Redox-mediated Transcriptional Activation in a CooA Variant*

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CooA, the carbon monoxide-sensing transcription factor from *Rhodospirillum rubrum*, binds CO at a reduced (FeII) heme moiety with resulting conformational changes that promote DNA binding. In this study, we report a variant of CooA, M124R, that is active in transcriptional activation in a redox-dependent manner. Where wild-type CooA is active only in the FeII + CO form, M124R CooA is active in both FeII + CO and FeIII forms. Analysis of the pH dependence of the activity of FeIII M124R CooA demonstrated that the activity was also coordination state-dependent with a five-coordinate, high-spin species identified as the active form and Cys75 as the retained ligand. In contrast, the active FeI + CO forms of both wild-type and M124R CooA are six-coordinate and low-spin with a protein ligand other than Cys75, so that WT and FeII M124R CooA are apparently achieving an active conformation despite two different heme coordination and ligation states. A hypothesis to explain these results is proposed. This study demonstrates the utility of CooA as a model system for the isolation of functionally interesting heme proteins.

All of the above gas-sensing proteins have in common a heme b prosthetic group that is directly involved in binding the effector, which leads to a conformational change in the protein that affects activity.

Upon binding CO, the homodimeric CooA undergoes a conformational change that allows it to bind to its cognate promoters in the coo regulon (13). The coo regulon contains two transcriptional operons whose polypeptide products are required for *R. rubrum* to anaerobically oxidize CO to CO2 with concomitant H+ reduction to H2 (14). CooA belongs to a family of transcriptional activators that include the cAMP receptor protein (CRP; Ref. 15) and the fumarate and nitrate reductase activator protein (FNR; Ref. 16). Recently the three-dimensional structure of the effector (CO)-free form of CooA has been solved (17), and its comparison to the three-dimensional structure of the effector (cAMP)-bound form of CRP (18) has indicated the conformational changes that take place upon effector-driven activation in this protein family. In addition, the structure of CooA indicated an unprecedented ligation arrangement for a heme protein wherein the N-terminal proline (Pro5, from the opposite subunit) and His77 serve as the heme-axial ligands in the FeII form. Interestingly a redox-mediated ligand exchange occurs in CooA (from Cys75 in the FeII form to His77 in the FeI form; Fig. 1) upon reduction of the heme iron (19, 20). Thus, CooA functions as both a CO sensor and a redox sensor because only the FeII form is competent to bind CO. The heme of WT CooA is hexacoordinate and low-spin in all oxidation and ligation states (21), indicating that incoming CO must displace one of the internal protein ligands. Picosecond time-resolved resonance Raman spectroscopy (22) and NMR studies (23) have indicated that His77 is the retained ligand in the FeI + CO form of CooA. A study of CooA variants showed that alteration of Pro5 did not significantly affect CooA activity; this is also consistent with the hypothesis of the retention of His77 in the FeII + CO form (24).

Similar to that of effector-bound CRP, the structure of effector-free CooA indicated that a single α-helix (designated the C-helix) in each monomer serves to create the intersubunit dimerization domain (18). In CRP and FNR, alterations of particular amino acids in the center of this helix have a variety of effects on activity (25–27). In this report, we have identified CooA variants altered in the C-helix that have a perturbed ligation structure in the oxidized (FeII) form. The FeII form of one of these variants can also bind specific target DNA and is competent in transcriptional activation. This novel activity can be modulated by the redox state of the heme iron, demonstrating the ability of CooA to serve as a model system for engineering unique sensing capabilities in heme proteins.

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‡ The abbreviations used are: CRP, cAMP receptor protein; FNR, fumarate-nitrate reductase activator protein; P-450, cytochrome P-450; P-450cam, camphor-bound cytochrome P-450; MOPS, 3-[N-morpholino]propanesulfonic acid; WT, wild type.
Experimental Procedures

Strains and Plasmids—The construction of strains bearing WT CooA and CooA variants in an E. coli overexpression system and in a β-galactosidase reporter system (19) have been described previously. Variants randomized in their amino acid sequences at selected locations in CooA were generated as described previously (24) or by modified oligonucleotide-directed polymerase chain reaction (28).

Purification of WT CooA and CooA Variants—The purification of WT CooA (to >95% homogeneity) was performed as described previously (19). Because of poor accumulation in the E. coli expression system, M124R CooA was purified to ~80% homogeneity. We also isolated M124R CooA under anaerobic conditions in the presence of sodium dithionite as described by Shaveler et al. (19) but observed little improvement in the yield of heme-containing CooA. When necessary, aliquots of the reduced anaerothetically isolated M124R CooA were chemically oxidized with a slight excess of potassium ferricyanide, which was then removed by G-25 gel filtration. The heme content of CooA preparations was quantified using the reduced pyridine-hemochromogen assay (29), and the protein content was measured using the BCA assay (Pierce).

In Vivo Activity Assays—In this study, we examined the in vivo activity of a CooA-dependent β-galactosidase reporter under different culture regimens that were designed to promote a particular oxidation state of the CooA protein. Strains containing a promoter of cooF gene (PcooF)-lacZ reporter fusion (19) were grown in 2× Luria broth with 0.9% (w/v) NaCl containing 100 μg/ml ampicillin. For assays of aerobic activity, inocula (250 μl of 500 m M buffer (pH 7.5, MOPS and pH 10.5, glycine/NaOH) to provide a final buffer concentration equal to 100 m M and a heme concentration of ~160 μM. Samples were then quickly degassed on an argon manifold to remove dissolved oxygen, frozen, and stored at 77 K. The EPR spectra were then recorded as described previously (24). Analysis of theoretical g values was performed using a rhombohomb diblock program computer (Rhombico Version 1.0; see Appendix in Ref. 32).

Results

In Vivo β-Galactosidase Assay—Following random mutagenesis of that portion of cooa encoding the Ser122-Cys123-Met124 residues, mutagenized clones were screened in a strain of E. coli in which CooA regulates lacZ expression (19). Some clones were noted that expressed β-galactosidase under aerobic growth conditions; this was not a property of clones expressing WT CooA. Similar to WT, however, these clones also showed activity under anaerobic conditions only when CO was present. Upon sequencing, these clones contained sequences that created M124R or M124K substitutions. The results with colonies were supported by quantitative β-galactosidase activity assays. Cells with WT CooA have ~1–3% activity under aerobic or anaerobic conditions compared with anaerobic conditions in the presence of CO. Cells with M124R CooA have activity similar to that of WT CooA when grown anaerobically in the presence of CO but substantially higher activity aerobically (~30%) and anaerobically (~13%). Cells with M124K CooA displayed aerobically independent activity several fold lower than did cells with M124R CooA, and cells with M124L, M124A, and M124I CooA were indistinguishable from those with WT CooA aerobically with slight differences anaerobically in the presence of CO (data not shown). To test whether M124R CooA was unique in its substantial activity under aerobic conditions, we mutagenically randomized the 124 position as we have done previously (24) and screened for aerobic expression of β-galactosidase. Variants M124R, M124K, and M124I were repeatedly isolated, although quantitative analysis of strains with M124K and M124I showed only low activity aerobically indicating that only the Arg substitution at position 124 has a substantial effect. Interestingly, P2Y and P2I CooA, which have similar spectral features in the FeIII form to that of M124R CooA (see below), showed no aerobic activity and only slightly perturbed activity in the presence of CO (24). It is important to note that CooA is in large excess in these in vivo assays, so that levels of specific activity below that of WT can allow maximal reporter activity. The in vitro characterization (see below) allows the manipulation of CooA levels.

Isolation of M124R CooA and Analysis of DNA Binding in Vivo—To understand the biochemical basis for the “aerobically active” phenotype, we isolated M124R and M124K CooA using the procedures described above. Because M124R CooA consistently showed a higher amount of aerobic activity when compared with that of M124K CooA, we chose to extensively characterize M124R CooA. Neither M124R nor M124K CooA accumulated well in the E. coli expression system (~5–10% of WT CooA). Although the isolated material was ~80% pure, the BCA protein assay combined with the heme assay revealed
that our M124R CooA preparations contained only 0.2 hemes/dimer compared with 1.6 hemes/dimer for the isolated WT protein (21). M124R CooA was also unstable in dilute solutions but was stabilized by addition of glycerol to 5–10% (v/v). Diluting stock FeIII M124R CooA into a solution at pH 7.0 caused instantaneous precipitation, which is not seen in WT CooA. Therefore, all manipulations were carried out at pH 8.0 in the presence of 5% glycerol. Although we were still unable to isolate M124R CooA containing greater than 0.2 hemes/dimer, we will demonstrate in this study that the ability of M124R CooA to bind DNA is both redox- and CO-specific, which is consistent with heme dependence.

To determine whether the FeIII M124R CooA activity was detectable in vitro, we tested isolated M124R and WT CooA in different redox states using the fluorescence polarization assay for CooA–DNA interactions. At saturating (40 μg/ml protein; 800 nM heme) concentrations of protein, FeIII WT CooA showed a basal value of anisotropy in this particular experiment (Fig. 2). Dithionite reduction to the FeII form had no effect in anisotropy, whereas addition of CO to the FeII WT CooA generated a large increase in the value of anisotropy as a result of CooA–DNA interaction. When an equivalent protein concentration (40 μg/ml protein; 80 nM heme) of FeIII M124R CooA in the FeIII form was added to the system, a large increase in the value of anisotropy was evident compared with FeIII WT CooA, indicating FeIII M124R CooA–DNA interaction. However, upon dithionite reduction to the FeII form, M124R CooA less effectively interacted with the DNA target. Finally, when CO was added to the sample, M124R CooA had an in vitro activity similar to that of WT CooA, indicating that with both FeII + CO samples the level of functional CooA had saturated the target DNA. These results indicate both a redox-dependent activity and a CO-dependent activity in M124R CooA.

To address the heme concentration dependence of M124R CooA–DNA interactions, we measured the fluorescence polarization of samples that contained increasing amounts of M124R CooA on a heme basis (Fig. 3). When based on heme-containing M124R CooA, FeIII M124R CooA (at pH 8.0) showed an increase in anisotropy reflecting a KD of ~90 nM. This value is approximately 5 times greater than the KD for FeII + CO M124R CooA, which was ~20 nM. In comparison, FeII + CO WT CooA has a KD of ~10 nM (Fig. 3), which is a value similar to that found in a previous study (24). Therefore, the CO-specific DNA binding of WT and M124R CooA to target DNA is roughly similar. The anisotropy value for FeII proteins was near baseline in this particular experiment but tended to increase at the higher concentrations used in this assay (data not shown) indicating the presence of nonspecific interactions at moderately high protein levels (for WT CooA, >500 nM heme = 25 μg/ml protein).

FeIII M124R CooA Has Unusual Coordination Properties—To determine whether the active phenotype of M124R CooA is correlated with structural perturbations near the heme center, we examined isolated M124R CooA using UV-visible absorption spectroscopy. Fig. 4A shows the UV-visible absorption spectra of FeIII M124R CooA compared with that of FeIII WT CooA. Significant spectral changes were observed in FeIII M124R CooA (Soret ~387 nm with a strong ligand-to-metal charge transfer band ~640 nm) when compared with that of FeIII WT CooA (Soret ~424 nm with a very weak ligand-to-metal charge transfer band ~650 nm) and are consistent with a high-spin, five-coordinate heme in FeIII M124R CooA. In contrast, the spectra of the FeII (Fig. 4B) and FeII + CO (Fig. 4C) forms of M124R CooA were indistinguishable from those of the FeII and FeII + CO forms of WT CooA, indicating that only the FeIII form of M124R CooA is significantly perturbed by this substitution. The five-coordinate, high-spin spectral signature of FeIII M124R CooA was similar to that observed for FeIII P2Y CooA (24). However, at pH 7.4, there was a higher percentage of the five-coordinate form in FeIII M124R CooA than that observed in FeIII P2Y CooA (24), indicating that their heme environments are somewhat different at identical pH.

In FeIII M124R CooA, the five-coordinate heme indicates that an open coordination should exist for exogenous ligands to bind. However, addition of cyanide, azide, or imidazole to ~1000-fold molar excess produced no significant changes in the UV-visible absorption spectrum of FeIII M124R CooA (data not shown), suggesting either electrostatic repulsion of these ligands or a relatively inaccessible heme iron. This result is in
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Fig. 4. UV-visible absorption spectroscopy of WT and M124R CooA. Spectra of FeIII forms (panel A), FeII forms (panel B), and FeII+ CO forms (panel C) of proteins were acquired as described under “Experimental Procedures.” Protein concentrations were ~5 μM (in heme) for WT CooA and ~3 μM (in heme) for M124R CooA.

contrast to that of FeIII P2Y CooA, which is also five-coordinate and high-spin yet binds imidazole albeit with a relatively low affinity (24). The differential ability of FeIII M124R CooA and FeIII P2Y CooA to bind exogenous ligands implies significant differences in their respective heme environments (see below).

pH Dependence of FeIII M124R CooA Spectra and in Vitro Activity—Because the UV-visible absorption spectrum of FeIII P2Y CooA, which contains a fraction of five-coordinate, high-spin heme, exhibited a pH dependence (24), we examined whether FeIII M124R CooA had similar behavior. The UV-visible absorption spectra of FeIII M124R CooA as a function of buffer pH are shown in Fig. 5A. At pH 8.0, FeIII M124R CooA exhibited spectral features that indicated a spin mix of the five-coordinate, high-spin form (Soret peak at 387 nm) and a shoulder at 423 nm, indicating the presence of the six-coordinate, low-spin form (contrast with Fig. 4A at pH 7.4). Increasing the pH of the solution increased the fraction of the six-coordinate, low-spin form with a clear isosbestic point at ~410 nm (Fig. 5A). At pH 10.5, the UV-visible absorption spectrum of FeIII M124R CooA was essentially identical to that of FeIII WT CooA at pH 7.4 (Fig. 4A). Fitting the ABS387 nm, (the Soret maximum of the five-coordinate, high-spin form) to the Henderson-Hasselbich equation and assuming a single ionization gave a pKᵣ of ~9.0 (Fig. 5B). This result suggests that hydroxide may be the sixth ligand in six-coordinate, low-spin FeIII M124R CooA at strongly basic pH.

Because FeIII M124R CooA undergoes a spectral transition with pH, we explored the possibility that the redox-mediated activity might also be influenced by pH. Fig. 5B shows the change in anisotropy as a function of pH and indicates that at a more basic pH FeIII M124R CooA becomes less active and is essentially incapable of binding target DNA at pH 10.5. This result indicates that not only is the DNA-binding ability of FeIII M124R CooA redox-dependent, but it also exhibits a coordination-state correlation as well. The pKᵣ of the transition for DNA binding activity was ~8.9, which is essentially identical to that found in the spectral transition (Fig. 5B). This result indicates that a five-coordinate, high-spin fraction of FeIII M124R CooA represents the species that is active in binding DNA. No pH-dependent effects (pH 8.0–10.5) were observed in the FeII+ CO M124R CooA (inactive) and FeII+ + CO M124R CooA (active) samples tested (data not shown). Under the same varied pH conditions, there was a slight decrease (~10%) in the in vitro activity of FeII+ + CO WT CooA at the higher pH range (data not shown).

EPR Spectroscopy of FeIII M124R CooA—EPR spectroscopy of FeIII M124R CooA corroborated the results of the UV-visible absorption spectroscopy. An increase in the pH of the solution resulted in a dramatic decrease in the intensity of high-spin forms of FeIII M124R CooA and a concomitant increase in the intensity of the low-spin form (Fig. 6). Inspection of the low-field region of the spectrum of FeIII M124R CooA revealed the presence of two distinct S = 5/2 systems. For the first spin system, analysis of the g values using a rhombogram computer program (32) for an S = 5/2 system with g = 2 indicates that the spectrum is composed of a system with theoretical g values of gₓ = 1.84 (assumed; see below), gᵧ = 4.30, and gᵣ = 7.53 (Table I) and are similar to those that are found in FeIII five-coordinate, high-spin thiolate-ligated hemes such as P-450cam (33) and the hypothesized arrangement of FeIII P2Y CooA (24). For the second spin system, theoretical g values of gₓ = 1.94 (assumed; see below), gᵧ = 4.99, and gᵣ = 6.96 (Table I) are similar to those found in FeIII five-coordinate, high-spin histidine-ligated hemes such as FixL (34) and soluble guanylyl cyclase (35). Concerning the former spin system, the signal at gₓ = 4.3 does not change significantly with pH, suggesting that a majority of this signal is arising from non-heme iron. Because Cys75 is the normal ligand in FeIII WT CooA, it is very likely that this residue is the source of the thiolate signal. The origin of the neutral nitrogen signal remains unclear, although Pro2 is a possible candidate.

The high-spin system has unusual relaxation properties in that it is only observable at relatively high power and very low temperature (200 microwatts and 4 K). Under these conditions, the low-spin (S = 1/2) features are saturated and appear as a dispersion line shape that dominated the high-field signals arising from the S = 5/2 system (data not shown). At lower powers and higher temperature (20–50 microwatts, 23 K), the low-spin system can be observed as well defined derivative-shaped features, although the high-spin features are completely unobservable under these conditions. Therefore, the gₓ values from the high-field region (S = 5/2) systems can only be assumed from the rhombogram analysis.

The six-coordinate, low-spin system exists in equilibrium with the two five-coordinate, high-spin systems and is evident even at near neutral pH (Fig. 6). This signal increases with pH and represents a thiolate/strong field ligation based on the g values (gₓ = 1.90, gᵧ = 2.26, and gᵣ = 2.45) that are identical to those of WT CooA (Table I). We note that there appears to be a higher proportion of low-spin signal in the EPR spectrum compared with that seen in the UV-visible absorption spectrum at similar pH values, and we propose that this reflects the very different temperatures at which these experiments were performed. Apparently the low temperature stabilizes the low-spin form for reasons that are presently unknown. The identity of the ligand trans to thiolate could be either H₂O or hydroxide, which is observed in P-450 (36), or presumably some nitrogen.
donor ligand (perhaps Pro2) provided by another residue in FeIII M124R CooA.

**DISCUSSION**

Residues in Regions Homologous to Position 124 of CooA Are Important for Activity in the CRP Superfamily—In the case of both CRP and FNR, there are different alterations that can cause effector-independent activity, but the region homologous to Cys123 and Met124 in CooA is particularly interesting. In FNR, substitutions at Asp154 (Asp154 is homologous to Cys123 in CooA) render the protein insensitive to redox, which is the primary effector for this protein. Normally FNR exists as a monomer under oxidizing conditions, and it is only under reducing conditions after the formation of Fe4S4 centers in each monomer that FNR can dimerize and thus bind palindromic DNA sequences with high affinity. The D154A substitution causes the protein to more readily dimerize under all conditions, although there is no evidence that it lies particularly close to the Fe4S4 center (25). The mechanism by which cluster formation leads to dimerization is a topic of central importance to understanding FNR action, but there is no reason to believe that it has any mechanistic similarities to CO binding to the heme of CooA. CRP is more similar to CooA in that it is a dimer under all conditions, and the binding of its effector, cAMP, leads to a conformational change that is presumably somewhat similar to that of CooA. Ser128 of CRP, which is homologous with Met124 in CooA, makes contact with the cAMP molecule bound to the other protein monomer (18). An S128P substitution severely reduces the ability of CRP to bind DNA in response to cAMP (26), and an S128T substitution results in a lack of discrimination between cAMP from cGMP as effectors (37). In addition, the binding of the cAMP to S128A CRP occurs with magnified negative cooperativity compared with that of WT CRP (26).

It seems paradoxical that these three proteins, with what appear to be completely different sites and mechanisms for effector recognition and response, should all be so strikingly affected by changes in this region of the protein. As noted below, it is our working hypothesis (17) that repositioning of the C-helices upon CO binding is important for activation of WT CooA, and preliminary results on alterations of the leucine zipper motif (38) along these helices are generally consistent with that hypothesis.2 However, the effects described for M124R CooA do not appear to be based on this specific mechanism because the M124R substitution should not have a dramatic effect on the leucine zipper motif. We therefore favor the hypothesis that the M124R substitution exerts its effects by a different mechanism of communication between the heme and the DNA binding domains; it is unclear whether this pathway is also present in WT CooA as well.

**Ligation Arrangement of FeIII M125R CooA**—The results of the UV-visible and EPR spectroscopy in this paper show that a portion of FeIII M124R CooA has a heme coordination that most closely resembles the five-coordinate, high-spin heme observed for P-450 cam (33), and the correlation between pK_a for the coordination state change and pK_a for activity strongly implies

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2 R. L. Kerby, unpublished data.
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that it is one or both of the five-coordinate, high-spin forms that are active. The EPR analysis also suggests that the majority of this species has a thiolate ligand that we assume to be Cys\textsuperscript{75} that is the normal ligand to the Fe\textsuperscript{III} form of WT CooA. It would therefore be tempting to conclude that the M124R substitution somehow causes the loss of the sixth protein ligand (Pro\textsuperscript{2}) to yield the five-coordinate Fe\textsuperscript{III} form with Cys\textsuperscript{75} as the protein ligand and that this form would be necessary and sufficient for the observed activity. This hypothesis is incorrect, however, as P2Y and P2H variants also have a substantial fraction of five-coordinate, high-spin heme in the Fe\textsuperscript{III} form, and at least a portion of this appears to have a thiolate ligand (24), yet these proteins display almost no activity in vivo or in vitro under oxidizing conditions (data not shown). Indeed, the P2Y and P2H variants are not significantly affected in their response to CO (24), indicating that the loss of the “Pro\textsuperscript{2} arm” of the N terminus (17) is not critical for the CO-dependent activation of WT CooA. We also note that the g values of these five-coordinate forms of M124R and P2Y CooA are not identical (Table I), and the difference in imidazole binding between these two proteins indicates some difference in their respective heme environments. It is therefore our working hypothesis that although both these variants have a five-coordinate Cys-ligated heme in the Fe\textsuperscript{III} form, the heme position is different in a subtle but functionally important way as described below.

Structural Consequences of the M124R Substitution—The recently published crystal structure of Fe\textsuperscript{II} WT CooA (17) provides a framework to form hypotheses about why M124R CooA might be active in the Fe\textsuperscript{III} form. It must be emphasized, however, that the published crystal structure of WT CooA is in the Fe\textsuperscript{II} form, which has a completely different ligation and coordination state than Fe\textsuperscript{III} M124R CooA and is inactive in DNA binding. Nevertheless, there are a few salient possibilities that arise from inspection of the published structure.

Inspection of the Fe\textsuperscript{II} WT CooA structure in Fig. 7 shows the orientations of the Met\textsuperscript{124} residues in relation to the hemes. Using the SwissPro structure-viewing program to analyze M124R gives a variety of different energetically reasonable conformers of the “new” amino acid. Two favored configurations have the guanidino cation of Arg\textsuperscript{124} hydrogen-bonded to either the 7-proprionate of the heme or to the peptide chain carbonyl of Ser\textsuperscript{78}. The former of these would have the potential of perturbing the heme itself, perhaps destabilizing the Pro\textsuperscript{2} ligand in the process, whereas the latter is immediately adjacent to the His\textsuperscript{77} and Cys\textsuperscript{75} region and possibly perturbs the heme directly or indirectly through that interaction. The current working hypothesis of CooA function (17) is that CO binding leads to a shift in heme position such that it now interacts with the nearby portion of the C-helices, and it is the repositioning of these helices with respect to each other that induces the conformational change at the opposite end of the protein. Consistent with this hypothesis, we assume that activity in the Fe\textsuperscript{III} form of M124R is caused by an interaction of the heme with the C-helices that is somewhat similar to that of the CO-bound heme of WT CooA. However, because the heme must itself be in a somewhat different position (it is still ligated to Cys\textsuperscript{75} rather than the expected His\textsuperscript{77} of Fe\textsuperscript{II} WT CooA), the heme-helix interactions for activity are certainly unlike the normal pathway in the WT protein; they simply have the same end effect. We rationalize the failure of P2Y and P2H to have similar activity because they presumably have the Fe\textsuperscript{III} heme in a slightly different position albeit in a roughly similar ligation state. The inability of Fe\textsuperscript{III} M124R CooA (in contrast in Fe\textsuperscript{III} P2Y) to bind imidazole is consistent with this steric association of the heme with the C-helices.

In summary, we have demonstrated that M124R CooA is active in transcriptional activation in the Fe\textsuperscript{III} form and that this activity is dependent upon both redox and coordination state of the heme. The M124R substitution certainly perturbs
the heme ligation and probably the heme position, which ultimately leads to this novel phenotype. The results strongly support the notion of extreme flexibility in the heme-protein contacts of CooA as suggested by the analysis of the crystal structure (17). This study and another on cyanide binding variants of CooA (39) demonstrate that unique sensing capabilities can be engineered into CooA and should encourage efforts to search for other variants of CooA that are responsive to other heme-site ligands.

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