THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 43, Issue of October 25, pp. 40930–40936, 2002
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

The Soft Metal Ion Binding Sites in the *Staphylococcus aureus* pI258 CadC Cd(II)/Pb(II)/Zn(II)-responsive Repressor Are Formed between Subunits of the Homodimer*

Received for publication, July 1, 2002
Published, JBC Papers in Press, August 9, 2002, DOI 10.1074/jbc.M206536200

Marco D. Wong, Yung-Feng Lin, and Barry P. Rosen‡

From the Department of Biochemistry and Molecular Biology, School of Medicine, Wayne State University, Detroit, Michigan 48201

The *Staphylococcus aureus* plasmid pI258 CadC is a homodimeric repressor that binds Cd(II), Pb(II), and Zn(II) and regulates expression of the *cadAC* operon. CadC binds two Cd(II) ions per dimer, with a tetrathiolate binding site composed of residues Cys^7, Cys^{11}, Cys^{58}, and Cys^{60}. It is not known whether each site consists of residues from a single monomer or from residues contributed by both subunits. To examine whether Cys^7 and Cys^{11} are spatially proximate to Cys^{58} and Cys^{60} of the same subunit or of the other subunit, homodimers with the same cysteine mutation in each subunit and heterodimers containing different cysteine mutations in the two subunits were reacted with 4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione, which cross-links thiol groups that are within 3–6 Å of each other. Cys^7 or Cys^{11} cross-linked only with Cys^{58} or Cys^{60} on the other subunit. The data demonstrate that Cys^7 and Cys^{11} from one monomer are within 3–6 Å of either Cys^{58} or Cys^{60} in the other monomer. The results of this study strongly indicate that each of the two Cd(II) binding sites in the CadC homodimer is composed of Cys^7 and Cys^{11} from one monomer and Cys^{58} and Cys^{60} from the other monomer.

The *cadCA* operon from *Staphylococcus aureus* plasmid pI258 confers resistance to the cations of the soft Lewis acids Cd(II), Pb(II), and Zn(II) (1). This operon encodes CadC (2), a 27-kDa trans-acting, homodimeric repressor that negatively regulates expression of CadA, a P-type Cd(II)/(Pb(II)/Zn(II)-translocating ATPase (3, 4). CadC has been shown to bind two soft metal ions per dimer in a site composed of cysteine residues (5, 6). However, it is unknown how these two metal binding sites are organized within the dimer.

CadC is a member of the ArsR family of metalloregulatory proteins (7). This family includes members such as the As(III)/Sb(III)-responsive ArsR repressor of the *ars* operon of *Escherichia coli* plasmid R773 (8), the Zn(II)-responsive repressors SmtB from the cyanobacterium *Synechococcus* PCC7942 (9), and ZiaR from *Synechocystis* PCC6803 (10). These proteins share the conserved sequence EL**C**V(**C**/G)D, where the cysteine residues are believed to be essential in metal sensing in ArsR, ZiaR, and CadC (Fig. 1). CadC, ZiaR, and SmtB each have an additional 25–40 amino acids at their N terminus with additional cysteine residues that may play a role in conferring metal ion specificity. Evidence that CadC residues Cys^7, Cys^{58}, and Cys^{60} are required *in vivo* for metal binding is derived from two-plasmid green fluorescent protein (GFP)[1] reporter assays and *in vitro* restriction enzyme protection assays (6). Spectroscopic studies indicated that four cysteine thiolates are involved in Cd(II) binding as a tetrathiolate complex formed by four cysteine residues with a Cd(II)-S distance of 2.5 Å (5). In such a structure the four sulfur atoms can be predicted to be ~4.5 Å from each other. Neither Cys^{11} nor Cys^{52} is conserved in CadC repressors, and neither is required for activity either *in vivo* or *in vitro* (6), so the fourth cysteine residue in the tetrathiolate complex has not been identified with certainty. However, modeling CadC on the structure of the SmtB aporepressor (11) suggests that Cys^{52} is 15–18 Å from Cys^{58} and Cys^{60}. Moreover, assuming the validity of the model, Cys^{52} would be predicted to be buried, and its thiolate would not be solvent-accessible. These considerations imply that the fourth cysteine residue is Cys^{11}.

CadC is a homodimer with two metal binding sites. By construction of heterodimers with one wild-type and one mutant subunit, we have shown that both metal binding sites are required for derepression *in vivo* and release from the operator DNA *in vitro* (12). These sites could be composed of Cys^7, Cys^{11}, Cys^{58}, and Cys^{60} from the same monomer (intrasubunit model) (Fig. 2*A*). On the other hand, in each site Cys^7 and Cys^{11} could be contributed by one monomer, and Cys^{58} and Cys^{60} from the other monomer (intersubunit model) (Fig. 2*B*). Intersubunit sites have been proposed (13), but no supporting data have been presented. The equivalent residues in the N terminus of SmtB are not visible in the crystal structure, so the location of CadC residues Cys^7 and Cys^{11} cannot be predicted.

To distinguish between these two possibilities, we constructed a series of homodimeric single, double, triple, and quadruple cysteine mutants of CadC and examined the ability of (4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione (dibromobimane) to form intersubunit cross-links. Dibromobimane is a fluorogenic, homobifunctional thiol-specific cross-linking reagent that becomes highly fluorescent when both of its alkylating groups react with cysteine residues that are within 3–6 Å of each other (14). Thus, dibromobimane can be used as a molecular ruler to identify cysteine residues that are in close proximity in a metal

* This work was supported by United States Public Health Service Grant AI45428. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, School of Medicine, Wayne State University, 540 E. Canfield Ave., Detroit, MI 48201. Tel.: 313-577-1512; Fax: 313-577-2765; E-mail: brosen@med.wayne.edu.

[1] The abbreviations used are: GFP, green fluorescent protein; dibromobimane, 4,6-bis(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org by guest, on February 18, 2010

```
                    7   11
                MKKKDT EIF YDEEKVNRIQGDLQTVDIS-GVSQILKAIADENRAKITYALCQDEECadC pI258
MTKPVL------QDGETVV QG--THAAIASELQ--AIAPEVAQSLAEFFAVLADPNRLRLLSLL-ARSESmtB Synechococcus PCC 7942
MSKSSLSKSQSCQNEEMPL DQPLVHLEQVRQVQPEVMSLDQAQQMAEFFSALADPSRLRLMSAL-ARQEZiaR Synechocystis PCC 6803
                                       MLQLTPLQLFKNLSDETRLGIVLLLREMGEArsR R773

58 60/61                                 103
L V   IANILGVTIANASHHLRTLYKQGVVNFRKEGKLALYSLGDE IRQIMMIALAHKKEVKVNVCadC pI258
L VG  LAQAIGVSESAVSHQLRSLRNLRLVSYRKQGRHVYYQLQDH IVALYQNHLDHLQECRSmtB Synechococcus PCC 7942
L V   LAAAMKVSESAVSHQLRILRSQRLVKYRRVGRNVYYSLADN VMNLYREVADHLQESDZiaR Synechococcus PCC 6803
L V   LCMALDQSQPKISRHLAMLRESGILLDRKQGKWVHYRLSP- IPSWAAQIIEQAWLSQQDDVQVIARKLASVNCSGSSKAVCIArsR R773
```

FIG. 1. **Alignment of CadC, SmtB, ZiaR, and ArsR.** Multiple alignments of pI258 CadC (GenBank™ accession number B32561), SmtB from *Synechococcus* sp. strain PCC 7942 (accession number BAA10706), ZiaR from *Synechocystis* sp. strain PCC 6803 (accession number Q55940), and ArsR from plasmid R773 (accession number CAA34168) were calculated with ClustalW (33). The positions of CadC residues of interest and the corresponding residues in homologues are *shaded*. The *numbers* above the residues refer to the sequence of CadC.

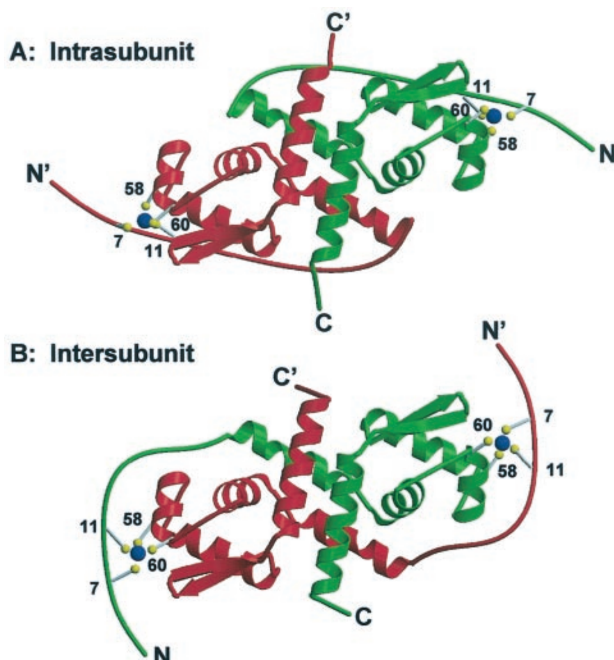

FIG. 2. **Intra- and intersubunit models for the formation of soft metal binding sites in the CadC homodimer.** Two possible models for CadC structure were generated from the crystal structure of the SmtB aporepressor (11) using MODELLER (34). Because the N-terminal regions of SmtB were not observed in the crystal structure, the homologous sequence of CadC was added with the only constraint that Cys^7 and Cys^{11} must be ~4.5 Å from Cys^{58} and Cys^{60} on either the same (*A*) or opposite (*B*) subunits. *A*, intrasubunit model. The N termini of the two CadC monomers were manually adjusted to bring Cys^7 and Cys^{11} into position relative to Cys^{58} and Cys^{60} on the same subunit to form a tetrathiolate Cd(II) binding site. *B*, intersubunit model. A tetrathiolate Cd(II) binding site formed by Cys^7 and Cys^{11} of one subunit and Cys^{58} and Cys^{60} of the other subunit was modeled by manual adjustment of the N termini of the two monomers. Strands and helices are drawn as *ribbons*. Cd(II) is shown as a *sphere* between the four sulfur atoms of Cys^7, Cys^{11}, Cys^{58}, and Cys^{60}, which are shown in *ball-and-stick form*. Images were generated with MOLSCRIPT (35) and RASTER3D (36).

site (15). Although the wild-type CadC forms fluorescent dimers, a quadruple mutant lacking Cys^7, Cys^{11}, Cys^{58} and Cys^{60} did not. All single mutants formed fluorescent dimers. Double mutants lacking either Cys^7 and Cys^{11} or Cys^{58} and Cys^{60} did not form dimers. To demonstrate unambiguously that Cys^7 or Cys^{11} interacts intermolecularly with Cys^{58} or Cys^{60}, heterodimers were constructed with two mutant subunits such that each monomer of the dimer had only a single cysteine residue. Dimers were formed by reaction with dibromobimane only when one subunit contained either Cys^7 or Cys^{11} and the other contained only Cys^{58} or Cys^{60}. This definitely demonstrates that Cys^7 and Cys^{11} in one subunit are ~4.5 Å of Cys^{58} and Cys^{60} in the other subunit. The data are consistent only with an intersubunit model of a tetrathiolate metal binding site composed of Cys^7 and Cys^{11} from one subunit and Cys^{58} and Cys^{60} from the other (Fig. 2*B*).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Reagents—For most experiments cultures of *E. coli* strain JM109(DE3) bearing the indicated plasmids were grown at 37 °C in LB medium (16). Kanamycin (40 μg/ml), chloramphenicol (40–80 μg/ml), and ampicillin (125 μg/ml) were added as required. For *in vivo* assay of the ability of mutant *cadC* genes to control *gfp* expression, cultures of *E. coli* strain BL21(DE3) *zntA*::*km* bearing the indicated plasmids were grown in a basal salts medium (17). Dibromobimane was purchased from Molecular Probes, Inc. All other chemicals were obtained from commercial sources.

Construction of CadC Mutants—The pMW1 series plasmids were constructed in pET28a (K_m^r) (Novagen) (6) by site-directed mutagenesis using either the Altered Sites™ *in vitro* mutagenesis system (Promega) or a QuikChange™ site-directed mutagenesis kit (Stratagene). The pMW1 series includes mutants C7G, C11G, Y12W, C58S, C60G, D61A, H103A, C58S/C60S, C7G/C11S, and Y12W/C7A/C58S/C60S. Note that a Y12W derivative was constructed to introduce a tryptophan residue for future use as an intrinsic spectroscopic probe into CadC and does not affect CadC function.[2] For the purposes of this study, it is used interchangeably with Tyr^{12}-containing CadCs. The pYSC2 series (12) was constructed by similar methodology in pET28b (K_m^r) (Novagen), which includes six histidine codons at the 3′-OH end. The pYSC2 series includes triple mutants C7A/C11S/C58A and C7A/C11S/C60A and the quadruple mutant C7A/C11S/C58S/C60S. The pYSCM series plasmids (12) were constructed in pACYC184 (18) (Cm^r) using similar methodology and include mutants C7A/C58S/C60S and C11S/C58S/C60S. All CadC mutants were sequenced with a Beckman Coulter CEQ 2000XL DNA Analysis System to ensure that additional mutations were not introduced. To produce CadC heterodimers containing one wild-type and one mutant subunit, the genes for both subunits were coexpressed in the same cells of *E. coli* JM109(DE3) that had been cotransformed with a pYSC2 series plasmid and a pYSCM series plasmid, which are compatible with each other (12).

Measurement of Regulation in Vivo—The gene for red-shifted GFP (19) was used as a reporter for monitoring the regulatory properties of the *cadC* gene product, as described previously (6). Briefly, cells contained two plasmids: pYSG1 (Ap^r) had *gfp* controlled by the *cad* operator/promoter, and pYSCM series plasmids carried *cadC* genes under control of the T7 promoter. Expression from the *cad* promoter was quantified from the fluorescence of red-shifted GFP with an emission wavelength of 507 nm and excitation wavelength of 470 nm in an SLM-Aminco Series 2 spectrofluorometer. The fluorescence intensity of GFP-containing cells was normalized to the fluorescence of cells carrying plasmids pYSG1 and pACYC184, which do not produce CadC.

Purification of CadC Homodimers and Heterodimers—Growth of cells and induction of the genes for homodimers or heterodimers were performed as previously described (6, 12). To produce heterodimers, LB medium with kanamycin (40 μg/ml) and chloramphenicol (40 μg/ml) was inoculated with a single colony of *E. coli* JM109(DE3) bearing two plasmids in which one *cadC* gene was in the background of plasmid pACYC184 and the other in the background of plasmid pET28b. The *cadC* gene expressed from pACYC184 did not encode a six-histidine tag, whereas the *cadC* expressed in the background of the pET28b plasmid contained the sequence for a six-histidine tag. The overnight cultures of these cells were used to inoculate 4 liters of prewarmed LB medium at 37 °C. At an absorbance of 0.6–0.8 at 600 nm, the cells were induced

[2] M. D. Wong and B. P. Rosen, unpublished results.

Downloaded from www.jbc.org by guest, on February 18, 2010

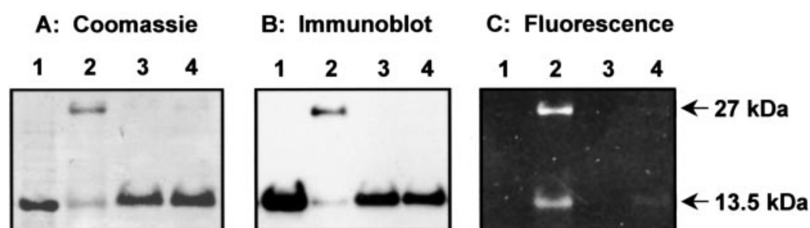

FIG. 3. **Reaction of CadC with dibromobimane.** Wild-type CadC (*lanes 1* and *2*) and quadruple mutant C7AC11SC58SC60S (*lanes 3* and *4*) were analyzed by SDS-PAGE on 16% polyacrylamide gels with (*lanes 2* and *4*) or without (*lanes 1* and *3*) reaction with dibromobimane. The gels were stained with Coomassie Blue (*A*), immunoblotted with anti-CadC (*B*), and visualized on a transilluminator for fluorescence (*C*). The positions of the 13.5-kDa monomers and 27-kDa dimers are indicated by *arrows*.

with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for an additional 3 h. Cells were harvested by centrifugation, washed with a buffer consisting of 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.137 M NaCl, and 2.7 mM KCl, pH 7.3, at 4 °C. Cell pellets were stored at −80 °C until use.

CadC homodimers and heterodimers were purified as described previously (6, 12) using buffers purged with argon. Protein solutions were sparged with argon for 0.5 h before applying to a 5-ml Probond Ni-affinity column (Clontech). Proteins were eluted with an imidazole gradient from 20 to 500 mM using an Automated Econo System (Bio-Rad). The eluates were collected in tubes containing small amounts of concentrated EDTA and DTT such that the final concentrations were each 10 mM.

Immunoblot Analysis—Purified wild-type and mutant CadC proteins were resolved by SDS-PAGE (20) on 16% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes at 100 V (21) followed by immunoblotting with a polyclonal antibody to CadC (Cocalico Biologicals, Inc., Reamstown, PA) (6) using anti-rabbit IgG (Sigma) as the secondary antibody. The membranes were also probed with monoclonal antibody to a C-terminal six-histidine tag directly conjugated with horseradish peroxidase (Invitrogen). Immunoreactive proteins were visualized by an enhanced chemiluminescence assay (PerkinElmer Life Sciences).

Assay of CadC Binding to the cad Promoter in Vitro—CadC binding to the *cad* promoter was assayed by protection of the single *Ssp*I site in the *cad* DNA from digestion (6, 12). Deprotection was examined by the addition of salts of soft metals. In some experiments CadC was removed by extraction with an equal volume of phenol. Samples were incubated at 37 °C for 30 min, following which they were mixed with 4 μl of a 6× sample solution (0.25% bromphenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in H_2O) and electrophoresed on 1.4% agarose gels containing 0.5 μg/ml ethidium bromide at 100 V for 60 min at 23 °C. Following electrophoresis the gels were soaked in 1 mM $MgSO_4$ for 30 min at 23 °C to remove excess ethidium bromide and photographed on a transilluminator using a Kodak DC120 scientific digital system. Immunoblot analysis of agarose gels was performed as described above for polyacrylamide gels.

Cross-linking Assays—Cross-linking studies with dibromobimane were described previously (15). Purified wild-type CadC and mutants were incubated with 70 mM DTT for 1 h at room temperature and dialyzed three times with 500 volumes of a buffer consisting of 50 mM MOPS, pH 7.0, 0.5 M NaCl, and 0.25 mM EDTA in an anaerobic glovebox to remove DTT. The proteins were quantified by using a protein assay kit (Bio-Rad) based on the method of Bradford (22). CadCs (16 μM) were incubated with 0.3 mM dibromobimane (Molecular Probes) for 15 min at 4 °C. The reactions were quenched with either 20 μM DTT or 0.3 mM tris(carboxyethyl)phosphine (Sigma), which was found to lower nonspecific cross-linking and fluorescence. Samples were analyzed by 16% SDS-PAGE. The gels were visualized under UV light at 365 nm and then stained with Coomassie Blue (GelCode® Blue Stain Reagent, Pierce).

RESULTS

Bimane Adduct Formation of CadC—Purified wild-type CadC migrates primarily as a monomer on SDS-PAGE (Fig. 3*A*, *lane 1*). If care is not taken to prevent oxidation, some CadC migrates as a non-reducible dimer (5). When treated with dibromobimane, the majority of protein migrated at the position of a CadC dimer (Fig. 3*A*, *lane 2*). The *upper band* reacted with antibody to CadC (Fig. 3*B*, *lane 2*) and was fluorescent (Fig. 3*C*, *lane 2*), demonstrating that it is a CadC dimer-bimane adduct. The fact that the fluorescent dimer was resistant to SDS denaturation strongly indicates that the cross-linking had occurred between cysteine residues on opposite subunits, as the intersubunit model would predict. The monomer also developed fluorescence slowly, which could result from formation of bimane adducts between Cys^7 and Cys^{11} and/or between Cys^{58} and Cys^{60}. In contrast, a quadruple mutant lacking Cys^7, Cys^{11}, Cys^{58}, and Cys^{60} did not dimerize when reacted with dibromobimane (Fig. 3, *A–C*, *lanes 4*), showing that bimane adduct formation requires CadC thiolates. Additionally, the monomer of the quadruple mutant did not develop fluorescence, even though it retains Cys^{52}. As shown below, the quadruple cysteine mutant bound to *cad* operator/promoter DNA, indicating that it does not have gross structural alterations. However, the quadruple mutant did not respond to addition of Cd(II), as shown below, consistent with the role of the cysteine residues in metal binding (6).

Cross-linking of CadC Homodimers with Single and Double Cysteine Mutations—Mutant CadCs C7G, C11G, C58S, and C60G have been shown to bind to the *cad* operator/promoter *in vivo* and *in vitro* (6). Although there was no apparent effect of the C11G mutation, alteration of Cys^7, Cys^{58}, and Cys^{60} each resulted in loss of metal responsiveness. These four mutant CadCs were purified and reacted with dibromobimane (Fig. 4). Elimination of any of the four did not prevent dimerization by reaction with dibromobimane; in each case formation of a fluorescent dimer was observed (Fig. 4, *lanes 2*, *4*, *6*, and *8*). This result could only occur if dimers were formed between cysteine residues on opposite subunits.

Two double mutants were constructed with substitutions of either the first two cysteines residues (C7G/C11S) or the second pair of cysteines (C58S/C60S). Both mutant proteins reacted with dibromobimane to form fluorescent monomers, but neither protein dimerized with dibromobimane treatment (Fig. 5). Similarly, a triple mutant, C7A/C58S/C60S, which contains only Cys^{11}, did not dimerize when treated with dibromobimane (data not shown).

Properties and Cross-linking of CadC Heterodimers—CadC heterodimers have been engineered in which one binding site was wild-type and the other had substitutions of the cysteine residues (12). These heterodimers retained their ability to bind to *cad* operator/promoter DNA but did not respond to addition of Cd(II), Pb(II), or Zn(II). Those results demonstrated that both subunits in the CadC dimer must have functional metal binding sites for derepression.

In this study heterodimers, in which the two subunits had different mutations and one subunit had a histidine tag, were purified. For convenience, a terminology for the heterodimers is used in which the first mutation is in the non-histidine-tagged subunit and the second is in the histidine-tagged subunit, and the residue number indicates which cysteine remains. For example, a "Cys^7-Cys^{58}" CadC heterodimer indicates the non-histidine-tagged subunit has only Cys^7, whereas the histidine-

Downloaded from www.jbc.org by guest, on February 18, 2010

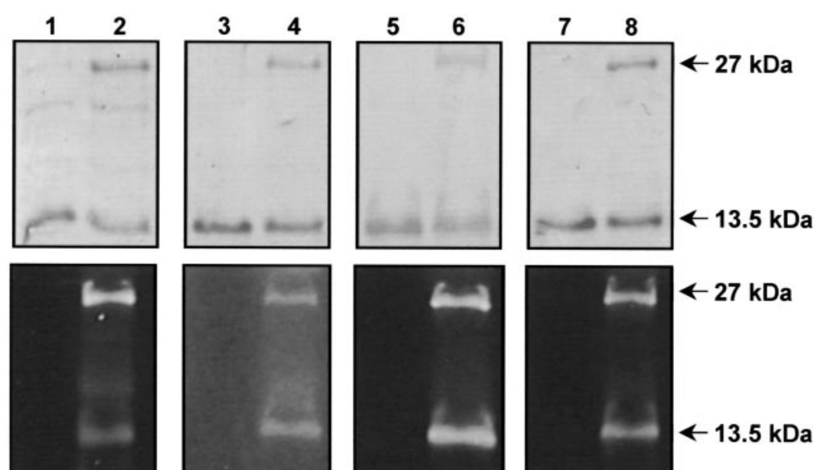

FIG. 4. **Dimerization of single cysteine mutants of CadC with dibromobimane.** CadC proteins with single cysteine substitutions C7A (*lanes 1* and *2*), C11G (*lanes 3* and *4*), C58S (*lanes 5* and *6*), and C60G (*lanes 7* and *8*) were analyzed by SDS-PAGE on 16% polyacrylamide gels with (*lanes 2*, *4*, *6*, and *8*) or without (*lanes 1*, *3*, *5*, and *7*) reaction with dibromobimane. The gels were stained with Coomassie Blue (*top*) and visualized on a transilluminator for fluorescence (*bottom*). The positions of the 13.5-kDa monomers and 27-kDa dimers are indicated by *arrows*.

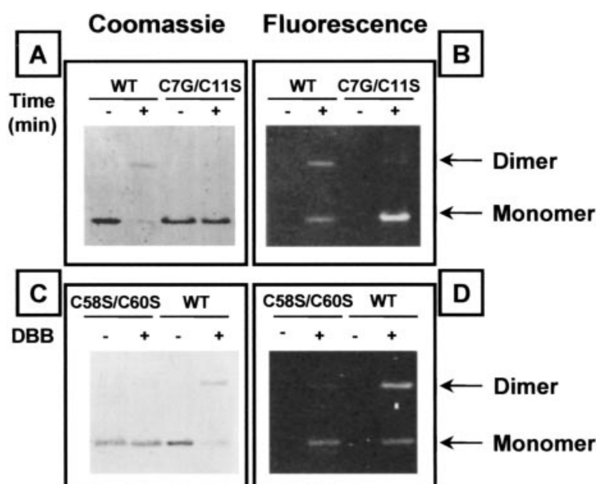

FIG. 5. **Bimane adduct formation of double cysteine mutants of CadC.** Wild-type CadC (*A–D*) and mutants with double cysteine substitutions C7G/C11S (*A* and *B*) and C58S/C60S (*C* and *D*) were analyzed by SDS-PAGE on 16% polyacrylamide gels with or without treatment with dibromobimane. The gels were stained with Coomassie Blue (*A* and *C*) and visualized on a transilluminator for fluorescence (*B* and *D*). Proteins were either not treated with dibromobimane (−) or reacted for 15 min (+). The positions of monomers and dimers are indicated by *arrows*.

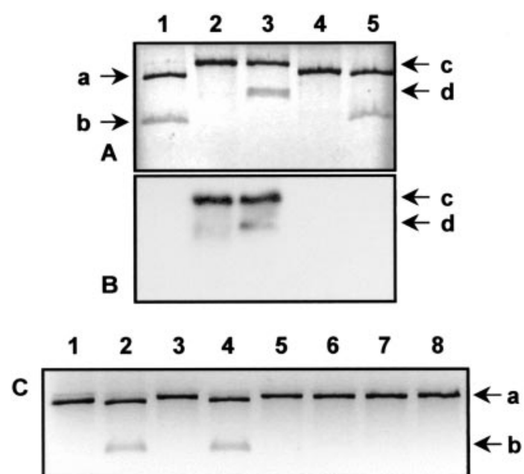

FIG. 6. **Binding of CadC to *cad* promoter DNA and metal responsiveness.** *Ssp*I restriction site protection assays were performed as described under "Materials and Methods." *A*, CadC remains bound to DNA following binding of Cd(II). Plasmid pYSG1 (4.6 kbp) has two *Ssp*I sites, one of which is located within the 108-bp *cad* operator/promoter fragment and the other in the vector. Digestion with *Ssp*I generated two restriction fragments of 3.6 kbp (*a*) and 1 kbp (*b*) (*lane 1*). In the presence of purified CadC, pYSG1 was cut only once by *Ssp*I, generating a single fragment (*lane 2*). Following addition of 20 μM $Cd(OAc)_2$, two fragments (*c* and *d*) were generated that migrated more slowly than their predicted sizes (*lane 3*). Following phenol extraction (*lanes 4* and *5*), the bands all migrated with the predicted mobilities. *B*, immunoblot of the CadC·DNA complex. The agarose gel was immunoblotted with anti-CadC. Reaction of fragments *c* and *d* (*lanes 2* and *3*) show that CadC remains bound to the DNA unless extracted with phenol (*lanes 4* and *5*). *C*, Cd(II) responsiveness of CadC mutants. Plasmid pYSG1 was digested with *Hin*dIII (*lane 1*) or *Ssp*I (*lanes 2–8*). Because there is only a single *Hin*dIII site, that enzyme produces a single fragment of 4.6 kbp. Wild-type CadC was added in *lanes 3* and *4*; *lanes 5* and *6* contained heterodimer Cys^{11}-Cys^{60}, and *lanes 7* and *8* contained the quadruple mutant C7A/C11S/C58S/C60S. 20 μM $Cd(OAc)_2$ was added in *lanes 4*, *6*, and *8*. Protein was extracted with phenol in *lanes 3–8*.

tagged subunit has Cys^{58}. Four heterodimers were purified, Cys^{7}-Cys^{58}, Cys^{7}-Cys^{60}, Cys^{11}-Cys^{58}, and Cys^{11}-Cys^{60}.

To examine the ability of the CadC heterodimers to bind to the *cad* operator/promoter DNA and to respond to Cd(II), a restriction protection assay was used (12). This assay measures DNA binding by the ability of CadC to protect the single *Ssp*I site contained within the *cad* operator/promoter from digestion with *Ssp*I. In this assay a 4.6-kbp plasmid that has two *Ssp*I sites, one within the 108-bp *cad* operator/promoter fragment and the other in the vector, is digested with *Ssp*I. This generates two restriction fragments of 3.6 and 1 kbp (Fig. 6*A*, *lane 1*). In the presence of purified wild-type CadC, the plasmid is cut only once by *Ssp*I, generating a single 4.6-kbp fragment (Fig. 6*A*, *lane 2*). Binding of Cd(II) induces dissociation, producing two fragments (Fig. 6*A*, *lane 3*). We have noted that both fragments produced by *Ssp*I in the presence of CadC and Cd(II) (Fig. 6*A*, *arrows c* and *d*) consistently migrate more slowly than the equivalent fragments in the absence of CadC (Fig. 6*A*, *arrows a* and *b*). SmtB, a Zn(II)-responsive homologue of CadC, has been shown to remain on the DNA after derepression by Zn(II) (23). The possibility that CadC remains bound to the DNA following Cd(II) binding was examined in two ways. First, the proteins on the agarose gel were electrophoretically transferred to a polyvinylidene difluoride membrane and then immunoblotted with anti-CadC (Fig. 6*B*). CadC remained bound to both *Ssp*I fragments. Second, following *Ssp*I digestion, the DNA was extracted with phenol to remove CadC (Fig. 6*A*, *lanes 4* and *5*). The two restriction fragments then migrated with the same mobility as the control (Fig. 6*A*, *lane 1*). Thus, under the conditions of this assay CadC remains bound not only to the *cad* operator/promoter following binding of Cd(II) but also binds to the half sites independently. It should be pointed out that this assay uses high concentrations CadC; whether the repressor remains bound to the operator/promoter *in vivo* following derepression is not known.

Binding of the Cys^{11}-Cys^{60} heterodimer (Fig. 6*C*, *lane 5*) to

Downloaded from www.jbc.org by guest, on February 18, 2010

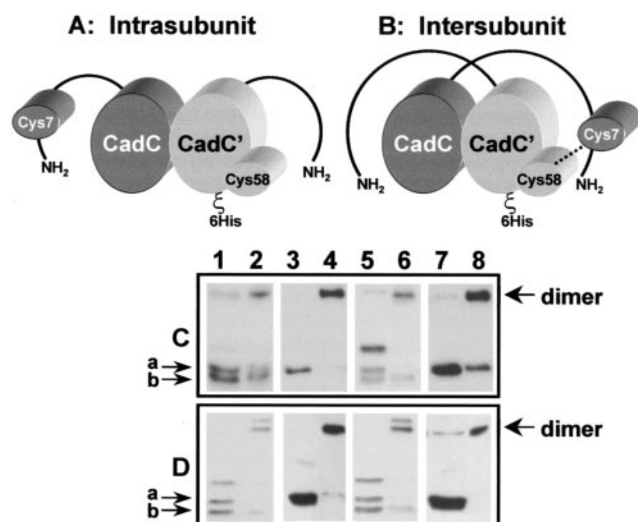

FIG. 7. **Mutant CadC heterodimers form cross-linked dimers with dibromobimane.** *A*, model of reaction of an intrasubunit Cys[7]-Cys[58] heterodimer with dibromobimane. An intramolecular heterodimer containing only Cys[7] on the non-histidine-tagged subunit and only Cys[58] on the six-histidine-tagged subunit will not form a cross-linked dimer with dibromobimane. *B*, model of reaction of an intersubunit Cys[7]-Cys[58] heterodimer with dibromobimane. In an intersubunit heterodimer, Cys[7] on one monomer is predicted to be 4.5 Å from Cys[58] on the other monomer and would form a bimane adducted dimer. *C* and *D*, reaction of mutant heterodimers with dibromobimane. Four mutant heterodimers, Cys[7]-Cys[58] (*C*, *lanes 1–4*), Cys[7]-Cys[60] (*C*, *lanes 5–8*), Cys[11]-Cys[58] (*D*, *lanes 1–4*), and Cys[11]-Cys[60] (*D*, *lanes 5–8*) were analyzed by SDS-PAGE without (*lanes 1*, *3*, *5*, and *7*) or with (*lanes 2*, *4*, *6*, and *8*) dibromobimane treatment. The samples were immunoblotted with anti-CadC (*lanes 1*, *2*, *5*, and *6*) or anti-six histidine tag (*lanes 3*, *4*, *7*, and *8*). The six-histidine-tagged (*a*) and non-histidine-tagged (*b*) monomers reacted with anti-CadC, whereas the six-histidine-tagged monomers (*a*) reacted with anti-six histidine tag. Additional cross-reacting species observed in some lanes may represent partially unfolded CadC polypeptides.

the *cad* operator/promoter DNA was compared with the wild-type (Fig. 6*C*, *lane 3*) and quadruple cysteine homodimers (Fig. 6*C*, *lane 7*). Each of the three CadCs protected the DNA from *Ssp*I digestion (Fig. 6*C*, *lanes 3*, *5*, and *7*), showing that the homodimer quadruple cysteine mutant and the heterodimer having only Cys[11] on one subunit and Cys[60] on the other was able to bind to the DNA. However, both types of mutants were unable to respond to Cd(II) (Fig. 6*C*, *lanes 6* and *8*). Although Cd(II) resulted in deprotection by the wild-type (Fig. 6*C*, *lane 4*), there was no effect of Cd(II) with either homodimeric or heterodimeric mutants (Fig. 6*C*, *lanes 6* and *8*). Note that in this assay the digests were extracted with phenol prior to electrophoresis, so that all of the bands migrated with the mobility of CadC-free DNA (*arrows a* and *b*). Although the data are shown for only the Cys[11]-Cys[60] heterodimer, the Cys[7]-Cys[58], Cys[7]-Cys[60], and Cys[11]-Cys[58] CadCs gave equivalent results. The fact that heterodimeric mutants retain the ability to bind to the *cad* operator/promoter indicates that these CadCs have sufficient native conformation to recognize their DNA binding site.

The definitive test of intramolecular *versus* intermolecular models was ability of heterodimeric CadC mutants to form bimane-dimer adducts following reaction with dibromobimane (Fig. 7). If the metal binding site was composed of cysteine residues from the same subunit (intramolecular model), then a heterodimer with (for example) only Cys[7] on one subunit and (for example) only Cys[58] on the other subunit should not form a bimane-dimer adduct (Fig. 7*A*). In contrast, this Cys[7]-Cys[58] heterodimer should form a fluorescent cross-linked dimer if the binding site is formed by residues from both subunits (intermolecular model) (Fig. 7*B*). In the absence of dibromobimane, each of the four heterodimers dissociated into monomers on SDS-PAGE. The two types of subunits could be differentiated by immunoblotting with anti-CadC (Fig. 7, *C* and *D*, *lanes 1* and *5*), which reacts with both subunits (*arrows a* and *b*), or anti-His tag (Fig. 7, *C* and *D*, *lanes 3* and *7*), which reacts only with the larger histidine-tagged subunit (*arrow a*). Each of the four heterodimers formed a dimer when treated with dibromobimane (Fig. 7, *C* and *D*, *lanes 2*, *4*, *6*, and *8*), which clearly support the intermolecular model: Cys[7] and Cys[11] on one subunit form bimane adducts with Cys[58] and Cys[60] on the other subunit. Thus each of the four cysteines thiolates must be within 3–6 Å of each other.

Contribution of Other Residues to the Metal Binding Site— The residues that contribute to the metal binding site in members of the ArsR family is a question of some interest. The above arguments make the assumption that the soft metal ion binding site in CadC has only the four protein ligands, Cys[7], Cys[11], Cys[58], and Cys[60]. In ArsR only three cysteine residues appear to be necessary for binding of As(III) or Sb(III) (24). However, in SmtB, residues contributing oxygen and/or nitrogen ligands may be involved in Zn(II) binding (11, 25). Asp[64] of SmtB is a Hg(II) ligand in the crystal structure and has been proposed to be part of the Zn(II) binding site (11). The corresponding residue is conserved as either an aspartate or glutamate in members of the ArsR family and could contribute an oxygen ligand for metal binding. In CadC this is Asp[61]. For this reason the effect of an D61A mutation was examined *in vivo* and *in vitro* (Fig. 8). *In vivo* the mutated *cadC* gene repressed expression of a *gfp* gene under control of the *cad* operator/promoter, and Pb(II) derepressed reporter gene expression, showing that the mutation did not alter the biological activity of CadC. Similar results were obtained with Cd(II) and Zn(II) (data not shown). The ability of purified D61A to bind to *cad* operator/promoter DNA *in vitro* was examined with an *Ssp*I protection assay (Fig. 8*B*). The mutant protein protected the DNA, and addition of 20 μM $Cd(OAc)_2$ produced deprotection with both wild-type and mutant. Pb(II) and Zn(II) similarly produced deprotection (data not shown).

In SmtB His[105] and His[106] are required residues (26) and are possible nitrogen-donating residues. These residues are located in a helix located in the dimerization domain of SmtB. CadC residue His[103] corresponds to SmtB residue His[106], so it is possible that it forms part of a metal binding site in CadC. Recently a second binding site for harder metals was identified at the interface of the two subunits of CadC (13). Although this site has been proposed to be an evolutionary relic, its participation in the biological activity of CadC has not been examined *in vivo*. To examine whether His[103] is required for CadC metal responsiveness, the alanine-substituted mutant H103A was constructed. The ability to respond to Pb(II) *in vivo* was examined using the *gfp* reporter assay. H103A repressed GFP expression in the absence of Pb(II) and derepressed in the presence of Pb(II) (Fig. 8*A*). In the *Ssp*I restriction enzyme protection assay, purified H103A CadC bound to *cad* promoter DNA, and addition of Cd(II) resulted in deprotection (Fig. 8*B*). The H103A mutant also responded to addition of Zn(II), both *in vivo* and *in vitro* (data not shown). Thus neither Asp[61] nor His[103] are essential for CadC function.

DISCUSSION

Members of the ArsR family of metalloregulatory proteins are homodimers with soft metal binding sites (7). In ArsR, an As(III)/Sb(III)-responsive repressor, the metal site is composed of Cys[32], Cys[34], and Cys[37], located at the first helix of the helix-turn-helix DNA binding domain (24). Binding of metal has been proposed to distort the helix, resulting in dissociation

Downloaded from www.jbc.org by guest, on February 18, 2010

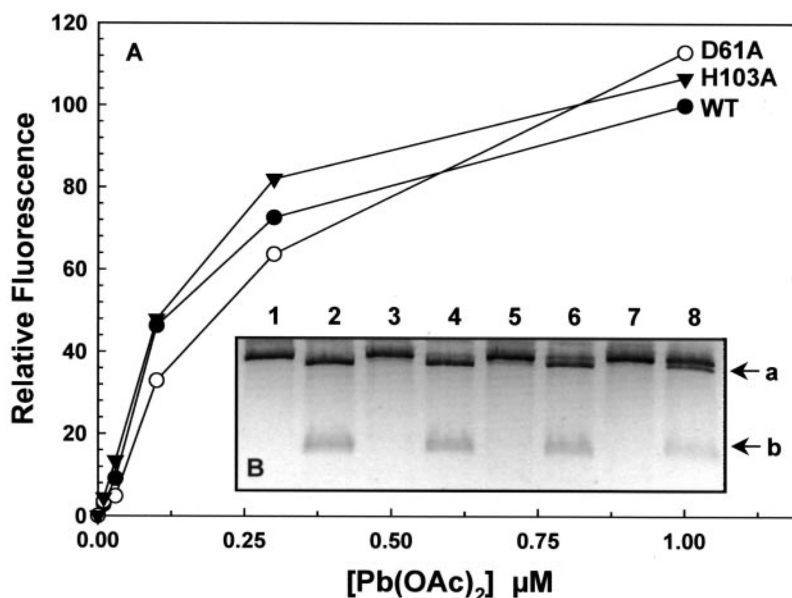

FIG. 8. **Neither Asp[61] nor His[103] residues are required for sensing of soft metal ions by CadC.** *A*, *In vivo* regulation of *gfp* expression from the *cad* operator/promoter. A two-plasmid system was used to measure the ability of wild-type CadC (●), D61A (○), and H103A (▼) to repress expression from the *cad* operator/promoter and to respond to the indicated concentration of $Pb(OAc)_2$, as described under "Materials and Methods." The mutant *cadC* genes were expressed as pYSC1 series plasmids in *E. coli* BL21(DE3) *zntA*::*km*. The same cells contained plasmid pYSG1, in which the gene for red-shifted GFP was under control of the *cad* operator/promoter. Cells were excited at 470 nm, and GFP fluorescence was measured at 507 nm. *B*, *in vitro* binding to *cad* operator/promoter DNA. The ability of D61A and H103A to protect the *cad* operator/promoter from digestion by the restriction enzyme *Ssp*I and to respond to Cd(II) was examined. pYSG1 was digested with *Hin*dIII (*lane 1*) or *Ssp*I (*lanes 2–8*) in the absence of CadC (*lanes 1* and *2*) or in the presence of wild-type CadC (*lanes 3* and *4*), D61A (*lanes 5* and *6*), or H103A (*lanes 7* and *8*). Following addition of 20 μM $Cd(OAc)_2$, fragments *a* and *b* were generated with wild-type CadC and mutants (*lanes 4*, *6*, and *8*). Samples were extracted with phenol prior to electrophoresis.

of the repressor from the DNA (7). Because the three cysteines are adjacent in the primary sequence, and there is no N-terminal extension with additional cysteine residues in ArsR, it is reasonable to conclude that each subunit has an As(III)/Sb(III) binding site composed of the three cysteines from the same ArsR monomer.

The situation is more complex in SmtB and CadC. One issue is that they may have more than one type of metal binding site. In SmtB two types of sites are observed in the crystal structure (11). One site is similar to the ArsR binding site in that it includes Cys[61] (25), which corresponds to Cys[32] in ArsR and Cys[58] in CadC, both of which are required for biological activity of their respective repressors (6, 27). It also includes Asp[64], which corresponds to Asp[35] in ArsR and Asp[61] in CadC (11). To examine whether CadC residue Asp[61] plays a role in metal sensing, a D61A mutant was created and was shown to respond to Cd(II), Pb(II), and Zn(II), both *in vivo* and *in vitro* (Fig. 8). Thus Asp[61] is not required for the biological activity of CadC.

The other putative metal binding site in SmtB is at the dimer interface (11) and includes His[106], which is essential for biological activity (26). In CadC His[103] corresponds to SmtB residue His[106]. Recently CadC has been reported to have a second metal binding site proposed to be at the dimer interface (13). From extrapolation from *in vitro* results it was suggested that this site is not required for CadC function *in vivo*. In this report His[103] was changed to alanine. *In vivo* H103A repressed expression of GFP under control of the *cad* operator/promoter, and addition of Pb(II), Cd(II), or Zn(II) produced normal derepression (Fig. 8). This confirms that a site containing His[103] is not involved in the biological activity of CadC.

A larger question is whether each of the two soft metal ion binding sites in CadC is composed entirely of residues from a single subunit or whether both subunits contribute residues to each site. CadC has two tetrathiolate binding sites per dimer for Cd(II) composed of four cysteine residues, Cys[7], Cys[11], Cys[58], and Cys[60] (5, 6, 13). Both CadC metal binding sites are required for its metalloregulatory properties (12). There are two possible ways in which the metal binding sites could be constructed: all four cysteine residues could be derived from a single CadC subunit (intrasubunit model, Fig. 2*A*) or Cys[7] and Cys[11] from one subunit could form a metal binding site with Cys[58] and Cys[60] from the other subunit (intersubunit model, Fig. 2*B*). As discussed above, the homologous ArsR repressor most likely has intrasubunit binding sites composed of three cysteine residues within a single subunit. On the other hand, intersubunit metal ion binding sites occur in other regulatory proteins. The unrelated homodimeric ArsD As(III)/Sb(IIII)-responsive repressor appears to have four intersubunit binding sites (28). In the MerR regulator, the single Hg(II) binding site is composed of cysteine residues from both subunits of the homodimer (29). Thus both models are reasonable possibilities.

The N terminus of apo-SmtB is not visible in the crystal structure, and therefore that structure sheds little light on the structure of the soft metal ion binding sites in the homologous CadC. To evaluate the intrasubunit possibility, CadC was modeled on the SmtB structure with the missing N-terminal residues added as an extended structure, where Cys[7] and Cys[11] were manually aligned with Cys[58] and Cys[60] on the same monomer (Fig. 2*A*). In this model the N terminus was just barely long enough to bring the two pairs of cysteines residues into proximity with each other. Thus, if the intrasubunit model were correct, the N terminus of CadC might be too extended to have secondary structure.

Because modeling alone cannot answer the question, an experimental approach was applied to determine the distance between the two pairs of cysteine residues. From the bond angles and distances in model compounds (30) and proteins with known tetrathiolate Cd(II) binding sites (31), the four sulfur ligands should be ~2.5 Å from the Cd(II) and 4.5 Å from each other. Cd K-edge x-ray absorption spectroscopy of the Cd(II)·CadC complex showed a distance of 2.53 Å between metal and sulfur atoms. To examine whether the distance from

Downloaded from www.jbc.org by guest, on February 18, 2010

the sulfur atoms of Cys[7] or Cys[11] on one subunit could be within 4.5 Å of the sulfur atoms of Cys[58] or Cys[60] on the other subunit, we used the well-known molecular ruler dibromobimane, which forms a fluorescent adduct linking two thiols that are more than 3 Å but less than 6 Å from each other (32). Wild-type CadC formed fluorescent dimers upon treatment with dibromobimane (Fig. 3). These dimers were resistant to reduction and denaturation with DTT and SDS, consistent with cross-linking between cysteines on the opposite subunits. If cross-linking had occurred between cysteines on the same subunit, only fluorescent monomers would be expected upon denaturation. In fact, both fluorescent monomers and dimers were observed, which might suggest that dibromobimane could produce both intra- and intersubunit cross-links. However, bimane labeling of double mutants that have only Cys[7] and Cys[11] or Cys[58] and Cys[60] produced fluorescent monomers but no dimers (Fig. 5). Thus the formation of fluorescent monomers is more likely due to cross-linking of Cys[7] with Cys[11], and Cys[58] with Cys[60], within one monomer than to formation of an intrasubunit soft metal ion binding site.

However, unambiguous confirmation of the intersubunit model comes from the ability to generate heterodimers with a single cysteine residue in each monomer. Because dibromobimane cross-linking occurred in heterodimers with the four possible combinations of cysteine residues (Fig. 7), both Cys[7] and Cys[11] on one monomer must be within the range of 4.5 Å of either Cys[58] or Cys[60] in the other monomer. The most reasonable interpretation of these results is that the two soft metal binding sites in the CadC homodimer are both assembled from Cys[7] and Cys[11] on one monomer and Cys[58] and Cys[60] on the other monomer.

Acknowledgments—We thank Jian Shen and Jun Ye for modeling of the CadC structure.

Addendum—We thank Dr. Giedroc for pointing out that the partially oxidized apo-CadC dimer was characterized by an inter- or intrasubunit disulfide bond between Cys[7] or Cys[11] and Cys[58] (not Cys[60]). This information is available in an electronic supplement to a report by Busenlehner *et al.* (37) to which we unfortunately did not have electronic access.

REFERENCES

1. Nucifora, G., Chu, L., Misra, T. K., and Silver, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3544–3548
2. Endo, G., and Silver, S. (1995) *J. Bacteriol.* **177,** 4437–4441
3. Tsai, K. J., Yoon, K. P., and Lynn, A. R. (1992) *J. Bacteriol.* **174,** 116–121
4. Tsai, K. J., and Linet, A. L. (1993) *Arch. Biochem. Biophys.* **305,** 267–270
5. Busenlehner, L. S., Cosper, N. J., Scott, R. A., Rosen, B. P., Wong, M. D., and Giedroc, D. P. (2001) *Biochemistry* **40,** 4426–4436
6. Sun, Y., Wong, M. D., and Rosen, B. P. (2001) *J. Biol. Chem.* **276,** 14955–14960
7. Xu, C., and Rosen, B. P. (1999) in *Metals and Genetics* (Sarkar, B., ed) pp. 5–19, Plenum Press, New York
8. San Francisco, M. J., Hope, C. L., Owolabi, J. B., Tisa, L. S., and Rosen, B. P. (1990) *Nucleic Acids Res.* **18,** 619–624
9. Morby, A. P., Turner, J. S., Huckle, J. W., and Robinson, N. J. (1993) *Nucleic Acids Res.* **21,** 921–925
10. Thelwell, C., Robinson, N. J., and Turner-Cavet, J. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10728–10733
11. Cook, W. J., Kar, S. R., Taylor, K. B., and Hall, L. M. (1998) *J. Mol. Biol.* **275,** 337–346
12. Sun, Y., Wong, M. D., and Rosen, B. P. (2002) *Mol. Microbiol.* **44,** 1323–1329
13. Busenlehner, L. S., Weng, T. C., Penner-Hahn, J. E., and Giedroc, D. P. (2002) *J. Mol. Biol.* **319,** 685–701
14. Kosower, N. S., Newton, G. L., Kosower, E. M., and Ranney, H. M. (1980) *Biochim. Biophys. Acta* **622,** 201–209
15. Bhattacharjee, H., and Rosen, B. P. (1996) *J. Biol. Chem.* **271,** 24465–24470
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY
17. Poole, R. K., Williams, H. D., Downie, J. A., and Gibson, F. (1989) *J. Gen. Microbiol.* **135,** 1865–1874
18. Chang, A. C., and Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156
19. Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M., and Youvan, D. C. (1995) *Bio/Technology* (*N. Y.*) **13,** 151–154
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685
21. Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
23. Kar, S. R., Lebowitz, J., Blume, S., Taylor, K. B., and Hall, L. M. (2001) *Biochemistry* **40,** 13378–13389
24. Shi, W., Wu, J., and Rosen, B. P. (1994) *J. Biol. Chem.* **269,** 19826–19829
25. VanZile, M. L., Cosper, N. J., Scott, R. A., and Giedroc, D. P. (2000) *Biochemistry* **39,** 11818–11829
26. Turner, J. S., Glands, P. D., Samson, A. C., and Robinson, N. J. (1996) *Nucleic Acids Res.* **24,** 3714–3721
27. Shi, W., Dong, J., Scott, R. A., Ksenzenko, M. Y., and Rosen, B. P. (1996) *J. Biol. Chem.* **271,** 9291–9297
28. Li, S., Rosen, B. P., Borges-Walmsley, M. I., and Walmsley, A. R. (2002) *J. Biol. Chem.* **277,** 25992–26002
29. Helmann, J. D., Ballard, B. T., and Walsh, C. T. (1990) *Science* **247,** 946–948
30. Rao, C. P., Dorfman, J. R., and Holm, R. H. (1986) *Inorg. Chem.* **25,** 428–439
31. Archer, M., Carvalho, A. L., Teixeira, S., Moura, I., Moura, J. J., Rusnak, F., and Romao, M. J. (1999) *Protein Sci.* **8,** 1536–1545
32. Mornet, D., Ue, K., and Morales, M. F. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 1658–1662
33. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
34. Sali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234,** 779–815
35. Esnouf, R. M. (1997) *J. Mol. Graph. Model.* **15,** 132–134, 112–113
36. Merrit, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **50,** 869–873
37. Busenlehner, L. S., Apuy, J. L., and Giedroc, D. P. (2002) *J. Biol. Inorg. Chem.* **7,** 551–559

Downloaded from www.jbc.org by guest, on February 18, 2010