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Coordination of Zn^{2+} (and Cd^{2+}) by Prokaryotic Metallothionein

INVOLVEMENT OF HIS-IMIDAZOLE*

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In mammalian metallothionein Zn^{2+} is exclusively coordinated to Cys-thiolate to form clusters in which the metal is thermodynamically stable but also kinetically labile. By contrast, little is known about coordination to prokaryotic metallothionein, SmtA. 3 nmol of Zn^{2+} $nmol^{-1}$ SmtA were displaced by 8 nmol of *p*-(hydroxymercuri)phenylsulfonate implicating eight of the nine Cys in the coordination of three metal ions. None of the Zn^{2+} associated with SmtA was accessible to 4-(2-pyridylazo)resorcinol prior to the addition of *p*-(hydroxymercuri)phenylsulfonate. An unusual feature of SmtA is the presence of three His residues, and we have investigated whether these contribute to metal coordination. Less Zn^{2+} was associated with purified SmtA(H40R/H49R/H55R), in which all three His residues were substituted with Arg, and approximately one equivalent of Zn^{2+} was immediately accessible to 4-(2-pyridylazo)resorcinol. Following incubation of SmtA with ^{111}Cd, three ^{111}Cd resonances were detected, two in a range expected for CdS_4 and the third indicative of either $CdNS_3$ or CdN_2S_2 coordination. Two-dimensional TOCSY ^1H NMR and ^{111}Cd-edited ^1H NMR showed two His residues bound to ^{111}Cd, confirming CdN_2S_2 coordination. The pH of half-dissociation of Zn^{2+} increased from 4.05 for SmtA to 5.37 for SmtA(H40R/H49R/H55R). Equivalent values for single His mutants SmtA(H40R), SmtA(H49R), and SmtA(H55R) were 4.62, 4.48, and 3.81, respectively, revealing that conversion of His^{40} or His^{49} to Arg impairs Zn^{2+} binding at the CdN_2S_2 and CdS_4 sites. Only approximately two equivalents of Zn^{2+} were associated with purified SmtA(H49R). The appearance of a fourth ^{111}Cd resonance at lower pH suggests that an alternative CdN_2S_2 site also exists.

MTs[1] bind metals of the copper and zinc triads, typically in metal thiolate clusters (reviewed in Ref. 1). Indeed, the presence of such clusters is often cited as a defining characteristic of MTs. In mammalian MTI and MTII, seven Zn^{2+} (or Cd^{2+}) ions are tetrahedrally coordinated to thiolate ligands in two distinct Zn_3S_9 and Zn_4S_{11} clusters. In comparison, little is known about metal coordination by bacterial MTs. Electronic absorption spectra suggested the presence of (some) metal thiolate coordination in MT purified from *Synechococcus* sp. (2), whereas the ^{113}Cd NMR spectrum of a low M_r MT-like protein from Cd^{2+}-resistant *Pseudomonas putida* was suggestive of Cys-thiolate and His-imidazole coordination (3). The *smtA* gene from the cyanobacterium *Synechococcus* PCC 7942 encodes an MT that contains three His residues (4), a feature uncommon among eukaryotic MTs. The possibility that these His residues may be involved in metal coordination has never been investigated. An atypical (for an MT) coordination chemistry could be significant in view of the unusually high affinity of SmtA for Zn^{2+} (5) and the exclusive role of SmtA in the sequestration/metabolism of Zn^{2+} (and detoxification of Cd^{2+}) but not copper ions (6).

A comparison of the pH at which 50% of metal ions dissociate is a criterion that has been used to distinguish MTs from other metal binding proteins. The pH of half-dissociation of Zn^{2+} from equine renal MT has been estimated to be 4.50 (7, 8), whereas values of 4.10 and 4.50 for a GST-SmtA fusion protein and equine renal MT, respectively, were obtained in a comparative study (5). This implies that SmtA has a higher affinity than other MTs for Zn^{2+}, whereas metal displacement curves for Cd^{2+} and copper ions have indicated that SmtA has a lower affinity than equine renal MT for these ions (5). Consistent with these observations, expression from the *smtA* operator-promoter is maximally induced by Zn^{2+}, in comparison with other metals at maximum permissive concentrations (4). Furthermore, mutants deficient in SmtA are hypersensitive to Zn^{2+} (and to some extent Cd^{2+}) but have normal tolerance to copper ions (6). In contrast, yeast mutants deficient in the MT gene *CUP1* are hypersensitive to copper ions but not Zn^{2+} (9), whereas transgenic animals in which *MTI* and *MTII* genes are disrupted are hypersensitive to hepatic poisoning by Cd^{2+} (10).

Here we describe analyses of metal coordination by recombinant SmtA and mutants thereof to determine the number of metal ions bound and the number of Cys residues involved. Most importantly, the results of experiments that implicate His-imidazole groups in metal binding by SmtA are reported.

EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant Polypeptides—DNA restriction and modification enzymes were supplied by New England Biolabs Inc., *Taq* DNA polymerase was supplied by Life Technologies, Inc., and other reagents were purchased from Sigma Chemical Co. All generated plasmid constructs were checked by sequence analysis as described previously (4), and reaction products analyzed using an Applied Biosystems 370A DNA sequence analyzer.

Plasmid pJHNR49 (4) was used as template DNA in PCR reactions

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[1] The abbreviations used are: MT, metallothionein; GST, glutathione *S*-transferase; PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)phenylsulfonate; TOCSY, total correlation spectroscopy; PCR, polymerase chain reaction.

using standard conditions with primers P1 (5′-CGCGGATCCTCATGACCTCAACA-3′) and P2 (5′-GGAGTCAAGCTTGGAAACAGTTGATTA-3′) designed to anneal to the 5′ and 3′ ends of *smtA*, respectively. The PCR amplification product, containing *smtA*, was ligated to pGEM-T (Promega Corp.) prior to subcloning into the *Bam*HI/*Sma*I site of the glutathione *S*-transferase gene fusion vector pGEX-3X (Amersham Pharmacia Biotech) to create pMDNR1.1.

A mutant of *smtA* was generated in which all three His codons were converted to Arg codons. A three-stage PCR reaction was performed (to minimize mispriming) using plasmid pJHNR49 as template DNA with primers P1 and P3 (5′-CCGGAATTCTGATTAGCCGCGGCAGTTACAGCCGGTGCGGCCGCAGCCTTTGCTACCACCGGTGCGGCCATCGGC-3′, designed to convert codons 40, 49, and 55 from CAC to CGC): Stage 1, three cycles of denaturation (95 °C, 1 min) and annealing (50 °C, 1 min) were performed with a 50-μl reaction containing pJHNR49 (100 ng) and P3 (2 mM) in PCR buffer; stage 2, the reaction volume was increased to 90 μl with the addition of *Taq* DNA polymerase (5 units) and dNTPs (0.22 mM) in PCR buffer, and three cycles of denaturation (94 °C, 1.5 min), annealing (45 °C, 1.5 min), and extension (73 °C, 2 min) were performed; stage 3, the reaction volume was increased to 100 μl with the addition of P1 (1 mM) in PCR buffer and 12 further cycles of PCR performed (as for stage 2). The PCR amplification product containing mutated *smtA* was digested with *Bam*HI and *Eco*RI and cloned into the *Bam*HI/*Eco*RI site of pGEX-3X to create pMDNR1.2.

Codons 40, 49, and 55 of *smtA*, which encode His, were also converted individually (from CAC to CGC) to encode Arg, thereby creating three separate mutants. Site-directed mutagenesis was performed via Quikchange (Stratagene) according to the manufacturer's protocols, with pMDNR1.1 as template. The primers used were: 5′-ACCACCGGTGCGGCCATCGGC-3′ with 5′-GCCGATGGCCGCACCGGTGGT-3′ to convert codon 40, creating pMDNR1.3; 5′-ACAGCCGGTGCGGCCGCAGCC-3′ with 5′-GGCTGCGGCCGCACCGGCTGT-3′ to convert codon 49, creating pMDNR1.4; and 5′-CCACACCGGCTGTAACTGCCGCGGCTAATCAACTGTTTCCA-3′ with 5′-TGGAAACAGTTGATTAGCCGCGGCAGTTACAGCCGGTGTGG-3′ to convert codon 55, creating pMDNR1.5.

Recombinant fusion proteins were expressed in *Escherichia coli* (JM101) grown in the presence of 0.5 mM Zn^{2+} and purified as described previously (11). Recombinant proteins and three residues of GST (Gly, Ile, and Leu), were released from glutathione-Sepharose-bound GST by incubation with factor Xa (Amersham Pharmacia Biotech). Proteins were resolved on 15% SDS-polyacrylamide gels and visualized with Coomassie Brilliant Blue. An aliquot of SmtA was hydrolyzed and analyzed for amino acid composition (Alta Bioscience, University of Birmingham) to allow calibration of colorimetric estimations of SmtA. Purified SmtA was concentrated (to 0.9 mM) for NMR spectroscopy using Centriprep-3 concentrators (Amicon) according to the manufacturer's protocols.

Metal Binding Studies—Purified recombinant proteins (in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$) were incubated at room temperature for 1 h with 353.52 kBq ^{65}Zn before fractionation on Sephadex G-25 equilibrated to pH 7.0 (in 0.05 M KH_2PO_4, 0.029 M NaOH). Fractions (0.5 ml) were collected, and aliquots (0.1 ml) were analyzed for protein content and radioactivity. Protein-containing fractions were pooled, and aliquots (625 μl) were incubated at room temperature for 1 h with 1.875 ml of 0.05 M KH phthalate/HCl (pH 2–5) or 0.05 M KH_2PO_4/NaOH (pH 5–7), followed by fractionation on Sephadex G-25 equilibrated with the same buffers. Fractions (0.5 ml) were collected, and aliquots (0.1 ml) were again analyzed for protein content and radioactivity.

The amount of Zn^{2+} bound to 1 nmol of recombinant protein (in 0.1 M sodium phosphate buffer, pH 7.0) was determined by addition of 0.67 mM PAR and incubation for 5 min with increasing amounts (1 nmol increments) of PMPS. The metallochromic indicator PAR is known to generate colored chelate compounds with Zn^{2+} (12). Metal ion release was monitored by the increase in absorbance at 492 nm, and the values were calibrated by reacting known amounts of Zn^{2+} with PAR.

NMR Spectroscopy—Samples were prepared by adding 4 mol eq of ^{111}Cd (as a 50 mM solution, prepared by dissolving ^{111}CdO in a minimum amount of HCl and diluted with D_2O) dropwise to 0.9 mM SmtA (in 50 mM Tris-HCl, pH 8.4) and equilibrated for >12 h. Excess ^{111}Cd was removed by ultracentrifugation (Amicon) and washed four times with 50 mM Tris-d_{11} (pH 8.4) for ~2 h.

^{111}Cd and $^{1}H\{^{111}Cd\}$ NMR spectra were obtained at 298 K on a Bruker DMX 500 MHz NMR spectrometer operating at 106.04 and 500.13 MHz for ^{111}Cd and ^{1}H, respectively. Typically parameters for ^{111}Cd were: spectral width, 240 ppm, centered at 620 ppm (relative to $Cd(ClO_4)_2$), with 16, 384 data points; pulse width, 8 μs (60 ° pulse); pulse delay, 0.6 s. Gated CPD ^{1}H decoupling (during acquisition time) was used to avoid negative NOEs. The total acquisition time for each spectrum was 6–8 h. An exponential function (equivalent to a line broadening of ~10–20 Hz) was used prior to Fourier transformation. Shifts are referenced to external 0.1 M $Cd(ClO_4)_2$ (0 ppm). ^{111}Cd-edited ^{1}H NMR spectra (4,096 data points) were obtained by using the first increment of a two-dimensional [^{1}H, ^{111}Cd] heteronuclear multiple quantum coherence experiment (13), and the coherence transfer was selected by pulsed field gradients (14). Data were acquired with different mixing times (1/2J[^{111}Cd, ^{1}H]) because of markedly different vicinal coupling constants ($^{3}J[^{111}Cd, ^{1}H]$) (15). ^{111}Cd GARP decoupling (centered at 620 ppm) (16) was carried out during the acquisition time to remove ^{111}Cd coupling. The ^{1}H spectral width was 8 ppm with the H_2O resonance in the center of the spectrum. Unshifted Gaussian functions were used for processing. Two-dimensional TOCSY spectra were acquired using a spin-lock time (MLEV-17) of 60 ms with 2,048 data points in the second frequency domain and 256 increments in the first frequency domain. Data were zero-filled to 2,048 × 1,024 data points. Typically 32 transients were acquired for each increment.

RESULTS

Production and Purification of Recombinant Polypeptides—Extracts from *E. coli* cells containing plasmids pMDNR1.1 and pMDNR1.2 were fractionated on glutathione-Sepharose 4B, and proteins of ~35 kDa, corresponding to the predicted size of GST-SmtA, were detected in fractions eluted with buffer containing 5 mM glutathione (data not shown). It was noted that GST-SmtA(H40R/H49R/H55R) migrated slightly faster than GST-SmtA, which does not correlate with a difference in molecular mass. Following overnight incubation of recombinant proteins immobilized on glutathione-Sepharose 4B with factor Xa, smaller proteins of ~6.5 kDa (corresponding to the predicted size of SmtA) were released in factor Xa cleavage buffer (devoid of glutathione).

The pH Stability of Zn^{2+} Binding to SmtA Is Reduced in a His Mutant—In vitro incubation of either SmtA or SmtA(H40R/H49R/H55R) for 1 h with ^{65}Zn gave rise to radioactivity and protein that co-chromatographed on Sephadex G-25 (Fig. 1, *A* and *B*). At pH 4.28 ^{65}Zn remained associated with SmtA but dissociated from SmtA(H40R/H49R/H55R) (Fig. 1, *C* and *D*). Fig. 1*E* shows the proportion of ^{65}Zn associated with either protein as a function of pH. The mean estimated pH values (and standard deviations) of half-dissociation of Zn^{2+} from SmtA and SmtA(H40R/H49R/H55R) from three independent experiments were 4.05 ± 0.09 and 5.37 ± 0.04, respectively. The former value is in good agreement with that reported previously (5), whereas the latter implies that substitution of His residues with Arg reduces the affinity for Zn^{2+}.

SmtA Binds 3 mol of Zn^{2+} mol^{-1} via Eight Cys Residues—Upon Cys modification of 1 nmol of SmtA in the presence of PAR, an increase in A_{492} (ΔA_{492}) was observed up to the addition of 8 nmol of PMPS (Fig. 2*A*). In each of three experiments using independent preparations of recombinant SmtA, no further increase in A_{492} was detected upon addition of more than 8 nmol of PMPS (Fig. 2*A*). This implies that only eight of the nine Cys residues are involved in Zn^{2+} coordination and that the remaining Cys residue is the least accessible to PMPS. Use of calibration curves for Zn^{2+} reacting with PAR gave estimates of 3.06 ± 0.37 nmol of Zn^{2+} released $nmol^{-1}$ of SmtA following the addition of saturating amounts of PMPS. This implies that each molecule of SmtA coordinates three Zn^{2+} ions.

One mol of Zn^{2+} mol^{-1} Protein Is Lost, and a Second Is Accessible to PAR, in a Triple His Mutant of SmtA—When SmtA(H40R/H49R/H55R) was incubated with PAR, an immediate color change (prior to the addition of PMPS) was observed. Thus, unlike Zn^{2+} associated with SmtA, a proportion of the Zn^{2+} associated with SmtA(H40R/H49R/H55R) is immediately accessible to PAR with no requirement for Cys modification. It is known that PAR, a metal chelator, can remove

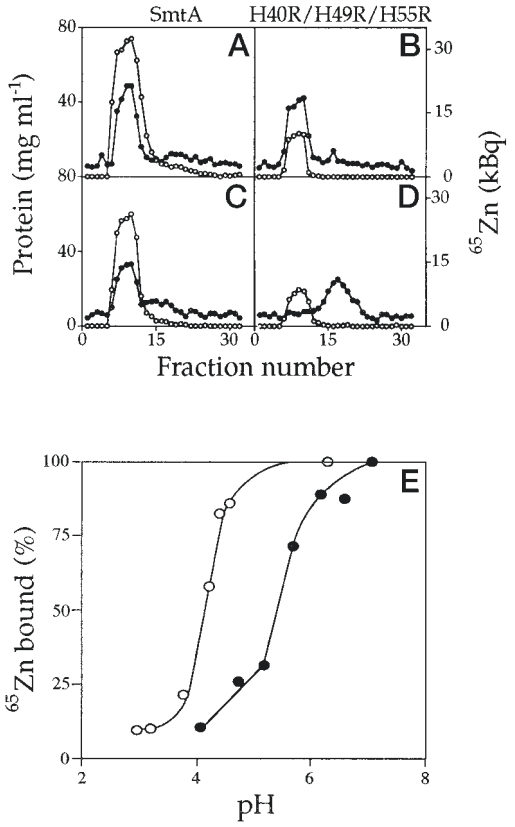

FIG. 1. **Hydrogen ion competition for metal binding to SmtA and its triple His mutant.** Purified SmtA or SmtA(H40R/H49R/H55R) were incubated for 1 h with ^{65}Zn at pH 7.0 and fractionated on Sephadex G-25 pre-equilibrated to pH 7.0 (*A* and *B*). Fractions were analyzed for protein (*open circles*) and ^{65}Zn (*closed circles*). Aliquots of pooled protein-containing fractions were then incubated for 1 h at pH 4.28 and fractionated on Sephadex G-25 pre-equilibrated to pH 4.28 (*C* and *D*). *E*, aliquots of ^{65}Zn-associated proteins were incubated for 1 h at the indicated pH. Free and bound ^{65}Zn were resolved by fractionation on Sephadex G-25 equilibrated with the same buffer. The proportion of ^{65}Zn bound to SmtA (*open circles*) and SmtA(H40R/H49R/H55R) (*closed circles*) at each pH is shown.

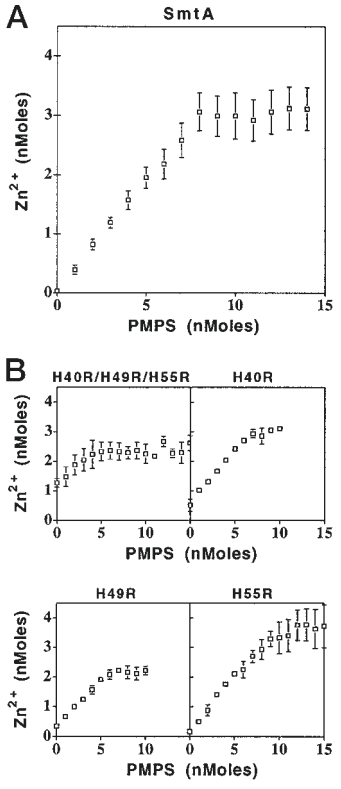

FIG. 2. **Release of metal ions from SmtA and His mutants of SmtA by titration with PMPS.** 1-nmol aliquots of protein were titrated with PMPS, and metal ion release was detected via the increase in absorbance at 492 nm following reaction with PAR. The amount of Zn^{2+} displaced from three independent preparations of SmtA (*A*) and triple or single His mutants of SmtA (*B*) is shown.

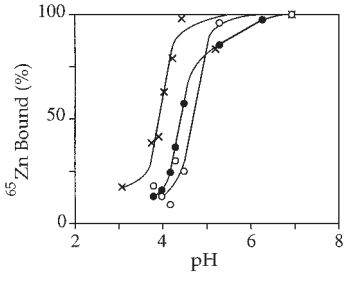

FIG. 3. **Hydrogen ion competition for metal binding to His mutants of SmtA.** ^{65}Zn-associated proteins were incubated for 1 h at the indicated pH, and free and bound ^{65}Zn was subsequently resolved by fractionation on Sephadex G-25 equilibrated with the same buffer. The proportion of ^{65}Zn bound to SmtA(H40R) (*open circles*), SmtA(H49R) (*closed circles*), and SmtA(H55R) (*crosses*) at each pH is shown.

Zn^{2+} from some proteins (17). The amount of Zn^{2+} removed from the mutant protein by PAR was estimated to be 1.22 ± 0.21 nmol Zn^{2+} $nmol^{-1}$ protein, a value close to one (Fig. 2*B*). These data suggest that the coordination of (at least) one Zn^{2+} ion is sufficiently weakened by the substitution of all three His residues by Arg such that PAR can now directly compete for this metal ion. The amount of metal subsequently displaced by PMPS was variable, possibly reflecting some variable loss of metal during purification, but the total stoichiometry is less than three and does not significantly deviate from 2 nmol Zn^{2+} $nmol^{-1}$ protein.

His^{40} and His^{49} but Not His^{55} Mutants Have a Reduced Apparent Affinity for Zn^{2+}—The pH lability (Fig. 1) and accessibility to PAR (Fig. 2) of Zn^{2+} associated with SmtA(H40R/H49R/H55R) implicates His residues in metal coordination. Mutants were therefore generated in which each His residue was individually substituted by Arg to define which of the three residues contribute(s) toward metal binding. Following overnight incubation with factor Xa, proteins of ~6.5 kDa (corresponding to the predicted size of SmtA) were released from glutathione-Sepharose 4B-immobilized extracts from *E. coli* containing plasmid pMDNR1.3, pMDNR1.4, or pMDNR1.5. Purified SmtA(H40R), SmtA(H49R), or SmtA(H55R) was incubated with ^{65}Zn, fractionated on Sephadex G-25, and then incubated and re-chromatographed in buffers of differing pH. Fig. 3 shows the pH displacement curves for each protein. The mean estimated pH values of half-dissociation of Zn^{2+} from SmtA(H40R), SmtA(H49R), and SmtA(H55R) from three independent experiments were 4.62 ± 0.22, 4.48 ± 0.2, and 3.81 ± 0.2, respectively. Replacement of His^{40} and His^{49} by Arg reduces the apparent affinity of SmtA for Zn^{2+}, whereas replacement of His^{55} confers a slight increase in stability of metal binding at reduced pH.

The amount of metal displaced by PMPS did not significantly deviate from 3 nmol of Zn^{2+} $nmol^{-1}$ protein for either SmtA(H40R) or SmtA(H55R), whereas the stoichiometry was close to 2 nmol of Zn^{2+} $nmol^{-1}$ SmtA(H49R) (Fig. 2*B*). An increase in A_{492} was observed with the addition of up to 6 nmol of PMPS $nmol^{-1}$ of SmtA(H49R). In each of three experiments using independent preparations of recombinant SmtA(H49R), no further increase in A_{492} was detected upon the addition of more than 6 nmol of PMPS. This implies that only six Cys residues coordinate Zn^{2+} in SmtA(H49R) and that the other

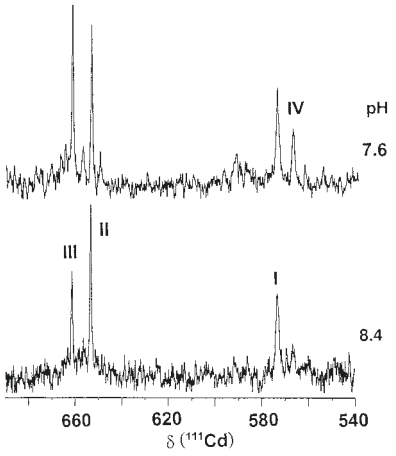

FIG. 4. **^{1}H {^{111}Cd} NMR analysis of SmtA.** Two peaks, II and III (654 and 661 ppm), are in the chemical shift range expected for CdS_4 coordination, and peak I (572 ppm) is in the range for $CdNS_3$ or CdN_2S_2 coordination. At pH 7.6 an additional peak (IV) is apparent at 567 ppm.

three Cys are less accessible to PMPS.

^{111}Cd and ^{1}H{^{111}Cd} NMR Spectra Show Cys-thiolate and His-imidazole Metal Coordination—The pH stability and accessibility to PAR of Zn^{2+} associated with His mutants of SmtA suggest that His-imidazole is involved in metal binding in addition to Cys-thiolate. To investigate the types of residues (especially His) involved in binding, ^{111}Cd was used to displace Zn^{2+} from the protein, and both ^{111}Cd (Fig. 4) and ^{111}Cd-edited ^{1}H (Fig. 5) NMR were used to probe the binding sites. Fig. 4 shows the ^{1}H{ ^{111}Cd } NMR spectrum of a solution of SmtA (0.9 mM in 50 mM Tris-d_{11}, 90% v/v H_2O) with resonances in the range expected for ^{111}Cd bound, in full or in part, by thiolate ligands (18, 19). Three major resonances were observed at pH 8.4, two with chemical shifts of 654 and 661 ppm and a third displaced at 572 ppm. At pH 7.6 a fourth resonance appeared at 567 ppm. No ^{111}Cd resonances were observed from 0 to 500 ppm. Fig. 5 shows ^{1}H{^{111}Cd} NMR spectra recorded with different mixing times (0.5/^{3}J[^{111}Cd, ^{1}H]). At very short mixing times, several resonances were observed around 2.7–3.0 ppm. This region is typical of the β proton resonances of Cys residues. Four apparent singlets at 6.65, 7.31, 7.78, and 7.98 ppm appeared in the aromatic region of the ^{1}H{^{111}Cd} one-dimensional heteronuclear multiple quantum coherence NMR spectrum. These peaks decreased in intensity with decreasing mixing times. A two-dimensional TOCSY ^{1}H NMR spectrum (with ^{111}Cd decoupling; mixing time, 65 ms) showed cross-peaks between the peaks at 6.65 and 7.78 ppm, 7.31 and 7.98 ppm, and 7.28 and 8.17 ppm (Fig. 5*A*), assignable to His CδH/CϵH connectivities. It is evident from Fig. 5*B* that only the two His residues giving rise to the former two sets of cross-peaks and not the latter are coordinated to Cd^{2+}. The ^{1}H NMR spectra of Cd^{2+}-SmtA and Zn^{2+}-SmtA (data not shown) show very similar well dispersed resonances in the aromatic and NH region (6 to 11 ppm) and low frequency-shifted methyl resonances in the aliphatic region (~0.5 ppm), features that are typical of folded proteins. This suggests that Cd^{2+}-SmtA and Zn^{2+}-SmtA proteins have similar structures.

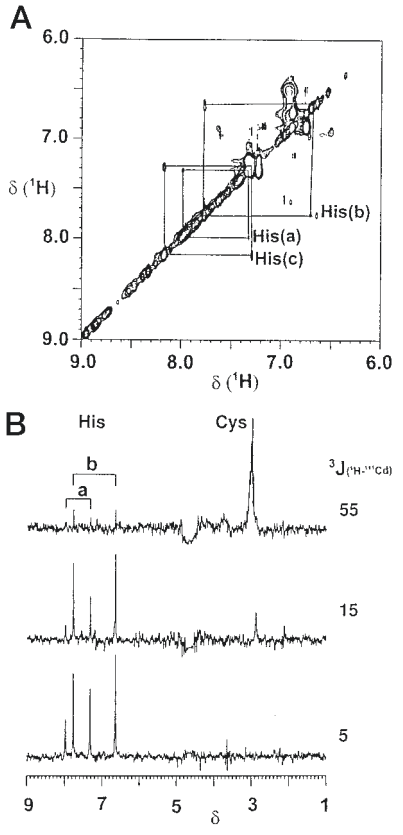

FIG. 5. **^{1}H NMR spectra of SmtA.** *A*, two-dimensional TOCSY ^{1}H NMR spectrum of SmtA showing connectives (CδH/CϵH) assignable to three His residues (*a*, *b*, and *c*). Residues *a* and *b* exhibit ^{111}Cd coupling. *B*, ^{111}Cd-edited ^{1}H NMR analysis of SmtA (50 mM Tris-d_{11} pH 8.4, 298 K) at various values of τ(=0.5/J). Peak intensities vary because of the large range of ^{111}Cd-^{1}H coupling constants. The spectra show that two of the three His residues (*a* and *b*, showing cross-peaks in *A*) and several Cys residues (β-CH_2 resonances near 3 ppm overlapping together) are bound to ^{111}Cd.

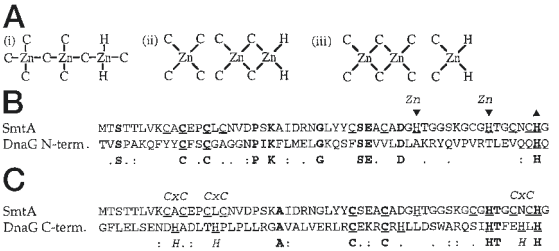

FIG. 6. **Zn^{2+} coordination by SmtA and similarities to Zn^{2+} binding domains of the product of the adjacent gene, *dnaG*.** *A*, three possible configurations for Zn^{2+} binding by eight Cys and two His residues of SmtA. *B*, alignment (generated using Clustal W) of SmtA with residues located toward the amino-terminal end of *Synechococcus* PCC 7942 DnaG that are implicated in Zn^{2+} binding. Identical and similar amino acids are indicated (below). Conversion of His^{40} or His^{49} to Arg reduced the apparent affinity of SmtA for Zn^{2+} (*downward arrowhead*), whereas conversion of His^{55} to Arg slightly increased the apparent affinity of SmtA for Zn^{2+} (*upward arrowhead*). *C*, alignment of SmtA with the Cys_2/His_2 type zinc-finger motif at the carboxyl terminus of *Synechococcus* PCC 7942 DnaG. Cys-Xaa-Cys motifs of SmtA (in *italics* above the sequence) align with His residues of DnaG (in *italics* below the sequence).

DISCUSSION

Our results show that SmtA coordinates to three Zn^{2+} ions via eight Cys residues. Analyses of metal binding to mutants of SmtA in which His residues were substituted by Arg, suggest that at least two His residues (specifically His^{40} and His^{49}) are also involved in the coordination of metal ions. ^{111}Cd and ^{111}Cd-edited ^{1}H NMR spectra confirmed that SmtA has at least three distinct metal binding sites. Two metal sites contain exclusively Cys-thiolate ligands, whereas the third contains both Cys-thiolate and His-imidazole ligands. This novel (for an MT) coordination chemistry is of significance in view of (i) the unusually high apparent affinity of SmtA for Zn^{2+} and (ii) observations that SmtA is exquisitely adapted to roles in the intracellular handling of Zn^{2+} rather than other metal ions.

^{111}Cd NMR has been used previously to probe metal coordination by eukaryotic MTs (19). The ^{111}Cd (or ^{113}Cd) NMR shifts of the CdS_4 clusters of eukaryotic MTs usually lie within the range of 600–700 ppm (18–20), whereas the shifts of isolated CdS_4 centers are usually at the high frequency end of this range (680–750 ppm) (21, 22). The ^{111}Cd NMR spectrum of ^{111}Cd-SmtA contains two high frequency peaks at 654 and 661 ppm, which are both in the chemical shift region normally associated with the CdS_4 clusters of eukaryotic MT. These two ^{111}Cd NMR peaks for Cd^{2+}-SmtA are relatively sharp. Unlike the spectra of eukaryotic MT, there is no evidence for Cd^{2+}-Cd^{2+} coupling (~29–48 Hz for MT) (19) that could suggest that clusters are not present in Cd^{2+}-SmtA. Alternatively the couplings may be small or the bridging ligands may be involved in fluxional processes, for example involving a dynamic equilibrium between structures *(ii)* and *(iii)* in Fig. 6*A*.

The third peak in the ^{111}Cd NMR spectrum lies at 572 ppm, outside the normal range for CdS_4 centers. Shifts of 630–660 ppm have been documented for $CdNS_3$ centers composed of Cys and a single His residue, and even lower shifts in the region of 400 ppm observed for centers that include two nitrogen atoms (19, 23, 24). The third peak for Cd^{2+}-SmtA is therefore suggestive of either $CdNS_3$ or CdN_2S_2 coordination. Two-dimensional TOCSY ^{1}H NMR and ^{111}Cd-edited ^{1}H NMR data reveal that two His residues are bound to ^{111}Cd, and therefore CdN_2S_2 coordination is inferred for the third site. In the only previous report of ^{113}Cd NMR of a prokaryotic MT-like protein isolated from *P. putida*, Higham *et al.* (3) reported ^{113}Cd NMR shifts of 615, 604, 483, and 476 ppm. These represent two groups of peaks separated by ~100 ppm, somewhat analogous to the present work.

The total number of Cys (nine) and His (three) residues in SmtA is sufficient for tetrahedral coordination of Zn^{2+} in three independent sites. However, the requirement for only eight equivalents of PMPS to displace all Zn^{2+} (Fig. 2) indicates that one Cys was not involved in metal binding. Furthermore, both the pH stability of Zn^{2+}-SmtA(H55R) (Fig. 3) and the ^{111}Cd-edited ^{1}H NMR spectrum (Fig. 5) imply that His^{55} does not stabilize metal binding. Thus, only configurations for metal coordination that involve eight Cys and two His residues are shown (Fig. 6*A*).

The reduced stoichiometry of SmtA(H49R) (Fig. 2*B*) suggests that the His coordinated ion is lost from this protein and hence that His^{49} is obligatory for the CdN_2S_2 site, whereas His^{40} (or His^{55}) is optional. Only six equivalents of PMPS are required to displace the Zn^{2+}, which remains associated with SmtA(H49R) supporting models *(ii)* and *(iii)* (Fig. 6*A*). Although only one metal ion is coordinated to His residues at pH 8.4 (Fig. 4), all metal ions are more readily displaced at low pH upon substitution of all three His with Arg (Fig. 1). Furthermore, the two Zn^{2+} that remain associated with SmtA(H49R) are also more readily displaced at low pH (Fig. 3), showing interaction between binding at the CdN_2S_2 and CdS_4 sites and/or showing that residual metal coordination is abnormal in the mutant proteins because of abnormal folding. NMR analyses of the mutant proteins would be required to resolve this.

Future investigations of metal coordination by SmtA should not only consider the basis of its unusual specificity in the detoxification of Zn^{2+} but also any specificity in the release of Zn^{2+} to apo-proteins. The nature of Zn^{2+} coordination by mammalian MT not only creates high affinity binding sites but also ones that are highly labile allowing rapid metal exchange (1). There is evidence to support a role for mammalian MT in the donation of Zn^{2+} to Zn^{2+}-binding proteins (25–30). It has been hypothesized that SmtA may donate Zn^{2+} to DNA primase (DnaG), which in *Synechococcus* PCC 7942 contains two Zn^{2+} binding sites and is encoded by a gene that is located next to the *smtA* gene (31). Similarities between metal binding domains provided a clue to the existence of interaction between a copper chaperone and its receptor (32). Alignments between SmtA and the metal binding domains of DnaG do show similarities in the spacing of some His and Cys residues (Fig. 6, *B* and *C*). Both alignments include His^{55}. It is formally possible that His^{55} and the ninth Cys contribute to transient metal coordination states that are favored during metal transfer.

The ^{111}Cd-SmtA NMR peak at 572 ppm (Fig. 4) is the most broad resonance, which is possibly a reflection of the kinetic lability of the CdN_2S_2 site. The pH dependence of the ^{111}Cd NMR spectrum of Cd $^{2+}$-SmtA was investigated only over a limited range in view of the potential instability of the protein and limited availability. However, at pH 7.6 an additional peak appeared at 567 ppm. Because no additional ^{111}Cd was added to the protein when the pH was lowered, this implies a redistribution of Cd^{2+} between available sites. This is consistent with flexible coordination modes involving a variable complement of the nine available Cys and three His ligands. Selective protonation of certain residues may encourage the redistribution of metal ions to other centers. We are intrigued by the possibility that such a process could "drive" metal release *in vivo*.

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