Unexpected NO-dependent DNA binding by the CooA homolog from Carboxydothermus hydrogenoformans

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Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved November 18, 2005 (received for review July 13, 2005)

CooA, the CO-sensing heme protein from Rhodospirillum rubrum, regulates the expression of genes that encode a CO-oxidation system, allowing R. rubrum to use CO as a sole energy source. To better understand the gas-sensing regulation mechanism used by R. rubrum CooA and its homologs in other organisms, we characterized spectroscopically and functionally the Fe(II), Fe(II)-NO, and Fe(II)-CO forms of CooA from Carboxydothermus hydrogenoformans. Surprisingly, and unlike R. rubrum CooA, C. hydrogenoformans CooA binds NO to form a six-coordinate Fe(II)-NO heme that is active for DNA binding in vitro and in vivo. In contrast, R. rubrum CooA, which is exclusively specific for CO, forms a five-coordinate Fe(II)-NO adduct that is inactive for DNA binding. Based on analyses of protein variants and temperature studies, NO-dependent DNA binding by C. hydrogenoformans CooA is proposed to result from a greater apparent stability of the six-coordinate Fe(II)-NO adduct at room temperature. Results from the present study strengthen the proposal that CO specificity in the CooA activation mechanism is based on the requirement for a small, neutral distal ligand, which in turn affects the relative positioning of the ligand-bound heme.

In addition to the well established roles of the heme cofactor in transporting and storing oxygen, mediating electron transfer processes, and catalyzing covalent transformations, a recently recognized function of this cofactor is as a gas-sensing regulator (1, 2). Examples of this class are NO-sensing soluble guanylyl cyclase (sGC), oxygen-sensing FixL, histidine kinase, and CO-sensing CooA. The primary function of heme in gas-sensing heme proteins is to selectively bind a small gas molecule and activate a protein response, typically by an allosteric conformation change that occurs upon a change in the heme environment (3, 4). For the transcription factor CooA, CO binding to heme enables CooA to bind DNA (3, 4), which results in the expression of a CO oxidation system that allows Rhodospirillum rubrum to grow using CO as a sole energy source (5).

CooA from R. rubrum is the paradigm of the CooA family. A member of the cAMP receptor protein and fumarate and nitrate reductase superfamily of transcriptional regulators, CooA is a homodimer that contains one h-type heme per 25-kDa monomer and is capable of sensing both redox state and CO (3, 4). Like other members of this family, CooA is arranged into two major domains: a regulatory, effector-binding domain that contains the hemes and a DNA-binding domain. Two long α helices (the C helices) form a leucine-zipper motif at the dimer interface of the protein, and play an important role in transmitting the CO signal from the effector-binding domain to the DNA-binding domain (6). Consistent with the redox requirements of the CooA-regulated CO dehydrogenase (7), CooA only binds to DNA under reducing conditions when exposed to CO (3, 4).

Each CooA heme is axially ligated by two endogenous donors in the Fe(III) and Fe(II) states, and CO binding to the Fe(II) state results in the displacement of the distal ligand (3, 4). In the Fe(II) state, Cys-75 is replaced by His-77 via a redox-mediated ligand switch, and Pro-2 is retained as the distal ligand. This ligand switch is reversible upon oxidation, as occurs upon reaction with molecular oxygen. Finally, CO addition displaces the distal Pro-2 ligand in the Fe(II) state (3, 4) and weakens the proximal Fe-His bond (9). Ultimately, changes to the heme coordination environment that occur upon CO binding enable CooA DNA binding via an activation mechanism that, although not fully understood, involves the relative repositioning of the heme and C helices (6, 9).

A recent analysis of 207 bacterial genomes yielded eight sequences in six organisms that were >50% identical to CooA from R. rubrum (10). Sequence alignment of these homologs showed significant conservation of R. rubrum CooA (Rr CooA) residues known to be critical for protein function, including the invariant histidine residue that serves as the proximal ligand in Fe(II) Rr CooA. However, only cooA from R. rubrum and one of the two cooA genes of C. hydrogenoformans (termed 2340 in the initial publication) encode the cysteine residue (Cys-75 in R. rubrum and Cys-80 in C. hydrogenoformans) that, in the case of Rr CooA, participates in a redox-mediated ligand switch (3, 4). When overexpressed in Escherichia coli, most of the CooA homologs displayed CO-dependent DNA binding in vitro and in vivo, providing evidence that these homologs are CooA proteins (10).

Initial characterization of C. hydrogenoformans ‘2340’ CooA (Ch CooA) indicated its heme environment is similar but not identical to that of Rr CooA. In the first investigations of the spectroscopic and functional properties of Ch CooA, several similarities and differences between the heme coordination environments of Ch and Rr CooA were observed (10, 11). Foremost, electronic absorption spectra of the Fe(II) and Fe(II)-CO states of Ch CooA were similar to analogous spectra of Rr CooA, suggesting the heme environments of the Fe(II) and Fe(II)-CO states of the two proteins are comparable (10). Key features in resonance Raman (rR) spectra of the Fe(II) and Fe(II)-CO forms of Ch and Rr CooA generally supported this conclusion (11). Based on mutational studies and sequence similarity, His-82 (His-77 in Rr CooA) and the N-terminal amino acid are likely to be the heme ligands in Fe(II) Ch CooA, analogous to Rr CooA (10, 11). Likewise, the distal ligand in Fe(II) Ch CooA is believed to be displaced upon CO binding. In contrast, the Fe(III) states of Ch and Rr CooA appear to be different from one another. Spectroscopic analyses of WT Ch CooA and variants determined that Fe(III) Ch CooA does not use Cys-80 as the proximal heme ligand, but instead retains His-82 as the ligand in the Fe(III) state (10, 11).

Consistent with the altered ligand arrangement of Fe(III) Ch CooA, the redox potential of the Ch CooA heme (approximately +250 mV) is dramatically higher than the Rr CooA heme (approximately −300 mV) (3, 4, 11). The pronounced difference between the
redox potentials of Ch and Rr CooA has been proposed to relate to the ability of Rr CooA, but not Ch CooA, to sense redox state at physiological conditions (11).

In this study, we report the unexpected discovery that Ch CooA is capable of sensing both NO and CO under standard assay conditions, unlike Rr CooA, which is exclusively specific for CO (12). Along with proteins such as soluble guanylyl cyclase (sGC) (1, 2) and possibly heme-regulated eukaryotic initiation factor 2α kinase (HRI) (13), Ch CooA is one of the few examples where NO binding to heme has been shown to substantially regulate protein function. Intriguingly, the ability of Ch CooA to form a six-coordinate Fe(II)-NO heme may be the critical factor that leads to the NO-dependent activation of Fe(II)-NO Ch CooA. Although NO sensing by Ch CooA is unlikely to be physiologically relevant, the reported spectroscopic and functional characterizations of Fe(II)-NO Ch CooA are of significance to the general heme-mediated activation mechanism of CooA proteins. The results from the studies described herein provide a more complete picture of the strategy by which CooA proteins ensure CO-specific activation, and thus add to our knowledge of functional mechanisms in gas sensing heme proteins.

**Results**

**CooA from C. hydrogenoformans Exhibits NO-Dependent DNA Binding.** Previous DNase I footprinting experiments and fluorescence anisotropy DNA-binding assays demonstrated that Fe(II) Rr CooA interacts with its target P_cooF promoter sequence only in the presence of the physiological CO effector (12). To determine whether Fe(II) Ch CooA also displays CO-specific DNA binding, fluorescence anisotropy assays were performed for Ch CooA samples treated with NO or CO gases. Unexpectedly, we discovered that Fe(II) Ch CooA binds to a P_cooF DNA sequence with high affinity when incubated with either CO or NO (Fig. 1). Fitting analyses indicated the DNA-binding affinity of NO-treated Fe(II) Ch CooA (K_d = 20 nM) is similar to the DNA-binding affinities of CO-treated Fe(II) Ch CooA (K_d = 25 nM) and Fe(II)-CO Rr CooA (K_d = 23 nM). As expected, Ch CooA did not bind to DNA in the Fe(II) state in the absence of effector gases, consistent with the previously observed behavior of Rr CooA (3). Also, the addition of 100 mM imidazole or CN^- failed to induce DNA binding (data not shown), consistent with earlier data that showed these ligands do not perturb the Fe(II) Ch CooA heme spectrum (10).

Results from in vivo β-galactosidase activity assays are also consistent with NO-dependent Ch CooA DNA binding. E. coli reporter strains that contained genes encoding the Ch or Rr CooA proteins were streaked on plates supplemented with differing levels of the NO-precursor NaNO_3_ and were assayed for β-galactosidase activity as visualized by a blue/white screen (Fig. 2). Several laboratories have provided evidence that the nonnitrifying bacterium E. coli likely generates trace amounts of NO during anaerobic nitrate respiration (14–16). As observed in Fig. 2, only E. coli strains having genes that encode for Ch CooA proteins turned blue during growth (showed β-galactosidase activity), implying that only Ch CooA displayed NO-dependent DNA binding, although all Ch or Rr CooA-containing strains turned blue if they were exposed to CO (Fig. 6, which is published as supporting information on the PNAS web site). Comparison of Fig. 2 A and B shows that the progression of the blue color correlates with NaNO_3_ concentrations. Finally, the in vivo response exhibited by Ch CooA proteins is likely caused by endogenous NO and not by nitrate ions directly; treatment of purified Ch CooA samples with 50 mM NaNO_3_ neither activated the protein for in vitro DNA binding nor induced changes in the heme electronic absorption spectrum, indicating that NO_3^- does not act as a heme ligand (data not shown).

**WT Ch CooA Forms a Six-Coordinate Fe(II)-NO Adduct.** Characterization of Fe(II)-NO Ch CooA by electronic absorption spectroscopy strongly suggests the NO-bound heme is six-coordinate in contrast to the case of Rr CooA, which forms a five-coordinate Fe(II)-NO heme. Although NO typically exerts a strong negative trans influence when bound to a heme iron and makes the formation of a five-coordinate Fe(II)-NO adduct more favorable...
by weakening the trans metal-ligand bond, the local protein environment can play a significant role in governing whether a five- or six-coordinate Fe(II)-NO heme is formed (16–19). Previous studies performed on Rr CooA showed that NO binding results in the release of both protein-derived axial ligands, yielding a five-coordinate Fe(II)-NO heme that is inactive for DNA binding (12). In contrast, anaerobic treatment of Fe(II) Ch CooA with NO gas resulted in the appearance of unique features in the electronic absorption spectrum that were distinct from those of Fe(II)-NO Rr CooA (Fig. 3). The shapes, intensities, and peak positions of the Soret, α, and β bands of Fe(II)-NO Ch CooA are like those observed for six-coordinate Fe(II)-NO hemes, suggesting that Fe(II)-NO Ch CooA is also six-coordinate (Table 1, which is published as supporting information on the PNAS web site).

EPR spectroscopy confirms the assignment made by electronic absorption spectroscopy and provides insight of the nature of the axial ligand trans to NO. Whereas EPR spectra of five-coordinate Fe(II)-NO hemes are dominated by an intense three-line pattern near g = 2.00, spectra of six-coordinate Fe(II)-NO hemes appear more rhombic, with features occurring at both g > 2 and g < 2 (20–22). In spectra of six-coordinate Fe(II)-NO hemes, the positions of the g values and the possibility of superhyperfine-coupling interactions between the unpaired electron and the trans donor can provide insight of the identity of the ligand trans to NO (23). The EPR spectrum of Fe(II)-NO Ch CooA exhibits features diagnostic of a six-coordinate Fe(II)-NO heme (Fig. 4 and Table 2, which is published as supporting information on the PNAS web site) and is similar to the spectrum reported for Fe(II)-NO myoglobin (20, 22). The EPR spectrum contains both a rhombic component (g values = 2.08, 2.04, 1.98) and a putative axial component based on the feature present at g = 2.04. For the globins, the axial and rhombic components are believed to arise from different orientations of the bound NO, although the nature of the axial component is still the subject of investigation (24). Like Mb and other globin proteins, the relative amounts of the rhombic and axial components of Fe(II)-NO Ch CooA varied with temperature, and the rhombic component became more apparent as the temperature was decreased (data not shown). The calculated g values and observed nine-line hyperfine/superhyperfine coupling pattern of the rhombic component of Fe(II)-NO Ch CooA provide compelling evidence that the trans ligand is a nitrogenous donor (Table 2). Finally, the amount of the rhombic component observed in Ch CooA samples varied substantially with sample preparations, reflecting possible protein conformational or structural heterogeneity that affects the local heme coordination structure. We believe that this heterogeneity may result from a steric constraint that is created if the nearby Cys-80 is S-nitrosylated in the presence of excess NO (25). In support of this explanation, we found that Fe(II)-NO C80S Ch CooA samples consistently yielded more uniform, well resolved rhombic EPR spectra (Fig. 7, which is published as supporting information on the PNAS web site).

In Ch CooA, the ligand trans to NO should either be His-82 or the distal axial ligand, which is believed to be the N-terminal amino group (10, 11). We favor the former hypothesis because (i) H82A Ch CooA loses heme during protein purification (11) suggesting that His-82 is critical for heme retention; (ii) a Ch CooA variant with a shortened N-terminal sequence forms a six-coordinate Fe(II)-NO adduct (data not shown) that is active for DNA binding (Fig. 2), consistent with the displacement of the N terminus by NO; and (iii) since the relative heme position is important for the activation of CooA, it is more likely that NO would form an adduct similar to that of CO, because they both lead to CooA activation.

**Protein Variant and Temperature Studies Investigating the Origin of NO-Dependent Ch CooA DNA Binding.** Because Ch CooA forms a six-coordinate Fe(II)-NO heme that is active for DNA binding, whereas Rr CooA forms a five-coordinate Fe(II)-NO heme that is inactive for DNA binding, we reasoned that a six-coordinate heme may be critical for achieving the active conformation. To test this hypothesis, we explored three possible factors that might lead to the preferential stabilization of the six-coordinate Fe(II)-NO Ch CooA species: residues near the bound NO, residues near the trans His ligand, and an inherently greater stability of Ch CooA.

The first hypothesis was that direct interactions between NO and neighboring amino acids on the distal side of heme might attenuate the trans influence of NO and account for the formation of a six-coordinate Fe(II)-NO heme in Ch CooA. Examination of the amino acid sequences expected to be in the distal heme environments of the two proteins revealed the presence of two lysine residues (Lys-119 and Lys-126) in the Ch CooA sequence that were not in the Rr CooA sequence (Fig. 8, which is published as supporting information on the PNAS web site). We hypothesized that electrostatic interactions between these lysine residues and the bound NO ligand might weaken the NO trans influence, lessening the ability of NO to displace His-82 in Ch CooA. To test this hypothesis, we characterized CooA variants having single or double substitutions, where the lysine residues in Ch CooA were replaced with corresponding or...
similar residues from *R. cooA* (K119A, K126A, K126T, and K119A K126A Ch CooA); we also examined T121K R. cooA. As illustrated by electronic absorption spectra (Table 1) and in vivo (Fig. 2) and *in vitro* (data not shown) activity assays, none of these substitutions altered the NO-response of the respective proteins. These results rule out the hypothesis that direct interactions between NO and neighboring lysines are the critical feature of *C. cooA*.

A second explanation for why *C. cooA* forms a six-coordinate Fe(II)-NO heme is that the conserved proximal histidine ligand (His-82) that is trans to NO in *C. cooA* might be more tightly bound to the heme iron than the analogous histidine ligand (His-77) in *R. cooA*. Theoretical studies of Fe(II)-NO heme models suggested that hydrogen-bonding interactions to the proximal histidine ligand affect whether the Fe-His bond is cleaved upon NO binding (18). There are no striking differences in the amino acid sequences of *Ch* and *Rr* CooA near the proximal heme environments, i.e., both homologs contain an asparagine residue (Asn-42 in *Rr* CooA) that is proposed to form a hydrogen bond with the proximal His-77 ligand in *Rr* CooA (26). However, *Rr* CooA, unlike *C. cooA*, undergoes a redox-dependent ligand switch (3, 4). Because the proximal heme environment of *Rr* CooA is likely to be more flexible than that of *C. cooA* due to this ligand switch, it is conceivable that the hydrogen bond formed between Asn-42 and His-77 in Fe(II) *Rr* CooA is weaker than the corresponding interaction in *C. cooA*. To optimize possible hydrogen-bonding interactions between His-77 and the residue at position 42 in *Rr* CooA, Asn-42 was mutated to an aspartic acid residue (N42D *Rr* CooA), and NO-binding studies were performed. Electronic absorption spectroscopy indicated that N42D *Rr* CooA does not form a six-coordinate Fe(II)-NO adduct upon NO addition, but instead forms a five-coordinate NO adduct like that of WT *Rr* CooA (Fig. 9, which is published as supporting information on the PNAS web site) (12). This result does not eliminate the possibility that His-77 is more weakly bound to the heme iron in *Rr* CooA than His-82 is bound to the heme iron in *C. cooA*; however, the result suggests that a stronger bond does not account for why *C. cooA* is capable of forming a six-coordinate Fe(II)-NO heme.

The final explanation tested for why *C. cooA* forms a six-coordinate Fe(II)-NO heme is that *C. cooA* is from a thermophilic organism and may be inherently more stable than *Rr* CooA at ambient temperature. To test this hypothesis, electronic absorption spectra of the Fe(II), Fe(II)-CO, and Fe(II)-NO states of *C. cooA* were recorded at several temperatures ranging from 25°C to ~70°C. Unlike Fe(II) and Fe(II)-CO *C. cooA*, which showed only modest changes as a function of temperature (data not shown), pronounced changes were observed in spectra of Fe(II)-NO *C. cooA* (Fig. 5). Most notably, the Soret feature diagnostic for a six-coordinate Fe(II)-NO heme at 418 nm decreased in intensity, and a new Soret feature concomitantly appeared near 400 nm, suggesting the formation of a five-coordinate Fe(II)-NO heme. Although temperatures ~70°C resulted in irreversible protein precipitation under our *in vitro* conditions and prevented observation of the fully converted five-coordinate species, temperature-dependent changes between 25°C and ~70°C were fully reversible, suggesting that His-82 is bound to the heme iron at room temperature and dissociates at elevated temperatures.

Although it is not practical to perform the *in vitro* DNA-banding assays at high temperatures because of multiple complicating temperature effects, we have shown that the coordination number of Fe(II)-NO *C. cooA* likely varies with temperature. Based on the known physiology of *C. hydrogenoformans* (27, 28), which almost certainly utilizes a CooA protein to regulate the expression of genes for CO oxidation, the observations that the five-coordinate Fe(II)-NO state of *C. cooA* appears to predominate at higher temperatures and that *Rr* CooA forms a five-coordinate Fe(II)-NO heme that is inactive for DNA binding are consistent with the hypothesis that Fe(II)-NO *C. cooA* will be unable to bind DNA at temperatures relevant for *C. hydrogenoformans* growth. An implication of this hypothesis is that NO-dependent DNA binding by *C. cooA* may result from its ability to form a stable five-coordinate Fe(II)-NO adduct at room temperature. To determine whether lowering the temperature affected WT *Rr* CooA heme coordination, we formed the Fe(II)-NO adduct of this protein at 4°C and room temperature by using standard buffer conditions and at ~6°C using 30% ethylene glycol as a cryogenic solvent. Although the five-coordinate species predominated at all temperatures tested, spectra of samples at 4°C and ~6°C showed a shoulder near 420 nm, suggesting that 10–20% of the sample may have converted to the six-coordinate Fe(II)-NO form (data not shown). These results are consistent with the view that the thermophile *C. cooA* forms a six-coordinate Fe(II)-NO heme at room temperature because of its increased relative overall stability.

**Discussion**

Effectors binding is responsible for changes to the CooA heme coordination structure that initiates the functional DNA-binding response. An intriguing feature of *Rr* CooA is its remarkable specificity for CO, and a principal goal of this study was to determine whether the recently identified CooA homolog from *C. hydrogenoformans* also exhibits this property. Here we report the unexpected discovery that NO binding to Fe(II) *C. cooA* enables high-affinity DNA binding and also results in the formation of a six-coordinate Fe(II)-NO heme, which is distinct from the five-coordinate Fe(II)-NO *Rr* CooA species. We considered three hypotheses to account for the differing coordination chemistry displayed by *Ch* and *Rr* CooA upon exposure to NO: (i) residue effects near the bound NO, (ii) residue effects near the proximal histidine ligand, or (iii) differential protein stability.

Distal amino acids influence the coordination number of NO-bound Fe(II) hemes (16, 19). For example, a tyrosine-phenylalanine substitution in close proximity to the Fe(II)-NO unit of the Sensor of Nitric Oxide (SONO) protein from *Clostridium botulinum* yielded a six-coordinate Fe(II)-NO heme, rather than the five-coordinate adduct observed for the native protein (16). Separately, studies of cytochrome c’ homologs have shown that the accessibility of the distal pocket plays a role in determining the coordination number of the Fe(II)-NO heme (19). Despite these precedents, we found that the two lysine residues unique to the distal heme environment of *Ch* CooA are...
not responsible for the difference in NO binding between this protein and Ch CooA.

Proximal ligand affinity is also a potential source of the coordination difference; theoretical calculations (18) and experimental studies (29) performed for Fe(II)-NO hemes showed a rough correlation between the strength of the Fe-His proximal bond and the likelihood of forming a six-coordinate Fe(II)-NO heme. Because these studies implied that hydrogen-bonding interactions to the proximal histidine ligand can be critical (18), we created N42D Rr CooA, a variant that should maximize hydrogen bonding with His-77 and thus increase the imidazolate character and strength of this ligand. However, this substitution failed to alter the phenotype of the native protein, suggesting that proximal effects are unlikely to be the critical differentiating factors between Ch and Rr CooA proteins.

The important difference between the Ch and Rr CooA proteins appears to be thermal stability. The coordination number of the heme iron in Fe(II)-NO Ch CooA changes from six- to five-coordinate with increasing temperature, and this change is fully irreversible. These observations reveal that Fe-His bond cleavage in Fe(II)-NO Ch CooA is temperature dependent, and suggests that the greater stability of the thermophilic Ch CooA homolog compared to Rr CooA may account for its ability to form a six-coordinate Fe(II)-NO heme that is active for DNA binding at room temperature. Similar temperature-dependent coordination changes have been reported for a SONO ortholog from the thermophile Thermoaeroaerobacter tengengensis (Tt SONO) (16). At room temperature, Fe(II)-NO Tt SONO is six-coordinate, whereas at 70°C, the Fe(II)-NO heme is five-coordinate. Because Rr CooA forms a five-coordinate Fe(II)-NO that is inactive for DNA binding, by analogy, Fe(II)-NO Ch CooA at ~70°C may also be unable to bind to DNA. Therefore, consistent with the known physiology of C. hydrogenoformans (27, 28), these data suggest that Ch CooA may be CO-specific at temperatures at which C. hydrogenoformans lives.

Activation of Ch CooA by NO at ambient temperature provides insights to important features of the general gas-sensing mechanism used by CooA. Together with results obtained from previous studies, the observation of NO-dependent DNA binding by Ch CooA suggests that the active form of CooA requires a six-coordinate heme with a histidine-bound proximal ligand and a small, neutral effector molecule as the distal ligand. Previous studies on Rr CooA suggest that the proximal histidine is critical to achieve the active form (12, 30), and that CO selectivity is based on the hydrophobicity of the distal heme pocket and size/shape of the effector ligand. Specifically, we list six key results that support this hypothesis. (i) Rr CooA variants with substitutions to the proximal His-77 ligand are unable to bind DNA, indicating that the proximal ligand must remain bound to heme to support activation (30). (ii) NO binding to Fe(II) Rr CooA causes the displacement of both axial ligands without inducing activation, also suggesting that active CooA must retain the proximal histidine ligand coordinated to the heme (12). (iii) The mere absence of the distal ligand is not sufficient to promote CooA DNA binding. For example, ΔP3R4 Rr CooA, which has an N terminus shorter by two residues and is substantially five-coordinate in the Fe(III) and Fe(II) states, only binds to DNA in the Fe(II)-CO state. This result implicates the heme-bound effector ligand in activation. (iv) The binding of bulky ligands, such as imidazole, to the available distal coordination site of Fe(III) or Fe(II) ΔΔ3R4 fails to activate DNA binding, suggesting that the size and shape of the resulting heme/ligand complex is an important factor for CooA activation. (v) The CN− ion, which is isoelectronic with CO, does not activate DNA binding of Fe(II) ΔΔ3R4 Rr CooA, suggesting that a neutral donor is critical. (vi) Analyses of CooA variants have shown that the activation of CooA by CO requires a hydrophobic distal heme pocket, consistent with the hypothesis that the positioning of the hydrophobic heme/ligand moiety in this pocket is important for activation. Taken together, these results imply that the size/charge of the effector ligand and retention of the proximal histidine are essential, and that NO satisfies these requirements if a six-coordinate form can be maintained. Although we expect differences in the shape of the NO- and CO-bound Fe(II) hemes because of typical differences in the Fe-C-O and Fe-N-O bond angles (31), NO-dependent DNA binding by Ch CooA strengthens the proposal that the active form of CooA requires a six-coordinate heme with a histidine-bound proximal ligand and a small, neutral effector molecule as the distal ligand.

In summary, these results significantly enhance our understanding of the effector-induced activation mechanism of CooA and also provide insight of how CooA proteins may prevent aberrant activation under physiologically meaningful conditions. Consistent with studies of other NO-binding or gas-sensing heme proteins (16, 19, 32, 33), the present work on Fe(II)-NO Ch CooA underscores how apparently subtle differences in protein stability and structure can substantially impact the coordination of the Fe(II)-NO heme. Such differences appear to have a profound impact on effector specificity and protein function in CooA and possibly in the broad class of gas-sensing heme proteins.

Materials and Methods

Expression and Purification of CooA Proteins. All CooA proteins were produced in an E. coli overexpression system as described (10, 30). WT Rr CooA was purified (>90%) by the reported protocol (30). All other proteins, including WT and variant Ch CooA proteins and Rr CooA variants, contained C-terminal His tags and were expressed with 0.5 mM isopropyl-β-D-galactopyranoside under limiting O2. His-tagged proteins were purified (>95%) with a Novagen nickel-nitrilotriacetic acid column. After precipitation with 50% (NH4)2SO4, His-tagged proteins were resuspended in 25 mM Mops/0.5 M KCl, pH 7.4 buffer and were frozen and stored at −80°C before use. The heme content of CooA preparations was measured by using the reduced pyridine-hemochromogen method (34).

Spectroscopic Methods. Samples for spectroscopic measurements were obtained by diluting purified proteins into 25 mM Mops, pH 7.5 solutions that typically contained 0.1–0.5 M NaCl. Electronic absorption spectra were recorded on a Varian Cary 4 Bio spectrophotometer with a spectral bandwidth of 0.5 nm. EPR spectra were recorded on a Bruker ESP 300E spectrometer that was equipped as described (35). Sodium dithionite (2 mM) or ascorbate (2–15 mM) solutions were used to produce Fe(II) proteins, as appropriate. Fe(II)-CO CooA samples were obtained as described (35), and Fe(II)-NO samples were formed by adding 0.5–1 ml of NO gas to the headspace of Ar-sparged cuvettes. NO gas was purchased from LIN AIR (99.5%) or obtained by the reduction of NaNO3 with sodium ascorbate. EPR samples were prepared under an argon atmosphere and frozen in liquid nitrogen. Specific conditions for recording EPR spectra are given in Figs. 4 and 7.

In Vivo Activity and in Vitro DNA-Binding Assays. In vivo plate assays were performed by using Mops-buffered medium plates (6), but containing 100 μg/ml ampicillin (sodium salt) and 25 μM IPTG. NaNO3-spotted plates were streaked with appropriate E. coli strains and were incubated anaerobically either in the presence or absence of 1% CO at 30°C for 2 days and then held at 4°C for 3 weeks for color development. In vitro DNA-binding assays were performed at room temperature by using a fluorescence polarization method (36) with a Beacon 2000 fluorescence resonance polarization detector (PanVera, Madison, WI) or an ISS PC1 photon counting fluorometer (ISS Instruments, AB, Boston).
Champaign, IL). Briefly, a Texas red-labeled \textit{R. rubrum} P\textsubscript{cooF} DNA fragment (26 bp, 6.4 or 8.0 nM) was titrated with either \textit{Rr} or \textit{Ch} CooA in a concentration range of 1–2,000 nM, and the fluorescence anisotropy was measured. Samples also contained 40 mM Tris-HCl (pH 8.0), 50 mM KCl, 5% glycerol, 6 mM CaCl\textsubscript{2}, 5 mM DTT, and 100 mM NaCl. Dissociation constants (K\textsubscript{d}) were calculated as described (37). The conditions for reduction, NO- and CO-treatment of the samples were similar to those used for obtaining electronic absorption spectra.

We thank Hwan Youn for creating strains of several \textit{Ch} and \textit{Rr} CooA proteins and for helpful discussions, Jose Serate for protein purification, and the Burstyn group for critical reading of the manuscript. This work was supported by National Institutes of Health Grants GM-53228 (to G.P.R.) and HL-66147 (to J.N.B.).