Redox-controlled Ligand Exchange of the Heme in the CO-sensing Transcriptional Activator CooA*

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Shigetoshi Aono‡, Kei Ohkubo, Takatoshi Matsuo, and Hiroshi Nakajima

From the School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Tatsunokuchi, Nomi-gun, Ishikawa 923-1292, Japan

The transcriptional activator CooA from Rhodospirillum rubrum contains a b-type heme that acts as a CO sensor in vivo. CooA is the first example of a transcriptional regulator containing a heme as a prosthetic group and of a hemeprotein in which CO plays a physiological role. In this study, we constructed an in vivo reporter system to measure the transcriptional activator activity of CooA and prepared some CooA mutants in which a mutation was introduced at Cys, His, Met, Lys, or Tyr. Only the mutations of Cys75 and His77 affected the electronic absorption spectra of the heme in CooA. The electronic absorption spectra, EPR spectra, and the transcriptional activator activity of the wild-type and mutant CooA proteins indicate that 1) the thiolate derived from Cys75 is the axial ligand in the ferric heme, but it is not coordinated to the CO-bound ferrous heme; 2) Cys75 is protonated or displaced in the ferrous heme; and 3) His77 is the proximal ligand in the CO-bound ferrous heme and probably also in the ferrous heme, but it is not coordinated to the ferric heme. NMR spectra reveal that the conformational change around the heme, which will trigger the activation of CooA by CO, takes place upon the binding of CO to the heme.

The purple, non-sulfur, photosynthetic bacterium Rhodospirillum rubrum can grow on CO as a sole energy source under anaerobic conditions in the presence of CO (1, 2). The expression (which is regulated at the transcriptional level) of the proteins coded in the cooFSCTJ and cooMKLXUH operons is induced under these conditions (3–5). The genes of key enzymes that gain energy for growth on CO such as CO dehydrogenase and hydrogenase are coded in the coo operons (3–5). The cooA gene product has been reported to be the transcriptional activator for regulation of the expression of the coo operons and to be a member of the CRP/FNR family of transcriptional regulators on the basis of amino acid sequence homology (3–5).

CRP is a homodimer of a 209-amino acid monomer that is composed of two domains (6–11). The small carboxyl-terminal domain contains a helix-turn-helix DNA-binding motif, and the large amino-terminal domain is responsible for subunit-subunit contact for dimerization and binds CAMP as the effector (9–13). The two domains are connected by a hinge region.

CooA contains a helix-turn-helix motif as the DNA-binding motif in its C-terminal region (3), which indicates that CooA is a DNA-binding transcriptional regulator protein. We have reported that the amino-terminal region from Met1 to Met131 is the heme-binding domain in CooA, which corresponds to the cAMP-binding domain in CRP (14). The homology between CRP and CooA suggests that they share a common mechanism of transcriptional regulation; however, the effectors of these regulatory proteins are completely different. The most interesting feature of the transcriptional regulation with CooA is that CO is required for the expression of the coo operons (2–5), indicating that CO acts as the effector of CooA.

It has been reported that recombinant CooA can be expressed in Escherichia coli and that it contains a b-type heme as a prosthetic group (15, 16). The ferrous heme in CooA can bind CO as an axial ligand (14–16). DNase I footprint analysis with CooA has shown that the protection of the target sequence on DNA is observed only in the presence of CO under anaerobic conditions (4, 5, 16), indicating that binding of CO to the heme in CooA causes the specific binding of CooA to the target DNA. These results show that the heme in CooA acts as a CO sensor in vivo and regulates the activity of CooA by the binding of CO. Although CO is widely used as a probe to study the biochemical and biophysical properties of hemeproteins, it does not have any physiological role in these cases. CooA is the first example of a hemeprotein in which CO plays a physiological role.

The function of the heme in CooA, which is the sensor of the effector, is a new one. Two hemeproteins, FixL (17–24) and soluble guanylate cyclase (25–34), have been reported as members of the hemeprotein family in which the heme acts as the sensor of the effector. The hemes in FixL and soluble guanylate cyclase are O2 and NO sensors, respectively, and are responsible for the regulation of the enzymatic activity with the effector (17–34). CooA is a new member of the “heme-based sensor proteins.” The coordination structure of the heme is an important factor in the regulation of the function of hemeproteins. In this study, we tried to determine the coordination structure of the heme in CooA by measurements of the electronic absorption spectra, EPR spectra, and the transcriptional activator activity of wild-type and some mutant CooA proteins prepared by site-directed mutagenesis. To measure the activity of CooA, we constructed an in vivo reporter system containing a cooF-lacZ operon fusion as the reporter gene. We propose that the heme in CooA exists in a six-coordinate form in ferric, ferrous, and CO-bound ferrous forms and that the exchange of the axial ligand takes place upon the change in the redox state of the heme. By measuring NMR spectra of ferrous and CO-bound ferrous CooA, we found that the conformational change around the heme, which will trigger the activation of CooA by CO, takes place upon the binding of CO to the heme.
EXPERIMENTAL PROCEDURES

Vectors and Strains—*R. rubrum* (IFO 3986) was obtained from the Institute for Fermentation (Osaka, Japan). Plasmid pRS551, ARS74 phage, and *E. coli* p90C (35) were kindly provided by Professor R. W. Simons (UCLA). Plasmid pKK223-3 and the TA cloning kit were obtained from Amersham Pharmacia Biotech and Invitrogen, respectively. *E. coli* strains were grown on LB medium. Ampicillin (50 mg/liter) and/or kanamycin (50 mg/liter) was added to the medium when necessary.

Construction of the Expression System—The expression vector pKK3CO5 was constructed as reported previously (15). To improve the yield of recombinant CooA, a new expression vector harboring two copies of cooa (pKK3CO6) was also constructed as follows. The DNA fragment containing cooa was excised from pCRCO (15) by digestion with EcoRI and was ligated with EcoRI-treated pKK223-3. The clone harboring pKK3CO6 was selected from the cells transformed by the above ligation mixture on LB agar plates containing ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside. The desired clone showed a deep orange-red color compared with the clone harboring pKK3CO5.

Expression and Purification of CooA—Recombinant CooA was expressed in *E. coli* JM109 as reported previously (15). Purification was carried out according a previous method (14, 15) with some modifications. A Q-Sepharose column was used in the first step instead of a butyl-Sepharose column. The fractions containing CooA eluted from the first column were combined and applied to a chelating Sepharose column charged with zinc ion. The chelating Sepharose column was pre-equilibrated with 20 mM Na₂HPO₄ buffer (pH 7.2) containing 1 mM NaCl. Adsorbed proteins were eluted by a 0–0.06 M imidazole linear gradient. The fractions containing CooA were combined and dialyzed against the appropriate buffer. Further purification on a gel filtration column (Sephacryl S-100) was carried out if necessary. The CooA solution after dialysis was concentrated by ultrafiltration with a YM-10 membrane (Amicon, Inc.) for spectroscopic measurements. CooA was dissolved in 50 mM Tris-HCl buffer (pH 8.0) unless otherwise noted.

All of the mutants constructed in this work were expressed and purified according to the wild type except for C75A CooA. As the amount of the expressed C75A CooA was less than one-fifth of that of the wild type and this mutant was labile compared with the wild type, C75A CooA could not be purified to homogeneity. Therefore, a crude sample of C75A CooA was used for the spectroscopic measurements.

Determination of the Heme Content—The concentration of CooA was determined by the pyridine ferrohemochrome method (36). A value of 54 mM⁻¹ cm⁻¹ (36) at the absorption maximum of the α band for the pyridine ferrohemochrome derived from the protoheme was used to calculate the concentration of CooA.

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Stratagene) or the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene). pKK3CO5 was used as the template for mutagenesis. The restriction fragments containing cooa promoter region, which is denoted as the COP region hereafter, was synthesized by the polymerase chain reaction according to the literature (37).

β-Galactosidase Assay—β-Galactosidase levels were determined by hydrolysis of o-nitrophenyl-β-D-galactopyranoside according to the method of Miller (38). The cell-free extract prepared from the reporter strain was used for the assay. *E. coli* λCOP bearing an appropriate CooA expression vector was grown in a 50-ml cultivation flask containing 10 ml of LB medium with 50 μg/ml ampicillin, 30 μg/ml kanamycin, and 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 8 h. The cultivation was carried out on a rotary shaker at 180 rpm. When the cells were grown with CO, 10 ml of a head-space gas in a flask sealed by a rubber septum was replaced by the same volume of CO with a gas-tight syringe before starting the cultivation. The collected cells after the cultivation were washed and resuspended in 4 and 1 ml of Z-buffer (38), respectively. The resuspended cells were broken by sonication and then centrifuged to prepare the cell-free extract for the measurement of β-galactosidase activity. The assay was carried out at 28 °C. The protein concentration of the cell-free extract was determined by the absorbance at 280 nm using bovine serum albumin as a standard.

The specific activity of β-galactosidase is expressed as nanomoles of o-nitrophenol-β-D-galactopyranoside hydrolyzed per min/mg of protein.

**EPR and NMR Measurements**—X-band EPR spectra were measured on a Jeol RE1X or RE3X apparatus. CooA was dissolved in 50 mM Tris-HCl buffer (pH 8.0) for the measurement of EPR spectra. Azide-bound metmyoglobin was used as a standard for quantitation of the low-spin heme. Myoglobin from horse heart was obtained from Sigma. The 1H NMR spectra were measured on a Varian Unity 750 (750 MHz) spectrometer at 25 °C. Chemical shifts were referenced to 2.2-dimethyl-2-silapentane-5-sulfonate. For the measurement of NMR spectra, CooA was dissolved in 50 mM KH₂PO₄/NaOH buffer (pH 7.6) containing 15% D₂O.

**RESULTS**

Electronic Absorption and EPR Spectra of Wild-type CooA—The electronic absorption spectra of wild-type CooA are shown in Fig. 1. While we reported previously that ferroc (oxidized) CooA purified using a butyl-Sepharose column showed the Soret absorption peak at 418.5 nm (15), CooA purified by the modified procedure described in this work revealed the Soret absorption peak at 410.0 nm (35), and in the phage lysate containing the recombinant phage was prepared. Lysogens were obtained by infecting *E. coli* JM109 with this resulting phage lysate and selected on LB agar plates containing kanamycin. A clone bearing a single copy of the recombinant λ prophage was selected and used in the following study. The resulting strain was named *E. coli* λCOP. Prophage copy number was determined by polymerase chain reaction according to the literature (37).

**Fig. 1. Electronic absorption spectra of wild-type CooA.** The thick solid line, broken line, and thin solid line represent the spectra of the ferric, ferrous, and CO-bound forms of CooA, respectively. CooA (6.8 μg dimer) was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enlarged spectra in the Q-band region are shown in the inset. The extinction coefficients of the Soret band are 108, 200, and 220 mM⁻¹ cm⁻¹ for the ferric, ferrous, and CO-bound forms of CooA, respectively.
Axial Ligand Exchange of the Heme in CooA

The EPR spectrum of ferric CooA showed a rhombic component with $g$ values of 2.46, 2.26, and 1.90 as shown in Fig. 2a. These values are typical of low-spin heme proteins, indicating that the heme in ferric CooA is in the six-coordinate low-spin form. The $g$ values of CooA are almost the same as those of substrate-free ferric cytochrome P450$_{cam}$ ($g = 2.45, 2.26,$ and 1.91) (39) and ferric cytochrome P420$_{cam}$ ($g = 2.45, 2.27,$ and 1.91) (40), in which a thiolate derived from a cysteine is the axial ligand of the heme.

The quantitation of the spin concentration revealed that the intensity of the signal of ferric CooA was 0.8 spins/CooA monomer. The content of the heme was also determined to be 0.8 mol/CooA monomer by the pyridine ferrohemochrome method. These results show that CooA contains 1 mol of protoheme/mol of CooA monomer, which was also consistent with the observations of Shelver et al. (16). We also confirmed by gel filtration experiments that CooA existed in a dimeric state in the ferric, ferrous, and CO-bound ferrous states as reported by Shelver et al. (16) (data not shown).

Electronic Absorption and EPR Spectra of CooA Mutants—To determine the axial ligands of the heme in CooA, we constructed some mutants by site-directed mutagenesis and measured their electronic absorption spectra and their transcriptional activator activity. Cysteine, histidine, methionine, lysine, and tyrosine were chosen as candidates for the axial ligand. CooA contains five Cys residues at positions 35, 75, 105, and 123; five His residues at positions 28, 77, 133, 146, and 200; and five Met residues except for the first one at positions 73, 76, 108, 124, and 131. We constructed all of the Cys-to-Ala (C35A, C75A, C80A, C105A, and C123A), His-to-Ala (H28A, H77A, H133A, H146A, and H200A), and Met-to-Leu (M76L, M76L, M108L, M124L, and M131L) CooA mutants at these positions. A His-to-Tyr mutant (H77Y CooA) was also constructed. For Lys-to-Ala and Tyr-to-Phe mutants, three (K26A, K30A, and K101A) and two (Y55F and Y67F) CooA mutants were constructed. All of the Lys and Tyr residues located in the heme-binding domain (131 residues from Met$^1$ to Met$^{131}$ (14)) in CooA were chosen as a target for mutagenesis.

The absorption maxima of the ferric, ferrous, and CO-bound ferrous forms of wild-type and mutant CooA proteins are summarized in Table I. Among the above 21 mutants, only C75A, H77A, and H77Y CooA showed different electronic absorption spectra from wild-type CooA. The other 18 mutants showed the same spectra as the wild type in the ferric, ferrous, and CO-bound ferrous forms.

The electronic absorption spectrum of C75A CooA in the ferric form is shown in Fig. 3. The Soret band was observed at 411.0 nm, which was blue-shifted by 12 nm compared with the wild type, and the clear $\alpha$ and $\beta$ bands in the Q-band region were not observed in ferric C75A CooA. The EPR spectra of C75A CooA are shown in Fig. 4. Ferric C75A CooA showed EPR signals in the $g = 6$ region at 4 K due to the high-spin heme, whereas it did not show any signals in the $g = 2$ region due to the low-spin heme at 4 and 77 K. These features are typical of five-coordinate, high-spin heme proteins, suggesting that Cys$^{75}$ is an axial ligand of the heme in ferric CooA. Ferrous C75A CooA showed the Soret, $\alpha$, and $\beta$ bands at 422.5, 570.0, 538.5, and 527.0 nm, respectively (Fig. 3). This spectrum is similar to that of the wild type, suggesting that the heme in ferrous C75A CooA is in the six-coordinate form, as is the wild type. CO-bound ferrous C75A CooA showed the Soret, $\alpha$, and $\beta$ bands at 420.5, 569.0, and 532.0 nm, respectively.

The electronic absorption spectra of H77A and H77Y CooA are shown in Figs. 5 and 6, respectively. Ferric H77A CooA showed the Soret, $\alpha$, and $\beta$ bands at 423.0, 557.0, and 525.0 nm, respectively (Fig. 5). This spectrum is similar to that of the wild type, suggesting that the heme in ferrous H77A CooA is in the six-coordinate form. The EPR spectra of H77A and H77Y CooA are shown in Figs. 2b and c, respectively. The EPR signals with $g$ values of 2.42, 2.25, and 1.92 were observed in H77A and H77Y CooA, respectively. These properties of ferric H77A and H77Y CooA with regard to
the electronic absorption and EPR spectra are almost the same as those of the wild type, indicating that the mutation at position 77 does not affect the coordination structure of the ferric heme, i.e. His77 is not thought to be coordinated to the ferric heme in CooA. Although the electronic absorption spectra of H77A and H77Y CooA in the ferric state were identical, the ferrous forms of these mutants showed different properties. Ferrous H77A CooA showed the Soret, \(a\), and \(b\) bands at 422.5, 557.5, and 528.0 nm, respectively. This spectrum is similar to that of wild-type CooA, although the Soret band was slightly blue-shifted by 2 nm compared with the wild type, and the molar extinction coefficients of the absorption maxima were different from those of wild-type ferrous CooA (see the legends of Figs. 1 and 5). On the other hand, the electronic absorption spectrum of ferrous H77Y CooA showed the Soret band and a single peak in the Q-band region at 424.0 and 558.5 nm, respectively, which resembled the spectrum of deoxymyoglobin (41) and was typical of five-coordinate, high-spin ferrous hemeproteins. Shelver et al. (16) have reported that H77Y CooA cannot be stably reduced by dithionite (although the data are not shown),

### Table I

| CooA     | Ferric |   | Ferrous |   | CO-bound |   |
|----------|--------|---|---------|---|----------|---|
|          | \(\delta\) | Soret | Visible | Visible | Soret | Visible | Visible | Visible | Visible |
| WT*      | 362.5  | 423.5 | 538.5  | 570.0  | 424.5  | 528.5  | 557.5  | 422.0  | 540.5  | 568.5  |
| H28A     | 363.5  | 423.0 | 539.0  | 570.0  | 424.5  | 528.5  | 558.5  | 422.0  | 540.0  | 568.5  |
| H77A     | 362.0  | 422.5 | 538.5  | 570.0  | 422.5  | 528.0  | 557.5  | 419.5  | 538.5  | 563.0  |
| H77Y     | 363.5  | 423.5 | 538.5  | 570.0  | 424.5  | 528.5  | 558.5  | 421.5  | 538.5  | 568.0  |
| H153A    | 361.5  | 422.5 | 539.5  | 570.0  | 425.0  | 528.5  | 558.5  | 422.0  | 540.0  | 568.0  |
| H146A    | 361.0  | 424.0 | 539.5  | 570.0  | 425.0  | 528.5  | 558.5  | 422.0  | 540.0  | 568.0  |
| H200A    | 359.5  | 424.0 | 540.0  | 570.0  | 424.5  | 528.0  | 557.5  | 422.0  | 540.0  | 568.0  |
| C35A     | 360.5  | 423.5 | 539.5  | 570.0  | 425.0  | 528.5  | 558.5  | 422.0  | 540.0  | 569.0  |
| C75A     | 411.0  | 423.5 | 540.5  | 570.0  | 425.0  | 528.5  | 559.0  | 422.5  | 540.0  | 567.0  |
| C80A     | 363.5  | 423.5 | 540.5  | 570.0  | 425.0  | 528.5  | 559.0  | 422.0  | 540.0  | 568.5  |
| C105A    | 358.5  | 423.5 | 539.0  | 570.0  | 425.0  | 528.5  | 559.0  | 422.0  | 540.0  | 567.0  |
| C123A    | 360.5  | 423.5 | 539.5  | 570.0  | 425.0  | 528.5  | 559.0  | 422.0  | 540.0  | 568.0  |
| M73L     | 364.5  | 423.0 | 539.0  | 570.0  | 424.5  | 528.5  | 557.5  | 422.0  | 540.0  | 568.5  |
| M76L     | 362.5  | 423.0 | 538.5  | 570.0  | 424.5  | 528.5  | 557.5  | 422.0  | 540.0  | 568.0  |
| M105L    | 360.0  | 424.0 | 539.0  | 570.0  | 424.5  | 528.5  | 559.0  | 422.0  | 540.0  | 568.0  |
| M124L    | 362.5  | 423.5 | 539.5  | 570.0  | 424.5  | 528.5  | 558.5  | 422.0  | 539.5  | 568.5  |
| M131L    | 362.5  | 423.5 | 539.5  | 570.0  | 424.5  | 528.5  | 558.5  | 422.0  | 539.5  | 568.5  |
| K26A     | 360.5  | 424.0 | 539.0  | 570.0  | 425.0  | 528.5  | 558.5  | 422.0  | 540.0  | 568.5  |
| K30A     | 360.0  | 424.0 | 539.0  | 570.0  | 425.0  | 528.5  | 559.0  | 422.0  | 540.0  | 568.5  |
| K101A    | 361.0  | 423.5 | 540.0  | 570.0  | 424.5  | 528.5  | 559.0  | 422.0  | 539.5  | 568.5  |
| Y55F     | 361.5  | 424.0 | 539.0  | 570.0  | 425.0  | 528.5  | 559.0  | 422.0  | 540.0  | 568.5  |
| Y67F     | 360.5  | 424.0 | 539.0  | 570.0  | 425.0  | 528.5  | 559.0  | 422.0  | 540.0  | 568.5  |

*WT, wild-type.

\(a\) The single peak in the Q-band region was observed.

\(b\) The clear peak was not observed in the Q-band region.

**Fig. 3.** Electronic absorption spectra of C75A CooA. The thick solid line, broken line, and thin solid line represent the spectra of the ferric, ferrous, and CO-bound forms of CooA, respectively. The enlarged spectra in the Q-band region are shown in the inset.

**Fig. 4.** X-band EPR spectra of C75A CooA at 77 K (a) and 4 K (b). The microwave power, modulation frequency, and modulation amplitude were 1 milliwatts, 100 kHz, and 1 millitesla \((mT)\), respectively. The signals at \(g = 2.01\) and 4.27 seem to be due to some organic radical in the sample or the contaminant proteins and an adventitious \(Fe^{3+}\), respectively.
which is inconsistent with our result. A reason for the difference may be that they used crude extracts prepared from E. coli. However, H77Y CooA in crude extracts can be reduced by dithionite in our preparation. The reasons for this difference are not obvious at present.

CO-bound ferrous H77A and H77Y CooA showed the Soret, α, and β bands at 419.5, 563.0, and 538.5 nm and at 419.5, 568.0, and 538.5 nm, respectively. The Soret absorption maxima of the His77 mutants were blue-shifted by 2 nm compared with the wild type, suggesting that there is some alteration of the surrounding structure of the heme in the CO-bound form when the mutation is introduced at His77.

**Axial Ligand Exchange of the Heme in CooA**

![FIG. 5. Electronic absorption spectra of H77A CooA. The thick solid line, broken line, and thin solid line represent the spectra of the ferric, ferrous, and CO-bound forms of CooA, respectively. H77A CooA (6.4 μM dimer) was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enlarged spectra in the Q-band region are shown in the inset. The extinction coefficients of the Soret band are 110, 144, and 180 mM⁻¹ cm⁻¹ for the ferric, ferrous, and CO-bound forms of H77A CooA, respectively.](image)

**FIG. 6. Electronic absorption spectra of H77Y CooA. The thick solid line, broken line, and thin solid line represent the spectra of the ferric, ferrous, and CO-bound forms of CooA, respectively. H77Y CooA (6.0 μM dimer) was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enlarged spectra in the Q-band region are shown in the inset. The extinction coefficients of the Soret band are 110, 112, and 216 mM⁻¹ cm⁻¹ for the ferric, ferrous, and CO-bound forms of H77Y CooA, respectively.**

![FIG. 7. 1H NMR spectra of ferrous CooA (a) and CO-bound CooA (b). CooA (700 μM dimer) was dissolved in 50 mM KH₂PO₄/NaOH buffer (pH 7.8).](image)

**Wild-type and Mutant CooA Transcriptional Activator Activities**—To measure the transcriptional activator activity of CooA in vivo, we constructed the reporter strain E. coli λCOP containing a single copy of the recombinant λ-prophage that contains a cooF-lacZ operon fusion. When E. coli λCOP transformed by the expression vector of wild-type CooA...
(pKK3CO5/E. coli λCOP) was grown in the presence of CO, the specific activity of β-galactosidase increased with cultivation time and then reached a constant value (Fig. 8, filled circles). On the other hand, β-galactosidase activity was scarcely observed when pKK3CO5/E. coli λCOP was grown in the absence of CO as shown in Fig. 8 (empty circles). These results show that recombinant CooA expressed in E. coli cells can act as the transcriptional activator only in the presence of CO, which is the same situation as that in R. rubrum, and that the transcriptional activator activity of CooA can be evaluated using the in vivo reporter system constructed in this study.

Immediately after the disruption of the cells, the crude extract prepared from E. coli cells expressing wild-type CooA showed the α and β bands at 557.5 and 528.5 nm, respectively, which were the same as those of ferrous CooA (data not shown). This result shows that the expressed CooA in E. coli exists in the ferrous form even when E. coli cells are grown aerobically. As ferrous CooA reacts readily with CO to form CO-bound CooA, CooA in E. coli cells grown in the presence of CO should exist in the CO-bound ferrous form. Therefore, the results shown in Fig. 8 indicate that CO-bound ferrous CooA is active as the transcriptional activator, but ferrous CooA is not.

The activity of the CooA mutants prepared in this study was measured, and the results are summarized in Table II. These mutants can be classified into three groups according to the dependence of the activity on CO as follows: group 1, the dominant-negative mutant that is inactive regardless of the presence and absence of CO; group 2, the dominant-positive mutant that is active regardless of the presence and absence of CO; and group 3, the mutant that is active in the presence of CO as shown in Fig. 8. These results show that some mutants show different activity compared with that of the wild type as described above, although their coordination structure of the heme was identical to that of the wild type. Among these mutants, M131L CooA was previously obtained as a dominant-positive mutant by random mutagenesis (14), and the possible mechanism by which this mutant is active even in the absence of CO is described below. The detailed properties of other mutants showing activity different from that of the wild type remain to be elucidated.

### DISCUSSION

Electronic absorption and EPR spectroscopies revealed that the heme in ferric CooA was in the six-coordinate, low-spin form. In the resonance Raman spectrum of ferric CooA, ν₂ and ν₄ bands have been observed at 1580 and 1501 cm⁻¹, respectively.² These values are consistent with the model that the ferric heme in CooA is in the six-coordinate, low-spin state. Ferric CooA shows a clear δ band and a weak charge transfer band at 362.5 and 760 nm, respectively, which are similar to absorptions in P450 cytochromes containing a thiolate as an axial ligand of the heme (42). The EPR spectrum of ferric CooA also resembles that of thiolate-ligated hemeproteins such as cytochromes P450 and P420, i.e. the g values observed in ferric CooA (g = 2.46, 2.26, and 1.90) are almost the same as those of substrate-free ferric cytochrome P450cam (g = 2.45, 2.26, and 1.91) (39) and ferric cytochrome P420cam (g = 2.45, 2.27, and 1.91) (40), in which a thiolate derived from a cysteine is the axial ligand of the heme. These results show that a cysteine is an axial ligand of the ferric heme in CooA.

Ferric C75A CooA shows the typical electronic absorption and EPR spectra of five-coordinate, high-spin hemeproteins. C75A CooA showed complex EPR signals in the g = 6 region as shown in Fig. 4, which may be the superposition of the signals due to the heme with a different conformation. As the mutation is introduced at the amino acid acting as the axial ligand in C75A CooA, the conformation of the heme will not be fixed. The spectroscopic properties of wild-type and C75A CooA indicate

The axial ligand exchange of the heme in CooA, we found that some mutants show different activity compared with that of the wild type as described above, although their coordination structure

### Table II

| CooA        | +CO⁵ | −CO⁶ |
|-------------|------|------|
| Control     | 0.05 | 0.05 |
| Wild-type   | 15.7 | 0.23 |
| H28A        | 9.9  | 0.16 |
| H77A        | 0.34 | 0.32 |
| H77Y        | 3.6  | 3.3  |
| H133A       | 6.7  | 0.05 |
| H146A       | 12.0 | 0.59 |
| H200A       | 14.7 | 0.28 |
| M73L        | 10.8 | 0.21 |
| M76L        | 9.6  | 0.20 |
| M108L       | 15.1 | 0.27 |
| M124L       | 14.4 | 0.25 |
| M131L       | 256  | 311  |
| C35A        | 9.5  | 0.20 |
| C75A        | 13.4 | 0.40 |
| C80A        | 13.4 | 0.90 |
| C105A       | 247  | 222  |
| C123A       | 6.2  | 0.15 |
| Y55F        | 17.3 | 0.23 |
| Y67F        | 4.0  | 0.11 |
| K26A        | ND¹  | ND²  |
| K30A        | 12.5 | 0.09 |
| K101A       | ND²  | ND²  |

⁵ E. coli λCOP was grown in the presence of CO.

² E. coli λCOP was grown in the absence of CO.

ND, not determined.

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² T. Uchida, S. Takahashi, K. Ishimori, I. Morishima, H. Nakajima, and S. Aono, manuscript in preparation.
that Cys\textsuperscript{75} is an axial ligand of the ferric heme in CooA. The electronic absorption spectra of the ferric CooA mutants at His, Met, Cys, Lys, or Tyr, except for Cys\textsuperscript{75}, are identical to that of the wild type, indicating that these residues are not the sixth axial ligand of the ferric heme in CooA. The EPR spectra of the His\textsuperscript{77} mutants (H77A and H77Y CooA), which are almost the same as that of the wild type, support that His\textsuperscript{77} is not coordinated to the ferric heme in CooA. A water molecule is a possible candidate for the sixth ligand of the ferric heme in CooA because ferric CooA shows almost the same EPR spectrum as the substrate-free cytochrome P450\textsubscript{Cam}, which contains a thiolate and a water as the axial ligands (39, 40). However, we cannot determine whether the sixth axial ligand in the ferric heme is a water or some amino acid residue.

Ferrous CooA shows the typical electronic absorption spectrum of the six-coordinate, low-spin hemeproteins. In the resonance Raman spectrum of ferrous CooA, \( v_2 \) and \( v_3 \) bands have been observed at 1579 and 1491 cm\(^{-1}\), respectively (43). These values are similar to those of the ferrous cytochrome \( b_2 \) (\( v_2 = 1583 \) and \( v_3 = 1493 \) cm\(^{-1}\)). These results indicate that the heme in ferrous CooA is in the six-coordinate, low-spin form. Ferrous H77Y CooA showed an electronic absorption spectrum similar to that of deoxymyoglobin that contains a five-coordinate heme. The resonance Raman spectrum of ferrous H77Y CooA shows the split frequency of 1579 and 1583 cm\(^{-1}\) (44). These results indicate that the heme in ferrous CooA is in the six-coordinate, low-spin form. On the other hand, ferrous H77A CooA shows an electronic absorption spectrum similar to that of wild-type CooA. The electronic absorption spectra of ferrous H77A and H77Y CooA are typical of six-coordinate and five-coordinate hemeproteins, respectively. These results suggest that His\textsuperscript{77} may be the axial ligand of the ferrous heme or may be located near the heme in ferrous CooA.

Cys\textsuperscript{75} is coordinated to the ferric heme in CooA as described above. Is it also coordinated to the ferrous heme? Ferrous low-spin, thiolate-ligated heme complexes and hemoprotein such as H450 (a soluble iron protoporphyrin IX-containing protein of unknown function (46)) typically exhibit Soret peaks at \( -445 \) nm (46-49), compared with the 424.5 nm Soret band exhibited by ferrous CooA. It has been reported that the blue shift in the wavelength of the Soret peak of ferrous H450 takes place from near 450 nm to \( -425 \) nm upon lowering the pH to 6, which requires that the thiolate (cysteinate) ligand is either protonated or displaced upon lowering the pH (46, 48, 49). In the case of ferrous CooA, therefore, Cys\textsuperscript{75} will be protonated or replaced by another amino acid residue to show the Soret peak at 424.5 nm.

The resonance Raman spectrum of CO-bound CooA has revealed that a histidine is the fifth ligand of the heme in the CO-bound form (43). The stretching modes of Fe–CO and C=O, \( \nu(\text{Fe–CO}) \) and \( \nu(c=O) \), have been observed at 487 and 1699 cm\(^{-1}\), respectively, in CO-bound CooA (43). Among the His mutants in CooA, only the mutation at His\textsuperscript{77} affected the properties of the electronic absorption spectra of CooA. These results indicate that His\textsuperscript{77} is the proximal ligand in CO-bound CooA. The properties of H77A and C75A CooA transcriptional activator activity support that His\textsuperscript{77} is the proximal ligand of CO-bound CooA, but Cys\textsuperscript{75} is not. The binding of CO to the heme activates CooA as the transcriptional activator by the conformational change around the heme and finally in the whole molecule as discussed below. Therefore, if the mutation is introduced in the proximal ligand in CO-bound CooA, it will cause the change in the transcriptional activator activity. C75A CooA shows activity similar to that of the wild type, whereas H77A CooA is inactive even in the presence of CO, which supports the above conclusion.

On the basis of the above discussion, the model of the coordination structure of the heme in CooA we propose is shown in Fig. 9. CooA shows the unique property that the axial ligand is exchanged upon the change in the redox state of the heme iron. Cys\textsuperscript{75} is coordinated to the ferric heme, but not to the CO-bound heme. On the contrary, His\textsuperscript{77} is coordinated to the CO-bound heme, but not to the ferric heme. The similar redox-controlled ligand exchange has been reported for cytochrome cd\textsubscript{1} from \textit{Thiophaera pantotropha} (51, 52). The exchange of the axial ligands is thought to be responsible for the adjustment of the redox potential to regulate the internal electron transfer and for the release of the reaction product (NO) from the d\textsubscript{1} heme (51, 52). In the case of CooA, the exchange of the axial ligand upon the change in the redox state of the heme may be concerned with the regulation of the redox potential of the heme.

Recombinant CooA expressed in \textit{E. coli} shows transcriptional activator activity when the reporter strain is grown with CO, when CooA exists in CO-bound form. It has been reported that CooA exhibits sequence-specific DNA binding and binds DNA only in the presence of CO under anoxic, reducing conditions (4, 5, 16), which shows that only CO-bound CooA can bind the target DNA to be active as the transcriptional activator. These results indicate that the binding of CO to the heme in CooA is a very important step in activating CooA as the transcriptional activator. CO should replace one of the axial ligands to bind the heme because the ferrous heme in CooA, which can bind CO, is in the six-coordinate form. NMR spectra of ferrous and CO-bound CooA reveal that the release of the axial ligand from the heme induced by the binding of CO causes some conformational change around the heme. This signal of the conformational change around the heme, which is induced by the binding of CO, will finally change the conformation of the
whole molecule to be adapted for the specific binding to the target DNA.

The properties of M131L CooA transcriptional activator activity suggest that the alteration of the relative orientation of the two domains, the heme-binding domain and the DNA-binding domain, will be involved in the activation process of CooA, which is triggered by the binding of CO. As Met^{131} is located at the end of the heme-binding domain and adjacent to the hinge region that connects the heme-binding domain and the DNA-binding domain (14), the replacement of Met by Leu at position 131 will cause the change in the relative orientation of the two domains by the change in the steric hindrance of the residue at position 131. M131L CooA is active even in the absence of CO probably because the conformation of M131L CooA in the absence of CO will be the same as that of wild-type CooA in the presence of CO. A similar effect of the mutations with that of the wild type, but the reasons for this up-regulation are not obvious at present.

CooA is a new member of the heme-based sensor protein family. So far, two hemeoproteins, FixL (17–24) and soluble guanylate cyclase, contain the five-coordinate heme and is different, especially at the initial stage. Whereas FixL and soluble guanylate cyclase, the signal transduction mechanism will be the common mechanism regulating the function of this suggests that the ligand-induced conformational change induced by the binding of the ligand has been reported with CooA and soluble guanylate cyclase. A similar conformational change triggered by the replace-soluble guanylate cyclase contain the five-coordinate heme and is different, especially at the initial stage. Whereas FixL and soluble guanylate cyclase, the signal transduction mechanism will be the common mechanism regulating the function of

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