Role of Cysteiny1 Residues in Sensing Pb(II), Cd(II), and Zn(II) by the Plasmid pI258 CadC Repressor

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The cadCA operon of Staphylococcus aureus plasmid pI258 confers resistance to salts of the soft metals lead, cadmium, and zinc. The operon is regulated by CadC, a member of the ArsR family of metal-responsive transcriptional regulators. In this study the role of the five cysteine residues of CadC in soft metal ion sensing was investigated. Cys-7, Cys-11, Cys-52, Cys-58, and Cys-60 were changed individually to glycine or serine residues. Cys-7, Cys-11, Cys-52, Cys-58, and Cys-60 resulted in reduced responses to soft metal ions. The results indicate that Cys-7, Cys-58, and Cys-60 were ligands to Pb(II), Zn(II), and Cd(II). Cys-7, Cys-11, Cys-52, Cys-58, and Cys-60 mutations each reduced or eliminated soft metal sensing. Wild-type and mutant CadC proteins were purified, and the effect of the cadC mutations was examined in Escherichia coli using a green fluorescent protein reporter system. Neither Cys-11 nor Cys-52 was required for in vivo response to Pb(II), Zn(II), or Cd(II). Cys-7, Cys-58, or Cys-60 mutations each reduced or eliminated soft metal sensing. Wild-type and mutant CadC proteins were purified, and the effect of the substitutions on DNA binding was determined using a restriction enzyme protection assay. Binding of wild-type CadC protected cad operator DNA from digestion at the single SspI site, and the addition of Pb(II), Zn(II), or Cd(II) resulted in deprotection. Chemical modification of the cysteine residues of CadC had no effect on protection but eliminated deprotection. C11G and C52G substitutions of Cys-7, Cys-58, and Cys-60 resulted in loss of soft metal-dependent regulation by CadC both in vivo and in vitro. The results suggest that these three residues are involved in sensing Pb(II), Zn(II), or Cd(II).

MATERIALS AND METHODS

Growth of Cells—Cells of E. coli were grown in LB medium (9) at 37 °C. Ampicillin (50 μg/ml), kanamycin (40 μg/ml), chloramphenicol (80 μg/ml), isopropyl-β-D-thiogalactopyranoside (0.1 mM), and 5-bromo-4-chloro-3-indoly-β-D-galactosidase (80 μg/ml) were added as required. For determination of metal ion responsiveness, a basal salts medium was used (10) with the omission of zinc salts. Growth was monitored from the absorbance at 600 nm.

Cloning of cadC—A 406-nucleotide base pair (bp) fragment from plasmid pYPK11 (11) containing the plasmid pI258 cadC gene was amplified by polymerase chain reaction (12). The polymerase chain reaction product was cloned into plasmid pGEM-T (Promega) generating plasmid pMW0. The entire cadC gene was sequenced using a Pharmacia Cy5-labeled autosquence kit (Amersham Pharmacia Biotech) and an ALFExpress apparatus using the method of Sanger et al. (13) to confirm that mutations had not been introduced during amplification. A 393-bp NdeI-PstI fragment then was isolated from pMW0 and ligated into the plasmid pT7-7 that had been digested with NdeI and PstI. A 448-bp XbaI-HindIII fragment, which includes both the ribosome-binding site from pT7-7 and cadC, was isolated from pMW0 and ligated into the plasmid pET-28a (Novagen) that was digested with XbaI and HindIII, generating plasmid pMW1. Plasmid pMW1 was transformed into E. coli HMG174(DE3) (14) for protein expression. In this plasmid, cadC is under the control of the T7lac promoter, which is repressed by a lacI gene, and induced with isopropyl β-D-thiogalactopyranoside. Production of CadC was examined by SDS-polyacrylamide gel electrophoresis on 18% acrylamide gels (15). The amount of CadC was estimated by Western blot analysis. Purified CadC was used to produce a polyclonal rabbit antiserum by Cocalico Biologicals, Inc. (Reamstown, PA). The proteins were transferred overnight onto a nylon membrane by immunoblotting.

Because the first cells could have evolved in hydrothermal vents rich in toxic soft metals such as cadmium and lead (1), the development of resistance mechanisms to such metals was essential for their survival. In bacteria soft metal resistance often is catalyzed by P-type ATPases (2, 3). The first to be identified was the cadmium resistance (cadCA) operon of Staphylococcus aureus plasmid pI258 (4). The cadA gene encodes a P-type ATPase that has been shown to transport Cd(II), Zn(II), and Pb(II) (5). The cadC gene encodes a transcriptional regulator that is a member of the ArsR family of metalloregulatory proteins (6). When expressed in S. aureus, CadC responds to metals (7, 8). In contrast, induction of the cad operon in vivo by cadmium or zinc was difficult to observe in Escherichia coli, which is intrinsically resistant to these metals. We have shown that resistance in E. coli is caused by expression of the chromosomally encoded zntA gene, and a zntA-disrupted strain exhibits increased sensitivity to zinc, cadmium, and lead (5). The disruption has been used as a background strain for examining cadC function in E. coli. In that study CadC was shown to respond to soft metals with the order of effectiveness Pb(II) > Cd(II) > Zn(II).

Here we investigate the role of cysteine residues in metal ion selectivity by CadC. CadC contains five cysteines at residues 7, 11, 52, 58, and 60. Each was altered by site-directed mutagenesis. The effect of the substitutions on activity in E. coli and in vitro was examined. None of the cysteine residues was required for repression in vivo or for binding to the cad promoter in vitro. Neither Cys-11 nor Cys-52 seems to be required for soft metal recognition by CadC. Substitution of Cys-7, Cys-58, or Cys-60 resulted in loss of soft metal-dependent regulation by CadC both in vivo and in vitro. The results suggest that these three residues are involved in sensing Pb(II), Zn(II), or Cd(II).

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1 The abbreviations used are: bp, base pair; kb, kilobase pair; GFP, green fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; MMTS, methyl methanethiosulfonate.
trocellose membrane at 25 V and probed with a polyclonal antibody to CadC using anti-rabbit IgG (Sigma) as the secondary antibody. Immunoblotting was performed using an enhanced chemiluminescence assay (PerkinElmer Life Sciences) and exposed on x-ray film at room temperature.

DNA Manipulation and Oligonucleotide-directed Mutagenesis—Plasmid DNA was prepared with a Wizard DNA purification kit (Promega). Endo- and exonuclease digestions, DNA isolation, ligation, transformation, and Klenow fragment fill-in reactions were performed according to standard procedures (9) unless otherwise noted. Oligonucleotide-directed mutations in cadC were introduced by site-directed mutagenesis using the Expand High Fidelity polymerase (Roche) according to the methods of Gold and von Hippel (21) using an extinction coefficient for GFP of 3.40. The fluorescent properties of the samples were determined from the fluorescence of red-shifted GFP as a function of detection wavelength using a Fluorolog 3 fluorometer (PerkinElmer Life Sciences) and exposed on x-ray film at room temperature.

Measurement of Regulation in Vivo—The gene for red-shifted green fluorescent protein (GFP) (17) was used as a reporter for monitoring the regulatory properties of the cadC gene product. A 108-bp fragment from plasmid pYPK11 containing the pL258 cad operator/promoter was amplified by polymerase chain reaction. The fragment was engineered using the same procedure as for the wild type. Cells were harvested by a single pass through a French Press cell at 20,000 p.s.i. Membrane and unbroken cells were removed by centrifugation at 150,000 g for 3 h at 4 °C. The cells were suspended in 20 ml of buffer A and broken by sonication using a SLM5000 spectrophotometer. The fluorescence of GFP-containing cells was measured by the addition of ethidium bromide and photographed on a transilluminator using a Kodak DC120 scientific digital system. The intensity of the bands was quantified by densitometry using UN-SCAN-ITgel (Silk Scientific, Inc.).

RESULTS

In Vivo Soft Metal Regulation of CadC—We have demonstrated previously that CadC repression of the plasmid pL258 cad promoter/cytochrome c repressor can be relieved by the addition of Pb(II), Cd(II), or Zn(II) (in order of effectiveness) (5). Derepression required expression in a lacZ-disrupted strain, which is unable to pump those soft metals out of the cytosol. In that study a lacZ gene was used as a reporter. Measurement of β-galactosidase activity requires permeabilization of the cells. In contrast, GFP fluorescence is noninvasive and can be assayed in vivo. For these assays a two-plasmid system was used. One plasmid contained a gfp gene under control of the cad promoter. The second plasmid had a wild-type or mutant cadC gene behind the T7 promoter. Although transcription of the T7 polymerase is under lacZ promoter control, there was sufficient basal level CadC expression to repress GFP in the absence of isopropyl-β-D-thiogalactopyranoside (Fig. 1A). In vivo soft metal responsiveness was estimated from the amount of GFP fluorescence after growth in the presence of varying concentrations of Pb(II), Zn(II), or Cd(II) (Fig. 1A). Each value represents the mean of three separate experiments, with the standard deviation shown by the error bars. Pb(II) was the most effective with half-maximal derepression at 0.1 μM Pb(OAc)2. About 10-fold more Zn(II) was required. Although Cd(II) derepressed, the response to Cd(II) was difficult to quantify because growth of the lacZ-disrupted strain was much slower in the presence of Cd(II) than the other two metals. Addition of other soft metals including Ni(II), Mn(II), Cu(II), and Zn(II) did not produce an increase in gfp expression (data not shown). It should be emphasized that the added metal ion concentrations may not be the same as the cytosolic concentrations. The free intracellular concentration may be influenced by the rates of uptake, binding, and efflux so that quantitative comparisons among the metals should be made with caution.

Requirement for Cysteine Residues for Repression and Derepression—CadC has five cysteines in residues 7, 11, 52, 58, and 60 (Fig. 2). In the homologous plasmid R773 ArsR repressor, Cys-32 and Cys-34 have been shown to respond for derepression by As(III) or Sb(III), where the thiolates form part of the metal binding site (24). ArsR residues Cys-32 and Cys-34 correspond to Cys-58 and Cys-60 in CadC. To investigate the possibility that cysteine thiol is ligands to...
Pb(II), Zn(II), or Cd(II) in CadC, each of the cysteine codons was mutated to glycine. CadCDS with glycine substitutions were able to repress expression of the gfp reporter from the cad promoter, except for the C58G CadC. However, a C58S CadC was able to repress and was used for subsequent studies.

Because the C7G, C11G, C52G, C58S, and C60G mutants repressed expression, their ability to respond to the addition of Pb(II) (Fig. 1B) or Zn(II) (Fig. 1C) to the growth medium was examined. Although each curve in Fig. 1, B and C, represents the results of a single experiment, each experiment was repeated 2–6 times with equivalent results. The Cys-11 mutation had no effect on metal responsiveness, and there was only a small effect of the Cys-52 mutation. In contrast, the Cys-7 and Cys-58 mutations exhibited a significant decrease in the ability to respond to either Pb(II) or Zn(II). The C60G mutant was unresponsive to either soft metal. Although it is possible that the mutations could have produced conformational changes in CadC that are only indirectly related to metal binding, these results suggest that Cys-7, Cys-58, and Cys-60, but not Cys-11 or Cys-52, are involved in metal sensing.

In Vitro Soft Metal Regulation of the cad Promoter by CadC—To investigate the DNA- and metal-binding properties of CadC, the repressor was expressed and purified from E. coli as described under “Materials and Methods.” CadC purified as a homodimer, and no evidence of monomer was observed upon molecular sieve chromatography (data not shown). This is consistent with the reported dimerization of the homologous ArsR (25) and SmtB (26). Upon SDS-polyacrylamide gel electrophoresis, CadC was present as both monomer and dimer (data not shown). If the amount of β-mercaptoethanol was increased to 1 mM, the protein migrated predominately as a monomer (data not shown). Thus CadC is a noncovalent homodimer that can form disulfide bonds in vitro.

The ability of purified CadC to bind to DNA containing the cad operator was measured by an enzyme protection assay (22). Plasmid pYSG1 has only a single HindIII site, so digestion with that enzyme generates a single restriction fragment of 4.6 kbp (Fig. 3A, lane 1). The plasmid 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 generates two restriction fragments of 3.6 and 1 kbp (Fig. 3A, lanes 2 and 7). In the presence of purified CadC, pYSG1 was cut only once by SspI (Fig. 3A, lanes 3 and 8). Protection from SspI digestion was specific for CadC; neither ArsR nor bovine serum albumin protected the cad DNA from SspI (data not shown). When Pb(II) (Fig. 3A, lanes 4 and 9), Cd(II) (Fig. 3A, lane 5), or Zn(II) (Fig. 3A, lane 6) was added, SspI digestion was again observed. This deprotection is consistent with the ability of these metals to alter the binding of CadC to the promoter. Hg(II), which we previously reported to act as a weak inducer of the cad operon (5), also reduced CadC protection (Fig. 3A, lane 14). Ni(II), Co(II), As(III), or Sb(III) had little or no effect on CadC protection (Fig. 3A, lanes 10–13, respectively). Because other members of the ArsR family bind these metals, it was important to demonstrate that CadC does not respond to them in vitro. The effect of Pb(II), Zn(II), or Cd(II) was quantified (Fig. 3B). In vitro, each of the three was nearly equally effective, varying only about 2-fold in contrast to the in vivo results, in which Pb(II) was 10-fold more effective than Zn(II). Each value represents the mean of three separate experiments, with the standard deviation shown by the error bars. As mentioned above, one possible reason for the differences between in vivo and in vitro results may be that the metals are accumulated differentially or bound in vivo.

Requirement for Cysteine Residues for CadC Binding and Release from the cad Operator DNA—The effect of the cysteine-
modifying reagent MMTS on CadC protection of cad DNA and metal-induced deprotection was determined. MMTS converts cysteine residues to Cys–S–S–CH₃ (23). Addition of the small metal-induced deprotection was determined. MMTS converts 14958 been identified. These repressors fall into several groups: ArsR repressors are As(III)/Sb(III)-responsive (29); CadC repressors are Pb(II)/Cd(II)/Zn(II)-responsive (5, 7, 8); and SmtB/ZiaR repressors are Zn(II)-responsive (30, 31). What is the molecular basis of metal ion sensing and selectivity in members of the ArsR family? The ions that derepress are all ions of soft Lewis acids, so it is likely ligands would be soft Lewis bases, which include the thiolates of cysteine residues and the imidazole nitrogens of histidines. An alignment of nine putative CadC proteins, ArsR, ZiaR, and SmtB identifies a number of conserved residues in some or all proteins (Fig. 2). Cys-7, Cys-58, and Cys-60 of the pI258 CadC are conserved in all CadCs. In the present study, mutagenesis of the three conserved cysteine residues in homologues is shaded. The multiple alignment was calculated with ClustalW (16).

**DISCUSSION**

The ArsR family is a rapidly growing set of repressor proteins involved in transcriptional regulation of an assortment of unrelated types of soft metal resistances (6), including P-type ATPases (4), ArsAB pumps (27), and metallothionines (28). At last count, 24 homologues in bacteria and 18 in archaea have been identified. These repressors fall into several groups: ArsR repressors are As(III)/Sb(III)-responsive (29); CadC repressors are Pb(II)/Cd(II)/Zn(II)-responsive (5, 7, 8); and SmtB/ZiaR repressors are Zn(II)-responsive (30, 31). What is the molecular basis of metal ion sensing and selectivity in members of the ArsR family? The ions that derepress are all ions of soft Lewis acids, so it is likely ligands would be soft Lewis bases, which include the thiolates of cysteine residues and the imidazole nitrogens of histidines. An alignment of nine putative CadC proteins, ArsR, ZiaR, and SmtB identifies a number of conserved residues in some or all proteins (Fig. 2). Cys-7, Cys-58, and Cys-60 of the pI258 CadC are conserved in all CadCs. In the present study, mutagenesis of the three conserved cysteine residues in homologues is shaded. The multiple alignment was calculated with ClustalW (16).
where the three sulfur atoms must be within 3.2–3.5 Å of each other (24). However, from the model of the ArsR aporepressor, this would not be possible without distortion of helix 1. If binding of metals to ArsR or CadC disrupts helix 1, the repressor would no longer be able to bind to DNA and would dissociate, allowing RNA polymerase to bind and transcription to proceed.

However, the CXC motif cannot account for selectivity if it is utilized for both As(III)/Sb(III)- and Pb(II)/Zn(II)/Cd(II)-responsive repressors. In ArsR, the third ligand to As(III), Cys-37, is not present in CadC. From the present results, it seems that Cys-7 of CadC is involved in Pb(II)/Zn(II)/Cd(II) sensing, and this residue has no counterpart in ArsR. Cys-14 in SmtB and Cys-20 in ZiaR may correspond to Cys-7 of CadC (Fig. 2), and Cys-14 and Cys-20 are required for zinc sensing in the respective repressors (30, 31). It seems likely that this conserved cysteine residue contributes to the selectivity of CadC, ZiaR, and SmtB. Our data are consistent with a model in which 1) Cys-7, Cys-58, and Cys-60 are metal ligands in CadC, and 2) Pb(II), Zn(II), and Cd(II) can be four-coordinate or higher, which implies that there could be additional protein residues involved in metal binding. Studies with SmtB suggest that
oxygen and/or nitrogen ligands may be involved in Zn(II) binding (33, 34). A reasonable candidate for a fourth ligand in CadC is Asp-61, which is conserved as either an aspartate or glutamate in all identified members of the ArsR family. Asp-61 corresponds to Asp-64 of SmtB, which is a Hg(II) ligand in the crystal structure (33). The quest for additional residues involved in metal sensing forms the basis of future studies on CadC.

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