer zinc from one protein to another 
in a defined reaction mixture, and this ability is likely to be important in 
regulating the availability of zinc to the membrane protein.

The mechanism by which AztD regulates the availability of zinc to AztC is 
not yet fully understood. However, it is clear that AztD is able to transfer 
zinc from one protein to another in a defined reaction mixture, and this 
ability is likely to be important in regulating the availability of zinc to the 
membrane protein.
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