Regulation of Cytochrome bd Expression in the Obligate Aerobe Azotobacter vinelandii by CydR (Fnr)

SENSITIVITY TO OXYGEN, REACTIVE OXYGEN SPECIES, AND NITRIC OXIDE*

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Azotobacter vinelandii is an obligately aerobic bacterium in which aerotolerant nitrogen fixation requires cytochrome bd. Regulation of cytochrome bd expression is achieved by CydR (an Fnr homologue), which represses transcription of the oxidase genes cydAB. cydAB mRNA was mapped by primer extension; the transcriptional start site was determined, and putative −10 and −35 regions were deduced. Two "CydR boxes," one at the +1 site and one upstream of the −35 region, were identified. Transcriptionally inactive, purified CydR was converted, by adding NiS, cysteine, and Fe2+, into an active form possessing acid-labile sulfide and spectra suggesting a [4Fe-4S]2+ cluster. Reconstituted CydR specifically bound both CydR boxes cooperatively, with higher affinity for the nearer consensus +1 site. Low concentrations of O2 or NO ([O2]/[CydR] or [NO]/[CydR] = 0.1–0.6) elicited loss of the 420 nm absorbance attributed to the [4Fe-4S]2+ cluster, formation of a 315 nm species, and loss of ability to retard DNA migration. Retardation by reconstituted CydR was enhanced by superoxide dismutase and/or catalase, suggesting a role for reactive oxygen species in CydR inactivation. The role of CydR in regulating cydAB expression in the supposedly anoxic cytoplasm of A. vinelandii and similarities to cydAB regulation by FnR in Escherichia coli are discussed.

Azotobacter vinelandii is an obligate aerobe that fixes nitrogen under a wide range of oxygen concentrations, even at air saturation (1). Nitrogen fixation is an energy-demanding process that consumes 16 mol of ATP to convert 1 mol of N2 to 2 mol of NH3 (2). This energy requirement can be met only by aerobic respiration, yet paradoxically, nitrogenase is notoriously sensitive to oxygen damage (3). One way to avoid this damage is "respiratory protection," i.e. the rapid utilization of oxygen to achieve subinhibitory levels of oxygen, thus allowing the coexistence in a cell of aerobic respiration and nitrogenase activity (1, 4). To scavenge traces of oxygen yet consume excess oxygen, A. vinelandii has a branched respiratory chain with at least two routes of electron transport to oxygen (1). One branch is

terminated by an oxidase closely resembling the cytochrome bd-type oxidase of Escherichia coli and certain other bacteria (5, 6). The oxidase comprises a low-spin cytochrome bo558 and two ligand-binding hemes, cytochromes d and bo595 (previously called cytochrome a1 (8)) (7). Another respiratory branch is terminated by an oxidase of the heme-copper superfamily (9), which is probably the oxidase referred to previously as cytochrome o (8, 10). However, a gene fragment from A. vinelandii has been independently sequenced and shown to resemble fixN or ccoN encoding a cb-type cytochrome c oxidase (11). It is not clear whether cytochrome c oxidase and cytochrome o are distinct oxidases.

Direct evidence for the essential role of cytochrome bd in respiratory protection of nitrogenase and the first molecular genetic analysis of respiratory metabolism in A. vinelandii were provided by Kelly et al. (12), who obtained mutants with transposon insertions in and around that region of the genome homologous to the E. coli cydAB genes. One class of mutants had insertions within the cydAB operon, had no spectroscopically detectable cytochrome bd, and significantly, could not fix nitrogen in air. Sequencing of the entire cydAB operon (13) revealed striking similarities to the E. coli cytochrome bd-type oxidase, in accord with spectral studies (5–9, 14). Purification of A. vinelandii cytochrome bd (15, 16) confirmed similarities in subunit composition, complement of redox centers, and reactivation mechanisms in these bacteria. However, a remarkable difference between the E. coli and A. vinelandii oxidases is that the former is synthesized maximally microaerobically (17), whereas synthesis of the A. vinelandii oxidase increases with oxygen supply (1, 18). Furthermore, and consistent with the patterns of regulation, cytochrome bd in A. vinelandii has a surprisingly low affinity for oxygen (apparent KM as low as 5 nM) (14), unlike the oxidase in E. coli, which has the highest affinity yet recorded (KM as high as 5 nm) for a terminal oxidase (19).

An explanation of the different responses in these bacteria of oxidase expression to oxygen supply is now beginning to emerge. In E. coli, regulation of cytochrome bd expression is complex and coordinated by the ArcAB two-component system and by FnR, major global regulators of the aerobic/anoxic switch (20, 21). ArcA activates cydAB gene expression at low-oxygen tensions (22, 23). As oxygen tension falls further, FnR is activated and represses cydAB expression (24). Recent work has identified two cydAB promoters, but the roles played by FnR and ArcA have not been fully elucidated. Lynch and Lin (25) found three sites for ArcA, one of which (site III) was located downstream of the previously identified cydAB promoter P1 (referred to hereafter as P1). A second promoter was found downstream of this site, but could not be detected by analysis of RNA extracted from aerobically grown cells, suggesting that cydAB P1 is used preferentially under such con-
ditions. It was suggested that ArcA-P (i.e. the active phosphorylated form) bound at site III activates cydAB anoxically when Fnr prevents transcription from P1 (21). Subsequently, Cotter et al. (26) demonstrated that a single site for ArcA-P upstream of P1 was sufficient for activation of cydAB expression. Significantly, two sites for Fnr were found, one at the start of cydAB transcription at P1 and another centered 53.5 bp upstream of cydAB. Significantly, two sites for Fnr were found, one at the start of cydAB transcription at P1 and another centered 53.5 bp upstream of cydAB. Fnr prevents transcription from P1 (25). Subsequently, Cotter et al. (27) demonstrated that two sites for Fnr were found, one at the start of cydAB transcription at P1 and another centered 53.5 bp upstream of the +1 site of P1. Thus, collectively, ArcA and Fnr afford maximal cydAB expression in E. coli growing in microaerobic environments, consistent with the finding that this quinol oxidase has a remarkably high affinity for oxygen (19).

A simpler pattern of cydAB expression in response to oxygen availability is observed at the physiological level in A. vinelandii. In this strict aerobe, cydAB transcription is up-regulated as the oxygen tension, and thus danger of nitrogense damage, increases (1, 3, 4, 14). Mutagenesis in the region of the A. vinelandii cydAB gene in the gene regulatory region revealed a region in which insertions resulted in marked overproduction of the cytochrome bd complex (12). These mutants failed to grow in a microaerobic atmosphere (1.5% O2) on defined medium either containing (BSN medium) or lacking (BS medium) a supply of fixed nitrogen in the form of ammonium ions (12). This region, separated from the downstream cydAB operon by ~1 kilobase pair, was sequenced by Wu et al. (27) and revealed a gene whose deduced product is highly similar to Fnr. The gene was named cydR (a) to indicate clearly its role in the regulation of the cyd operon (this being the only operon in A. vinelandii so far demonstrated to be CydR-regulated) and (b) because the term fnr appears inappropriate since neither fumarate nor nitrate respiration occurs in A. vinelandii (27). It was postulated that CydR is a repressor of cydAB transcription (27).

Fnr senses anaerobiosis via an oxygen-labile [Fe4S4]+ cluster that promotes dimerization of the protein and enhances site-specific DNA binding (20, 21, 28–31). Homologues of Fnr control a variety of physiological functions in a diverse range of phylogenetically distinct prokaryotes (21). They are characterized by the presence of four essential cysteine residues that act as ligands for the [Fe4S4]+ cluster and the amino acid sequence EXXSR in the DNA-binding region, which confers specificity for the Fnr “box” or -binding site with the consensus sequence TT-GAT . . . ATCAA. Both of these features are conserved in CydR.

In this paper, purified CydR is shown to be an oxygen-responsive, DNA-binding protein with an exceptionally high affinity for oxygen (19). The bacterial strains and plasmids used in this study are listed in Table I. E. coli and A. vinelandii cultures were grown at 37 and 30 °C, respectively. The growth medium used for E. coli was 2× YT broth (32), and that for A. vinelandii was BSN (12).

### Table I

| Strain/plasmid | Relevant genotype/properties | Source/Ref. |
|---------------|-----------------------------|-------------|
| A. vinelandii MK8 | cydR8 | 12 |
| E. coli DH5α | supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | 12 |
| E. coli RKP3363 | DH5α/pRKP1082 | This work |
| Plasmids | | |
| pGEX-KG | GST fusion plasmid | Amersham Pharmacia Biotech |
| pG333 | cydR+ in pWSK29 | 27 |
| pMK4 | Cloned promoter of cydAB | This work |
| pMK41 | +1 CydR box mutated | This work |
| pMK435 | −50.5 CydR box mutated | This work |
| pMK4351 | Both CydR boxes mutated | This work |
| pRKP1025 | PCR fragment generated by primers RP38 and RP39 using pMK4 as template and cloned into pGEM-T-Easy | This work |
| pRKP1026 As above, but pMK41 used as template | This work |
| pRKP1028 As above, but pMK435 used as template and insert reversed | This work |
| pRKP1029 As pRKP1025, but pMK4351 used as template | This work |
| pRKP1092 Neo-HindIII fragment containing cydR cloned in pGEX-KG | This work |

* PCR, polymerase chain reaction.

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### Experimental Procedures

**Strains and Plasmids**—The bacterial strains and plasmids used in this study are listed in Table I. E. coli and A. vinelandii cultures were grown at 37 and 30 °C, respectively. The growth medium used for E. coli was 2× YT broth (32), and that for A. vinelandii was BSN (12).

**General DNA Manipulations**—Standard procedures were used for DNA isolation and manipulation (32). Enzymes for DNA manipulation were obtained from Promega, Stratagene and MBI Fermentas. DNA isolation and labeling was done with [α-32P]dCTP (Amersham Pharmacia Biotech) and Klenow fragment of DNA polymerase for use in the DNase I footprinting and gel retardation assays (see below). DNA sequencing was performed using an ABI 373A sequencer (Applied Biosystems).

**Construction of Mutant Promoters**—Plasmid pMK4 contains the entire cydAB promoter (12, 27). Site-directed mutagenesis was done using the QuickChange™ site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions. Oligonucleotides RP30 (5'-CTTGTTTATTTAACCTGGATTAACCTGCGTTAATTGCCCCC-3') and RP31 (complementary to RP30) were used to change the +1 CydR box, and oligonucleotides RP32 (5'GCAGGTTCATTACCGGCCGTTATGTGGCACC-3') and RP33 (complementary to RP32) were used to change the −50.5 CydR box. The resulting plasmids were pMK41 (mutated +1 site), pMK435 (mutated −50.5 site), and pMK4351 (both sites mutated). All mutations were confirmed by sequencing. Polymerase chain reaction fragments were generated from these plasmids with primers RP38 (5'-ATCGATGGATCCATGGTTTAGCAGCCTGCTACCCCTCC-3'), incorporating the underlined site for BamHI and RP39 (5'-CATATGATCACCCGGGACGCTCTAAAAAGGTGGATGCCC-3'). To prepare high-quality DNA for footprinting, the wild-type and mutated promoter sequences were finally cloned into the Promega pGEM-T-Easy vector according to the manufacturer's instructions. Plasmids pRKP1025 (wild-type), pRKP1026 (mutated +1 site), pRKP1028 (mutated −50.5 site), and pRKP1029 (both sites mutated) were thus obtained (Table I). The insert in pRKP1028 is in the opposite orientation from that in the other three. DNA fragments containing wild-type and mutated CydR-binding sites were isolated from plasmids pRKP1025, pRKP1026, and pRKP1029 after cutting with BamHI and ApaI and from pRKP1028 after cutting with BamHI and SacI.

### Mapping of the cydAB Transcript

The cydAB transcript was mapped by extracting total RNA by the hot phenol method (33) from a culture of A. vinelandii strain MK8. This strain carries a Tn5-B2000 mutation in cydR and consequently overproduces cytochrome bd (12, 27). It was grown under conditions of high aeration (50 ml of culture in a 1-liter conical flask shaken at 200 rpm for 7 h before harvest). Primer extension was performed according to Yague et al. (34). The primer used was purified by running a 15% sequencing gel, and the band was visualized using a "shadowing technique" against the background of a thin-layer chromatographic plate under UV light and isolated from the gel using standard procedures (32). Ten ng of 32P-end-labeled 34-mer oligonucleotide RP141 (5'-AAGATGGGATGGTTTAGCTTTAGACATGTGGGC-3') was used for each reaction. The extension was performed according to Yague et al. (34). The primer used was purified by running a 15% sequencing gel, and the band was visualized using a "shadowing technique" against the background of a thin-layer chromatographic plate under UV light and isolated from the gel using standard procedures (32). 

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1 The abbreviations used are: bp, base pair(s); GST, glutathione S-transferase.
AGCTCCCAA-3′) were mixed with 5 μg of total RNA in 30 μl of primer extension buffer (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 μM each dNTP, and 0.5 mM spermidine), heated at 75 °C for 3 min, and then cooled at room temperature for 10 min. Twenty units of Moloney murine leukemia virus reverse transcriptase (Promega) containing 5% deoxyribophosphate (1.8 μl of 125 mM solution) were added to the annealed primer/RNA mixture. The mixture was incubated at 42 °C for 30 min, and the nucleic acids were precipitated with ethanol. The DNA was resuspended in formamide loading dye and resolved on a denaturing 6% polyacrylamide gel. The sizes of the primer-extended products were determined by running a known sequence ladder (M13mp18 DNA sequenced with the M13-40 primer; U.S. Biochemical Corp.) in parallel.

Subcloning of cydR in the Gene Fusion Vector pGEX-KG—To clone cydR into the pGEX-KG vector, a NcoI site was introduced at the ATG initiation codon of cydR on plasmid pGW33 (Table I) using site-directed mutagenesis directed by oligonucleotides RP34 (5′-CAGAGAAAGTT-3′) and RP35 (complementary to RP34). These primers additionally result in changes in the first two amino acid residues of CydR from Met-Ser to Met-Ala. The plasmid pGW33 was cloned into pGEX-KG to give plasmid pRP1082.

Purification and Reconstitution of CydR—CydR was purified from French press extracts of aerobic cultures of E. coli strain RKP3363 (DH5α containing plasmid pRP1082) grown in 2× YT broth (32) plus supplements (300 μg/ml). Three hours after inoculation with an overnight culture (1.6% of the final volume), the expression of GST-CydR fusion protein according to the manufacturer’s instructions. The column was washed with Tris-buffered saline (25 mM Tris-HCl and 138 mM NaCl (pH 7.5)) containing 0.1% Triton X-100 (10% of the resin bed volume), and CydR was cleaved from GST by thrombin protease for 10 min at room temperature. For reconstitution of CydR (30), Nifs (purified from A. vinelandii) and dithiothreitol (2.5 mM final concentration) and (NH₄)₂SO₄ (10 mM) in the anaerobic cabinet. Protein concentrations were estimated by the Bio-Rad dye binding procedure with bovine serum albumin as the standard.

Spectroscopy and Inactivation of CydR—A Beckman DU Series 600 spectrophotometer or a Perkin-Elmer spectrophotometer with Winlab software was used for optical spectroscopy. CydR samples were transferred from the anaerobic cabinet in sealed cuvettes. Air-saturated H₂O (23 °C) and freshly prepared NO solution (1.9 mM in H₂O at room temperature) were prepared as described by Poole et al. (36) and were added to the sample by injection in the anaerobic cabinet.

Gel Retardation Assays—For determining CydR binding to the cydAB promoter, reconstituted CydR protein was diluted in 10 mM Tris-HCl (pH 7.0) and incubated anaerobically for 10 min with labeled promoter DNA (20–50 counts/min/μl). 150 ng of salmon sperm DNA, 2.5 μg of bovine serum albumin, 5 mM dithiothreitol, and band-shift buffer (20 mM Tris-HCl (pH 7.5), 5% glycerol, and 10 mM KCl) in a final volume of 5 μl. Sensitivity of CydR to O₂ was studied by precubing 10 μM protein (monomer) for 20 min in 10 mM Tris-HCl (pH 7.0) in the presence of mixtures of anoxic water and air-saturated water to give final concentrations of up to 60 μM O₂. Sensitivity of CydR to NO was studied by adding 100 μM NO or NaNO₂ to give final concentrations of up to 60 μM NO or NaNO₂. Superoxide dismutase (5 units; Sigma) and/or catalase (5 units; Sigma) was added to these reactions where indicated. O₂ concentrations quoted refer to those in the precubination mixture, not to the incubation with DNA. To allow CydR-DNA complex formation, 1-μl samples of the reactions were then incubated for 10 min as described above in the final volume of 5 μl.

FIG. 1. Mapping of the 5′-end of the cydAB transcript by primer extension. M13mp18 DNA sequenced with the −40 primer was used as a sequence ladder to determine the size of the extended product, which is indicated by the arrow. nt, nucleotides.

The loading of samples was performed at a voltage of 20 V after pre-running at 120 V for 5 min. Promoter DNA and purified CydR protein interactions were visualized on 5% polyacrylamide gels (19:1 acrylamide/bisacrylamide) with 25 μM Tris and 250 mM glycine (pH 8.3). The gel was run for 1 h at 60 mA for (two gels). Quantitation of shifted and non-shifted bands was performed by determining density expressed as counts/mm² using a Bio-Rad Model GS-25 Molecular Imager® system.

DNase I Footprinting—DNase I footprinting using purified CydR and DNA restriction fragments containing the cydAB promoter was performed according to Green et al. (28) and Rhodes (37), except that reactions were not extracted with phenol/chloroform. The details of the binding conditions are presented under “Results.” The C lane was included as a sequence reference to determine the location of the binding sites.

RESULTS

Mapping the Transcriptional Start Site by Primer Extension—It has been reported (38) that the cydA and cydB genes are cotranscribed and that the transcriptional start site is 275 bp upstream of the ATG initiation codon of cydA. An oligonucleotide (RP141) starting 188 bp upstream of the cydAB ATG initiation codon was therefore used for primer extension experiments. Fig. 1 shows that the transcriptional start site is actually 268–269 bp upstream of the ATG codon of cydA.

Putative −10 and −35 regions were sought. The sequence TTTATT (labeled −10 motif in Fig. 4; see later) has four matches with the −10 motif (TATAAT) characteristic of E. coli promoters. Transcriptional start sites by primer extension. The −35 sequence (TTGTCAG) that also has four matches with the E. coli consensus sequence TTGACA. However, as shown in Fig. 4, the gap between our −10 sequence and the putative −35 sequence is 24 bp, considerably exceeding the usual distance in E. coli, for example, of 17 bp.

Two sequences similar to the E. coli Fnr boxes (TTGACT . . . ATCCAA) were located in the promoter region of cydA (see Fig. 4 below), which we designate here as the +1 CydR box (TTGACT . . . ATCCAA) and the −50.5 CydR box (TTGACT . . . TTCAAA), the latter being centered 50.5 bp away from the more highly conserved +1 sequence. As marked in Fig. 4. The +1 site has one mismatch compared with the Fnr consensus sequence (39), whereas the −50.5 site has two mismatches, one in each half-site. From the beginning of one CydR box to that of the other, the distance is exactly five turns of the helix, which suggests that CydR molecules that bind to these two CydR boxes are located on the same face of the DNA and may therefore interact with each other.
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Overexpression and Purification of CydR—Using a GST fusion vector, CydR was first expressed as a GST fusion protein (GST-CydR) at levels up to 15% of soluble cell protein (Fig. 2, lane 1). Purification was then achieved by glutathione-Sepharose 4B affinity chromatography. Most of the fusion protein in the supernatant was adsorbed by this column in 30 min (Fig. 2, lane 2). After washing with Tris-buffered saline, GST-CydR was purified to near-homogeneity (Fig. 2, lane 3). CydR was then completely cleaved from GST by thrombin protease in ~30 min at room temperature (Fig. 2, lane 4). Typically, growth in the 30-liter airlift fermentor produced ~120 g of cells (wet weight), from which ~100 mg of CydR could be obtained. The cleaved CydR protein contains 15 extra amino acids at its N terminus; these do not prevent demonstration of the aerobic/analoerobic transcription switch in vitro in the case of E. coli Fnr (30).

Reconstitution of the Fe-S Cluster in CydR—Since apo-Fnr can be reconstituted to form an active protein containing two \([4\text{Fe-4S}]^{2+}\) clusters/dimer (29, 30), the same procedure was used in an attempt to reconstitute CydR. Reconstitution of CydR could be achieved in ~3 h at room temperature, but since we found that CydR is very sensitive to high temperature and denatured instantaneously at 37 °C, CydR was routinely reconstituted at 4 °C overnight. Purified Fnr protein is reported to be contaminated with some nucleic acid (30), and the high absorbance of the CydR preparation at 260 nm (data not shown) suggested the same. However, after the reconstituted CydR protein was purified through a column packed with Toyopearl ether-650M hydrophobic interaction resin, it was a straw-brown color, and the absorbance at 260 nm was greatly reduced, indicating that contaminating nucleic acids that might inhibit promoter binding were largely eliminated.

Spectra recorded during a typical reconstitution experiment are shown in Fig. 3. The signal at 420 nm attributed to reconstitution of an Fe-S cluster increased in intensity with time. The model compound \([\text{Fe}_4\text{S}_4(\text{S-Et})_4]^{2+}\) has an \( \epsilon_{280} \) of 17,200 m\(^{-1}\) cm\(^{-1}\) and an \( A_{230}/A_{280} \) ratio of ~0.7 in methylformamide, which shows only small variations with changes in solvent or thiol ligand (30). Assuming the presence of one \([4\text{Fe-4S}]^{2+}\) cluster/monomer in the reconstituted CydR protein, as in Fnr, the absorbance of the 420 nm species corresponds to a concentration of \([4\text{Fe-4S}]^{2+}\) clusters of ~60 \( \mu \text{M} \), i.e. ~40% of the anticipated concentration of protein after reconstitution. However, the final spectrum in Fig. 3, taken at 2.5 h, does not reveal the full intensity of the signal, but analysis was frustrated after longer incubations by the formation of a fine black precipitate.

The ratio \( A_{230}/A_{280} \) can also serve as a useful index of the iron-sulfur cluster content of a protein. The CydR protein contains 14 phenylalanines (\( \epsilon_{280} = 220 \text{ m}^{-1}\) cm\(^{-1}\)) and one tyrosine (\( \epsilon_{274} = 1440 \text{ m}^{-1}\) cm\(^{-1}\)), which contribute to the absorbance at 280 nm, and no tryptophan. Based on studies with Fnr (11 phenylalanines and five tyrosines) (30), we estimate that \( \epsilon_{280} \) for CydR, with a much lower tyrosine content than Fnr, is on the order of 3000. Hence, it can be calculated that the \( A_{230}/A_{280} \) ratio for CydR containing one \([4\text{Fe-4S}]^{2+}\) cluster/monomer should be ~0.62. This value is close to that calculated for Fnr (0.56) because the Fe-S cluster makes a 4–8-fold larger contribution to the absorbance at 280 nm than does the protein. The highest ratio determined experimentally for CydR was ~0.5, although as for Fnr, determination was frustrated by the persistence of absorbance at 260 nm due to nucleic acids, which artifically raises the protein assay, and by slow precipitation of material after reconstitution (see below), which contributes a turbidity “base line” to the uncorrected absorbance spectrum. Nevertheless, these values are 1.6-fold higher than the ratio measured for Fnr (30).

The reconstituted CydR protein contained ~7–8 atoms of acid-labile sulfur (mean = 7.7, S.D. = 1.9) per mol of CydR monomer, ~1.8-fold higher than measured for Fnr (30), which is thought to possess one \([4\text{Fe-4S}]^{2+}\) cluster/monomer. The unreconstituted, inactive CydR protein contained only 0.4 atoms of acid-labile sulfur (S.D. = 0.1) per mol of CydR monomer.

Interaction of CydR with Wild-type and Mutant Target Sequences—In Fnr, the paradigm for such protein-DNA binding site studies, Glu-209, Ser-212, and Arg-213 in the second helix of the helix-turn-helix motif have a significant role in the recognition of an Fnr box (39). These amino acid residues are conserved in CydR, suggesting that CydR and Fnr will recognize very similar DNA-binding sequences. To determine whether the putative CydR boxes are actually recognized by CydR, mutations were made (Fig. 4) in which the central G, which interacts with Glu-209, was mutated to A, and the corresponding C, in the second half of the box, was changed to T. The interaction of CydR with its target sequences was then analyzed by gel retardation. Fig. 5 shows that the specific retardation was seen only with the wild-type sequence at CydR concentrations as low as ~5 \( \times \) \( 10^{-7} \) and ~5 \( \times \) \( 10^{-6} \) M (monomer). Little retardation could be detected when the +1 CydR box, the −50.5 box, or both were mutated even at a CydR monomer concentration of ~5 \( \times \) \( 10^{-5} \) M. The unreconstituted
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**Fig. 4.** The promoter region of cydAB showing putative wild-type and mutant sites for CydR binding. Plasmid pMK4 contains the wild-type sequence; pMK41 is mutated in the +1 CydR box; pMK435 is mutated in the −50.5 CydR box; and pMK4351 has mutations in both CydR boxes. The black circles indicate transcriptional start sites identified by primer extension analysis. The thick bar labeled “−10” shows the probable −10 motif, consistent with the experimentally determined start site (see “Results”); the thin bar labeled with a question mark shows the putative −35 site identified in Ref. 38.

**Fig. 5.** Gel retardation assays with radiolabeled wild-type and mutant cydAB promoter DNAs. Four 203-bp fragments were amplified by polymerase chain reaction from corresponding plasmids with primers RP38 and RP39 (see “Experimental Procedures”). Plasmids were digested with NcoI and end-labeled. Lanes labeled A, B, C, and D were obtained using target DNAs from plasmids pMK4 (wild-type), pMK41 (mutations in the +1 CydR box), pMK435 (mutations in the −50.5 CydR box), and pMK4351 (mutations in both CydR boxes), respectively. Lanes labeled 0 show migration in the absence of CydR protein. Lanes labeled 8, 7, 6, and 5 show migration in the presence of CydR protein at final concentrations of 0.05 × 10⁻⁵ M, 0.5 × 10⁻⁵ M, 0.5 × 10⁻⁶ M, 0.5 × 10⁻⁷ M, and 0.5 × 10⁻⁸ M (calculated as monomer), respectively.

CydR protein (up to 0.5 × 10⁻⁵ M) did not retard the same DNA fragment (data not shown).

DNase I footprint analysis confirmed that CydR protects the CydR boxes in the +1 and −50.5 regions (Fig. 6). The concentration necessary for protecting both the CydR boxes (pMK4) was −2 × 10⁻⁷ M (monomer). DNA protection was lost when the G or C nucleotides were changed in the two half-sites of the CydR boxes (pMK435). Interestingly, mutation of the +1 CydR box modified CydR protection at the −50.5 region. In pMK41, a higher CydR concentration (2 × 10⁻⁶ M) was required to protect the −50.5 CydR box. In contrast, mutation of the −50.5 site (pMK435) did not change the CydR concentration required for protecting the +1 site. These results allow us to distinguish between a primary and a secondary binding site, with CydR showing a higher affinity for the +1 CydR box.

**Interaction of CydR with Oxygen**—In Fnr, reaction with oxygen of the [4Fe-4S]²⁺ cluster in the reconstituted protein produces a non-DNA-binding, transcriptionally inactive form (31). Since A. vinelandii is an obligate aerobe that must maintain an effectively anoxic cytoplasm for nitrogenase function, it was of interest to compare the sensitivity of CydR and Fnr to oxygen. Sequential additions of O₂ (as O₂-saturated buffer) were made to the reconstituted CydR protein in a volume of 0.4 ml in a sealed cuvette and monitored by UV-visible electronic spectroscopy (Fig. 7A). The same samples were subsequently used for gel retardation experiments after suitable dilution. Slight precipitation of material during the titration caused absorbance at all wavelengths to rise, but particularly at lower wavelengths, consistent with the increased turbidity. Nevertheless, an obvious change superimposed upon the turbidity signals was the increase of a peak centered at 420 nm and a decrease of the peak at 420 nm tentatively attributed to a [4Fe-4S]²⁺ cluster. To minimize the effect of the base-line shift, changes in absorbance at 315 nm were expressed as the absorbance difference between 315 and 295 nm, and the changes at 420 nm were expressed as the difference between 420 and 500 nm. Fig. 7A shows that nearly half of the absorbance change at 420 nm was lost on adding oxygen to give an O₂/[CydR] ratio of −0.03. Adding oxygen to give an O₂/[CydR] ratio of −0.1 almost eliminated the A₄₂₀ signal. The absorbance at 315 nm increased as the preparation was exposed to small amounts of air, but was not seen when reconstituted CydR was fully exposed to air. The maximal absorbance was reached when the O₂/[CydR] ratio was −0.1.

**Interaction of CydR with NO and Reactive Oxygen Intermediates**—Redox-sensitive metal clusters of proteins are sensitive...
not only to O\textsubscript{2}, but may also be sensitive to degradation by radicals of biological significance such as NO (40). In light of this and considering that \textit{A. vinelandii}, a soil bacterium, is likely to encounter NO produced by denitrifying bacteria, we studied the effects of NO on CydR. Successive additions of NO (as a strictly anoxic gas-saturated solution) and monitoring of spectral changes (Fig. 7B) showed that the effects of NO were very similar to those of oxygen, both with respect to the nature of the spectral changes and the \([NO]/[CydR]\) ratio required for abolition of the 420 nm signal (Fig. 7B).

Parallel gel retardation experiments demonstrated the sensitivity of the DNA-binding properties of CydR to O\textsubscript{2}, NO, and partial reduction properties of O\textsubscript{2}. (Fig. 8). Reconstitution of CydR using the procedures described above gave a preparation that significantly retarded DNA migration (Fig. 8A, compare lane 2 with lanes 1 and 3). However, the presence of catalase (Fig. 8A, lane 4), superoxide dismutase (lane 5), or both (lane 6) significantly enhanced DNA retardation, suggesting a role for reactive oxygen intermediates in modulating CydR binding.

Increasing concentrations of oxygen, introduced before protein-DNA interaction, progressively inhibited CydR-DNA interactions (Fig. 8, A, lanes 7, 9, and 11; and B, bars 7, 9, and 11). An \([O_2]/[CydR]\) ratio of \(-0.6\) (6.25 \(\mu\)M O\textsubscript{2}) was sufficient to prevent DNA retardation. Again, catalase and/or superoxide dismutase provided protection (Fig. 8, A, lanes 8, 10, and 12; and B, bars 8, 10, and 12). Intermediate \([O_2]/[CydR]\) ratios gave intermediate results (data not shown).

An \([NO]/[CydR]\) ratio of 0.6 (6.25 \(\mu\)M NO) was equally effective in preventing DNA retardation by CydR. The presence of O\textsubscript{2} in NO solutions forms nitrite ion, and so control lanes (Fig. 8A, lanes 13–18) were set up with nitrite at concentrations similar to those of NO. No significant effects were observed in these cases.

**DISCUSSION**

An absolute requirement for aerotolerant nitrogen fixation in \textit{A. vinelandii} appears to be synthesis of the quinol oxidase cytochrome \textit{bd} (1, 12). We have previously shown genetically that transcription of the cyd\textit{AB} operon, encoding the two subunits of cytochrome \textit{bd}, is repressed by CydR (27) and that mutation of CydR causes elevation of oxidase synthesis (12, 27). Cytochrome \textit{bd} levels, as well as cytochrome \textit{bd}-specific mRNA, increase in a wild-type strain when the oxygen concentration increases under non-nitrogen-fixing conditions (18, 27). The converse is true in \textit{cydR} mutants, i.e. the cytochrome \textit{bd} concentration increases sharply when the oxygen concentration decreases. The trends are the same in cells grown under nitrogen-fixing conditions.2

The transcriptional start site of cyd\textit{AB} was mapped in this work to 268–269 bp upstream of the cyd\textit{AB} ATG translational initiation codon by primer extension. This method is probably more precise than the ribonuclease protection assay (38), which placed the transcriptional start site at 275–277 bp upstream of the cyd\textit{AB} translational initiation site. We and Moshiri \textit{et al.} (38) have not obtained any evidence for a second promoter for

\[ ^2 \text{S. E. Edwards, S. Hill, and R. K. Poole, unpublished results.} \]
the cydAB operon in *A. vinelandii*. The transcriptional start site is the same under both nitrogen-fixing and non-nitrogen-fixing conditions (38). Putative −10 and −35 regions were identified based on similarity to the promoters recognized by *E. coli* σ^54^. However, *A. vinelandii* RNA polymerase may recognize different promoter sequences, and further promoter analysis is required. Moshiri *et al.* (13) showed that the cloned *A. vinelandii* cydAB genes in *E. coli* could reconstitute cyanide-insensitive respiratory chain activity from NADH to O_2, but not succinate- or lactate-dependent respiration, and that cytochrome *d* was detectable spectroscopically. In another study, however, the cloned cydAB genes did not complement an *E. coli* mutant for growth on Zn^2+^- and azide-containing medium, and no cytochrome *d* was detected spectroscopically (41). Moshiri *et al.* (38) demonstrated that the cydAB genes are up-regulated under nitrogen-fixing conditions in a σ^54^-dependent manner. However, the promoter of cydAB does not resemble the typical σ^54^-dependent promoter, but is more similar to the *E. coli* σ^70^-dependent promoter. It is possible that σ^24^ regulates the expression of cytochrome *bd* indirectly.

The CydR protein has now been purified by glutathione-Sepharose 4B affinity column chromatography to near-homogeneity. The aerobically purified CydR protein can be reconstituted into an active form, as can the aerobically purified *E. coli* Fnr protein (29, 30), and only in this state binds to target sequences in the cydAB promoter identifiable by footprinting studies and similarity to Fnr boxes. Two CydR-protected regions were seen at this promoter, as was also revealed in *E. coli* (26) by DNase I footprinting studies of Fnr binding to the cydAB regulatory region. One region of 25 bp extends from positions −17 to +7 and thus overlaps the transcrptional start site (+1). Another region of 24 bp extends from positions −61 to −38 and is centered at position −50.5 relative to the same start site. This arrangement of CydR (Fnr)-binding sites is very similar to that of the cydAB promoter in *E. coli*, in which the Fnr sites extend from positions −13 to +10 and from positions −67 to −45, respectively, with reference to the start of P1 transcription. Only one putative Fnr site was considered by Lynch and Lin (25). Thus, in both *A. vinelandii* and *E. coli*, an Fnr-like protein acts directly to repress cydAB gene expression. In *E. coli*, however, both sites are bound by Fnr with similar affinity, and the sites become occupied simultaneously as the protein concentration is increased (26). In *E. coli*, the upstream site centered at position −53.5 is identical to the consensus sequence, whereas the site at the start of cydAB transcription has a single mismatch. In *A. vinelandii*, however, CydR binds both sites, but with higher affinity for the +1 CydR box. This may reflect the closer match of the +1 site to the Fnr consensus sequence. “Anaerobic” cydAB repression in *A. vinelandii* may involve the binding of two pairs of CydR monomers over the +1 and −50.5 regions, which prevents essential RNA polymerase-DNA contacts. CydR binding to the primary (high-affinity) +1 site could cooperatively help CydR binding to the secondary (low-affinity) −50.5 site.

Thus, even in this obligately aerobic bacterium, cydAB transcription is still regulated in response to O_2_. However, whereas the absorbance loss at 420 nm of the Fnr protein in *E. coli* requires an [O_2]/[Fnr] ratio of −1 (31), a ratio of only −0.1 is sufficient to cause loss of the distinctive 420 nm band of the Fe-S cluster in CydR. Furthermore, an [O_2]/[CydR] ratio of −0.6 significantly prevents the retardation by CydR of its target DNA, whereas a ratio of 3 is needed to abolish the retardation by Fnr of its target DNA (31). This observation is perhaps not surprising given the requirement in this organism that the cytoplasmic oxygen tension should be maintained at very low levels, despite ready penetration of oxygen through the cytoplasmic membrane from an external growth environment that may be air-saturated. Although intracellular oxygen levels have not been measured in *A. vinelandii* (or any other bacterium), CydR appears to be a highly sensitive monitor of cytoplasmic oxygen, as anticipated for continued operation of nitrogenase under highly aerobic growth conditions. We envisage that, during growth under microaerobic conditions, intracellular oxygen concentrations are sufficiently low to allow nitrogenase function and that CydR would be active, repressing cydAB expression. Under conditions of stress imposed by high oxygen, the repressed levels of cytochrome *bd* may not maintain the essentially anoxic state of the cytoplasm that is required for nitrogenase and CydR will be inactivated; this in turn derepresses cytochrome *bd* synthesis, which provides respiratory protection.

Several lines of evidence indicate that cysteine-rich motifs of metal-binding proteins and redox-sensitive metal clusters of metalloproteins are natural biosensors not only of O_2_ and Fe (40), but also of NO (42). Fe-S-containing proteins like dehydratases have long been known as targets of O_2_ and H_2O_2_ (43) and are also targets of NO. NO forms complexes with Fe-S clusters in model compounds (44), and mitochondrial Fe-S enzymes are inhibited by NO (45). Aconitase is especially sensitive to NO, but it has recently been proposed that the peroxynitrite anion (ONOO⁻), formed in the reaction of NO with O_2_, is the inactivating species (46, 47). Regulatory Fe-S-containing proteins like SoxR and mammalian iron-responsive element-
binding protein 1 have also been shown to be NO-sensitive (40, 48, 49), but it was not known whether Fnr is sensitive to NO or insensitive, as is the molybdenum-metalloenzyme xanthine oxidase (50). We now show for the first time that a member of the Fnr family is inactivated by NO as well as by oxygen. The mechanism of this inactivation needs further study. The physiological function of the effects of NO on CydR, if any, are unclear. However, although A. vinelandii is not itself a denitrifying bacterium, it inhabits environments where other bacteria produce NO as an intermediate in this pathway. NO may derepress cytochrome bd so that nitrogenase is protected by respiration and able to exploit the end product of denitrification, namely dinitrogen.

The peak observed in absorbance spectra at 315 nm formed after adding O2 or NO may be a breakdown product of the [4Fe-4S]2+ cluster or reflect the presence of substoichiometric iron levels in the protein (51). Several studies of Fnr including recent Mössbauer spectroscopy (52) show that the loss of the 420 nm-absorbing form of Fnr is due to conversion of the [4Fe-4S]2+ cluster to a [2Fe-2S]2+ cluster. Only stoichiometric amounts of O2 are needed for this inactivation, whereas ferri-cyanide is required in considerable excess (31), suggesting that Fnr is a true O2 sensor. The greater sensitivity of CydR to oxygen than of Fnr suggests that even substoichiometric amounts of O2 are adequate for cluster inactivation. A plausible mechanism that accounts for the oxygen sensitivity of the [4Fe-4S]2+ clusters of Fnr-like proteins and the biphasic nature of the response (40) requires a rapid oxygen-driven, redox-balanced conversion of [4Fe-4S]2+ to [2Fe-2S]2+ via a [3Fe-4S]1+ intermediate. In this process, O2 oxidizes [4Fe-4S]2+ to [4Fe-4S]1+, releasing Fe2+ and generating superoxide anion. The superoxide (generated close to the [3Fe-4S]1+ cluster) reduces it, yielding [2Fe-2S]2+ and Fe2+ and regenerating O2, which is free to attack further [4Fe-4S]2+ clusters. That the presence of superoxide dismutase and catalase enhances DNA damage may be a breakdown product of the [4Fe-4S]2+ cluster or reflect the presence of substoichiometric iron levels in the protein (51). Several studies of Fnr including recent Mössbauer spectroscopy (52) show that the loss of the 420 nm-absorbing form of Fnr is due to conversion of the [4Fe-4S]2+ cluster to a [2Fe-2S]2+ cluster. Only stoichiometric amounts of O2 are needed for this inactivation, whereas ferri-cyanide is required in considerable excess (31), suggesting that Fnr is a true O2 sensor. The greater sensitivity of CydR to oxygen than of Fnr suggests that even substoichiometric amounts of O2 are adequate for cluster inactivation. A plausible mechanism that accounts for the oxygen sensitivity of the [4Fe-4S]2+ clusters of Fnr-like proteins and the biphasic nature of the response (40) requires a rapid oxygen-driven, redox-balanced conversion of [4Fe-4S]2+ to [2Fe-2S]2+ via a [3Fe-4S]1+ intermediate. In this process, O2 oxidizes [4Fe-4S]2+ to [4Fe-4S]1+, releasing Fe2+ and generating superoxide anion. The superoxide (generated close to the [3Fe-4S]1+ cluster) reduces it, yielding [2Fe-2S]2+ and Fe2+ and regenerating O2, which is free to attack further [4Fe-4S]2+ clusters. That the presence of superoxide dismutase and catalase enhances DNA damage may be a breakdown product of the [4Fe-4S]2+ cluster or reflect the presence of substoichiometric iron levels in the protein (51). Several studies of Fnr including recent Mössbauer spectroscopy (52) show that the loss of the 420 nm-absorbing form of Fnr is due to conversion of the [4Fe-4S]2+ cluster to a [2Fe-2S]2+ cluster.
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