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

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 cadCA operon. CadC binds two Cd(II) ions per dimer, with a tetrathiolate binding site composed of residues Cys7, Cys11, Cys58, and Cys60. It is not known whether each site consists of residues from a single monomer or from residues contributed by both subunits. To examine whether Cys7 and Cys11 from one monomer are accessible. These considerations imply that the fourth cysteine residue in the tetra-thiolate complex has not been identified with certainty. However, modeling CadC on the structure of the SmtB aporepressor (11) suggests that Cys52 is 15–18 Å from Cys58 and Cys60. Moreover, assuming the validity of the model, Cys52 would be predicted to be buried, and its thiolate would not be solvent-accessible. These considerations imply that the fourth cysteine residue is Cys11.

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 Cys7, Cys11, Cys58, and Cys60 from the same monomer (intramer model) (Fig. 2A). On the other hand, in each site Cys7 and Cys11 could be contributed by one monomer, and Cys58 and Cys60 from the other monomer (intersubunit model) (Fig. 2B). 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 Cys7 and Cys11 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 inter-subunit 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...
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Cysteine residue. Dimers were formed by reaction with dibromobimane such that each monomer of the dimer had only a single Cys60 did not. All single mutants formed fluorescent dimers. The Cd(II) binding site formed by Cys 7 and Cys11 of one subunit and Cys 58 and Cys60 of the other subunit. The data are consistent only with a tetrathiolate inter- or intrasubunit model of a tetrathiolate Cd(II) binding site. Cys7 and Cys11 must be homologous sequence of CadC was added with the only constraint that Cys 7 or Cys 11 interacts intermolecularly with Cys 58 or Cys60 in one subunit to form a metal binding site composed of Cys7 and Cys11 from one subunit and Cys58 and Cys60 from the other (Fig. 2B).

**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 (31) 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 Cys7 and Cys11 must be 4.5 Å from Cys58 and Cys60 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 Cys7 and Cys11 into position relative to Cys58 and Cys60 on the same subunit to form a tetrathiolate Cd(II) binding site. B, intersubunit model. A tetrathiolate Cd(II) binding site formed by Cys7 and Cys11 of one subunit and Cys58 and Cys60 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 Cys7, Cys11, Cys58, and Cys60, which are shown in ball-and-stick form. Images were generated with MOLSCRIPT (35) and RASTER3D (36).

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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 Na₂HPO₄, 1.4 mM KH₂PO₄, 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 Ni²⁺ affinity column (Clontech). Proteins were eluted with an imidazole gradient from 20 to 500 mM using an Automated Econo System (Bio-Rad). 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 electrothermally 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 (Pierce).

**Assay of CadC Binding to the cad Promoter in Vitro**—CadC binding to the cad promoter was assayed by protection of the single Sep1 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 H₂O) 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 MgSO₄ for 30 min at 23 °C to remove ethidium bromide and photographed on a transilluminator using a Kodak DC1200 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 mM NaCl, and 0.25 mM EDTA in an anaerobic glovebox (data not shown). 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. 3A, 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. 3A, lane 2). The upper band reacted with antibody to CadC (Fig. 3B, lane 2) and was fluorescent (Fig. 3C, 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 Cys² and Cys¹¹ and/or between Cys⁵⁸ and Cys⁶⁰. In contrast, a quadruple mutant lacking Cys⁷, Cys¹¹, Cys⁵⁸, and Cys⁶⁰ 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 Cys⁵². 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**—Purified 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 Cys⁷, Cys⁵⁸, and Cys⁶⁰ 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 cysteine 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 Cys¹¹, 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 “Cys²⁷-Cys⁵⁸⁷” CadC heterodimer indicates the non-histidine-tagged subunit has only Cys²⁷, whereas the histidine-
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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 or without treatment with dibromobimane. The gels were stained with Coomassie Blue (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. SspI 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 SspI sites, one of which is located within the 108-bp cad operator/promoter fragment and the other in the vector. Digestion with SspI 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 SspI, 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 HindIII (lane 1) or SspI (lanes 2–8). Because there is only a single HindIII 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 Cys⁵⁸-Cys⁶⁰, 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 Cys⁵⁸. Four heterodimers were purified, Cys⁷-Cys⁸⁵, Cys⁷-Cys⁹⁰, Cys¹¹-Cys⁸⁵, and Cys¹¹-Cys⁹⁰.

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 SspI site contained within the cad operator/promoter from digestion with SspI. In this assay a 4.6-kbp plasmid that has two SspI sites, one within the 108-bp cad operator/promoter fragment and the other in the vector, is digested with SspI. This generates two restriction fragments of 3.6 and 1 kbp (Fig. 6A, lane 1). In the presence of purified wild-type CadC, the plasmid is cut only once by SspI, generating a single 4.6-kbp fragment (Fig. 6A, lane 1). Binding of Cd(II) induces dissociation, producing two fragments (Fig. 6A, lane 3). We have noted that both fragments produced by SspI in the presence of CadC and Cd(II) (Fig. 6A, arrows c and d) consistently migrate more slowly than the equivalent fragments in the absence of CadC (Fig. 6A, 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 trans-ferred to a polyvinylidene difluoride membrane and then immunoblotted with anti-CadC (Fig. 6B). CadC remained bound to both SspI fragments. Second, following SspI digestion, the DNA was extracted with phenol to remove CadC (Fig. 6A, lanes 4 and 5). The two restriction fragments then migrated with the same mobility as the control (Fig. 6A, 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 Cys¹¹-Cys⁹⁰ heterodimer (Fig. 6C, lane 5) to
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 Cys7 on one subunit and (for example) only Cys58 on the other subunit should not form a bimane-dimer adduct (Fig. 7A). In contrast, this Cys7-Cys58 heterodimer should form a fluorescent cross-linked dimer if the binding site is formed by residues from both subunits (intermolecular model) (Fig. 7B). 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: Cys7 and Cys11 on one subunit form bimane adducts with Cys58 and Cys60 on the other subunit. Thus each of the four cysteines thiolates must be within 3–6 Å of each other.

FIG. 7. Mutant CadC heterodimers form cross-linked dimers with dibromobimane. A, model of reaction of an intrasubunit Cys7-Cys58 heterodimer with dibromobimane. An intramolecular heterodimer containing only Cys7 on the non-histidine-tagged subunit and only Cys58 on the six-histidine-tagged subunit will not form a cross-linked dimer with dibromobimane. B, model of reaction of an intersubunit Cys7-Cys58 heterodimer with dibromobimane. In an intersubunit heterodimer, Cys7 on one monomer is predicted to be 4.5 Å from Cys58 on the other monomer and would form a bimane adducted dimer. C and D, reaction of mutant heterodimers with dibromobimane. Four mutant heterodimers, Cys7-Cys58 (C, lanes 1–4), Cys7-Cys60 (C, lanes 5–8), Cys11-Cys58 (D, lanes 1–4), and Cys11-Cys60 (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.

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 Cys32, Cys37, and Cys47, 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
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 Asp106/His103/Slb111(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 Cys61 (25), which corresponds to Cys32 in ArsR and Cys68 in CadC, both of which are required for biological activity of their respective repressors (6, 27). It also includes Asp61, which corresponds to Asp35 in ArsR and Asp61 in CadC (11). To examine whether CadC residue Asp61 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 Asp61 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 His106, which is essential for biological activity (26). In CadC His103 corresponds to SmtB residue His106. 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 His103 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 His103 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, Cys7, Cys11, Cys58, and Cys60 (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 (intrabinding site, model, Fig. 2A) or Cys7 and Cys11 from one subunit could form a metal binding site with Cys58 and Cys60 from the other subunit (intrabinding site, model, Fig. 2B). As discussed above, the homologous ArsR repressor most likely has intrabinding site. From the Cd(II) and 4.5 Å from each other. Cd K-edge x-ray absorption spectroscopy of the CdII·CdC complex showed a distance of 2.53 Å between metal and sulfur atoms. To examine whether the distance from...
the sulfur atoms of Cys\textsuperscript{7} or Cys\textsuperscript{11} on one subunit could be within 4.5 Å of the sulfur atoms of Cys\textsuperscript{58} or Cys\textsuperscript{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 denaturation. In fact, both fluorescent monomers and dimers are more than 3 Å but less than 6 Å from each other (32).

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 inter-subunit cross-links. However, bimane labeling of double mutants that have only Cys\textsuperscript{7} and Cys\textsuperscript{11} or Cys\textsuperscript{58} and Cys\textsuperscript{60} produced fluorescent monomers but no dimers (Fig. 5). Thus the formation of fluorescent monomers is more likely due to cross-linking of Cys\textsuperscript{7} with Cys\textsuperscript{11}, and Cys\textsuperscript{58} with Cys\textsuperscript{60}, within one monomer than to formation of an intra-subunit 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\textsuperscript{7} and Cys\textsuperscript{11} on one monomer must be within the range of 4.5 Å of either Cys\textsuperscript{58} or Cys\textsuperscript{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\textsuperscript{7} and Cys\textsuperscript{11} on one monomer and Cys\textsuperscript{58} and Cys\textsuperscript{60} on the other monomer.

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