Heme Environmental Structure of CooA Is Modulated by the Target DNA Binding

EVIDENCE FROM RESONANCE RAMAN SPECTROSCOPY AND CO REBINDING KINETICS*

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In order to investigate the gene activation mechanism triggered by the CO binding to CooA, a heme-containing transcriptional activator, the heme environmental structure and the dynamics of the CO rebinding and dissociation have been examined in the absence and presence of its target DNA. In the absence of DNA, the Fe-CO and C=O stretching Raman lines of the CO-bound CooA were observed at 487 and 1969 cm$^{-1}$, respectively, suggesting that a neutral histidine is an axial ligand trans to CO. The frequency of ν(Fe-CO) implies an open conformation of the distal heme pocket, indicating that the ligand replaced by CO is located away from the bound CO. When the target DNA was added to CO-bound CooA, an appearance of a new ν(Fe-CO) line at 519 cm$^{-1}$ and narrowing of the main line at 486 cm$^{-1}$ were observed. Although the rate of the CO dissociation was insensitive to the additions of DNA, the CO rebinding was decelerated in the presence of the target DNA, but not in the presence of nonsense DNA. These observations demonstrate the structural alterations in the heme distal site in response to binding of the target DNA and support the activation mechanism proposed for CooA, which is triggered by the movement of the heme distal ligand to modify the conformation of the DNA binding domain.

CooA is one of the heme proteins that act as a DNA-binding transcriptional activator (1, 2). The purple, nonsulfur, phototrophic bacterium Rhodospirillum rubrum synthesizes a series of enzymes that oxidize carbon monoxide (CO) into carbon dioxide, which is coupled to the evolution of molecular hydrogen (3). Unlike other bacteria capable of oxidizing CO anaerobically, R. rubrum expresses the CO-oxidizing enzymes only in the presence of atmospheric CO (4). A gene region designated as cooa was demonstrated to be responsible for the regulation, whose product, protein, CooA, shows high homology with the known transcriptional regulators, such as cyclic AMP receptor protein (CRP)$^1$ (28% identical, 51% similar) (4). Like the mechanism of CRP activation by cyclic AMP, CO is assumed to bind with CooA (3, 5–10), which causes the protein-CO complex to bind specifically to the target DNA, resulting in the expression of CO oxidizing enzymes (7, 10).

CooA is a homodimer containing 222 amino acid residues in each subunit (2, 7). Recent success of the high level expression of CooA in Escherichia coli has enabled us and other researchers to obtain enough homogeneous protein for spectroscopic investigations (1, 2). It was revealed that each subunit of CooA contains a single b-type heme as a prosthetic group, which can bind exogenous CO reversibly, as is evidenced by its optical absorption spectra (1, 2). The significant homology between CooA and CRP suggests that CooA consists of two domains, N-terminal effector binding domain and C-terminal DNA-binding domain containing the helix-turn-helix motif (11–13). Our recent mutagenesis work confirmed that the N-terminal region of CooA consisting of amino acid residues from Met-1 to Met-131 is the heme binding domain (14). Alteration at the heme center caused by the CO binding should be transmitted to the DNA-binding domain, which increases the affinity of CooA with the target DNA. The molecular mechanism of this process remains to be elucidated and would be an important contribution to the understanding of the transcriptional activation in prokaryotes.

The CO binding to the heme has special importance for characterization of heme proteins. The vibrational frequencies of the Fe-CO unit detected by resonance Raman and infrared spectroscopies are used to characterize the axial ligand trans to the bound CO as well as the polarity of the distal heme pocket (15–18). The rebinding kinetics of CO after photodissociation from CO-bound hemes reflect the environment around the hemes. The characterization of CO-bound CooA is of special interest, since it is the CO-bound form of CooA that plays crucial and physiological role in its gene activation (1–10), and provides a clue to elucidate the activation mechanism of CooA by CO. We have, therefore, undertaken the resonance Raman investigation on the CO-bound and reduced forms of CooA. CO rebinding kinetics of CooA were also determined by a laser photolysis method. It has been confirmed that the ferrous protein is in a six-coordinated form with two axial ligands, one of which is suggested to be a histidine residue (2). Addition of CO to the reduced protein causes the replacement of the other axial

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$^1$ The abbreviations used are: CRP, cyclic AMP receptor protein; Mb, myoglobin.
ligand by CO and creates the open distal pocket. The conformation of the distal heme site was modulated by the specific interaction with the target DNA. These results are consistent with the signal transduction mechanism proposed for CooA that the specific DNA binding is induced by the movement of the distal ligand triggered by the CO binding (1, 2).

**EXPERIMENTAL PROCEDURES**

The protein expression and preparation were performed as described previously (1, 14). In brief, recombinant CooA was expressed in *E. coli* JM109 and purified using consecutive column chromatographies; a Q-Sepharose ion-exchange column (Amersham Pharmacia Biotech) and a Chelating Sepharose column (Amersham Pharmacia Biotech, HR 10/10). After removing salts by dialysis against an appropriate buffer, the sample was used for spectroscopic measurements. Reduced CooA was prepared by adding a slight excess of freshly prepared dithionite solution under argon atmosphere into the protein solution. CO-bound CooA was prepared by introducing gaseous CO into the reduced sample.

The resonance Raman spectra were obtained by excitation with 413.1-nm light from a Kr+ laser (Spectra Physics, model 2016). The scattered light was dispersed with a single polychromator (Ritsu, DG-1000) equipped with a cooled CCD camera (Astromed, CCD3200). The spectral slit width was 6 cm⁻¹. The sample solutions for Raman measurements were sealed in quartz cells which were rotated at 1000 rpm at room temperature. Typically, the sample aliquots were 5 μl on the basis of heme content dissolved in 50 mM Tris/HCl buffer at pH 8.0. Raman shifts were calibrated using heat indene, CCl₄, and an aqueous solution of potassium ferrocyanide as frequency standards, providing accuracy of ±1 cm⁻¹ for intense isolated lines. The laser light was focused into the cell so that the laser power was −0.6 milliwatts for CooA-CO to avoid photolysis of the iron-bound CO, and −3 milliwatts for ferrous CooA. Absorption spectra were measured both prior to and after Raman measurements, and no degradation was detected under the experimental condition applied in this study.

The kinetics of CO rebinding were obtained by a laser photolysis apparatus (19). The absorption changes were monitored at 420 nm using a photomultiplier (Hamamatsu, R2949) and digitized by a storage oscilloscope (Tektronix, TDS-520A). The second harmonic (532 nm, 6-ns pulse width) of a Q-switched Nd:YAG laser (Continuum, Surelite-I) was used for the photolysis. The incident power was about 10 mJ. The CO concentrations were varied every 0.2 nm from 0.2 to 1.0 nm by using a gas mixer (Stec, SGD-XX). The apparent rate constants, kₘ₃, calculated from the kinetic traces were plotted against CO concentrations to estimate the bimolecular recombination rate constant (kₘ₃ = kₘ₃(CO)). The CO dissociation rate constants (kₘ₃) were determined by rapidly mixing CooA-CO with equal volume of a concentrated (20 mM) potassium ferrocyanide (20, 21) with a stopped-flow mixer (Unisoku). The time courses were monitored at 566 nm with a spectrophotometer (PerkinElmer, Lambda19) or a rapid scanning monochrometer (Olis, RSM-1000). The sample solutions used for the kinetic measurements typically contained 20 μM CooA in 50 mM Tris/HCl buffer at pH 8.0, 20 °C. A representative DNA sequence recognized by CooA is as follows (10).

**SEQUENCE 1**

5'-AAGCTGTCACTGCAGGCACAGCAGGGG-3'  
3'-TTGACAGTAGACCGGTGTCTGCCCCGG-5'

2 The NO-bound ferrous CooA is unstable, which precludes us from establishing the coordinated histidine by ESR spectroscopy and estimating the kₘ₃ rate of CO by the conventional NO-replacement method.

**RESULTS AND DISCUSSION**

The high frequency resonance Raman spectra (1200–1700 cm⁻¹) of the dithionite-reduced (spectrum A), and CO-bound (spectrum B) forms of CooA are presented in Fig. 1, and the observed frequencies of the major lines are compared with those of cytochrome c (22), cytochrome c with Met-80 → Cys mutation (23), cytochrome b₅ (24), and myoglobin (Mb) (25) in Table I. It is established that the lines in the high frequency region can be used as sensitive markers of the oxidation state (ν₁) and spin and coordination states (ν₂ and ν₃), respectively (polarization data not shown), whose frequencies indicate that the heme iron is six-coordinate low spin as found for cytochrome b₅. As we will discuss later, CO-bound CooA is likely to have a histidine as a fifth ligand, which should also be one of the axial ligands in the reduced protein. Coordination of methionine or cysteine as the other axial ligand can probably be excluded, since the frequency of ν₂ usually appears at around 1590 cm⁻¹ for His/Met or His/Cys ligand pairs, 10 cm⁻¹

**FIG. 1. High frequency resonance Raman spectra of CooA in the dithionite-reduced (A) and CO-bound (B) states.**

**TABLE I**

| Protein                  | ν₁   | ν₂   | ν₃   | ν(Fe-C) | ν(C=O) | Ref.     |
|--------------------------|------|------|------|---------|--------|---------|
| CooA (Fe²⁺)              | 1579 | 1491 | 1359 | 487     | 1969   | a       |
| CooA-CO                  | 1580 | ND²  | 1371 | 22      | 22     | a       |
| Cytochrome c (Fe²⁺)      | 1592 | 1489 | 1363 | 23      | 23     | a       |
| Cytochrome c M80C (Fe²⁺) | 1592 | 1494 | 1360 | 24      | 24     |       |
| Cytochrome b₅(Fe²⁺)      | 1583 | 1498 | 1361 | 25      | 25     |       |
| Myoglobin (Fe²⁺)         | 1564 | 1473 | 1356 | 26      | 26     |       |
| Myoglobin-CO             | 1585 | 1501 | 1373 | 27      | 27     |       |

a This work.  
b ND, not detected.
higher than that for CooA (22, 23, 27). Furthermore, the absorption maxima of the $a$, $b$, and Soret absorption bands for ferrous CooA are 559, 529, and 425 nm, respectively (1), which are distinct from those of cytochrome $c$ (550, 522, and 445 nm) (28) or imidazole-bound cytochrome P450 (566, 538, and 445 nm) (29). Since the absorption peaks in the optical spectrum are rather similar to those of cytochrome $b_5$ (556, 526, and 423 nm) (30), the sixth ligand of ferrous CooA is likely to be a neutral ligand.

Addition of CO to the reduced CooA forms a stable CooA-CO complex, which is indicated by a strong Soret absorption band at 422 nm (1, 2). The resonance Raman spectrum for the CooA-CO complex (spectrum B, Fig. 1) is characteristic of a low spin six-coordinated heme and resembles that of Mb-CO. Fig. 2 shows resonance Raman spectra in the Fe-CO stretching and the C-O stretching frequency regions of the natural abundance CO (spectrum A) and 13CO-labeled CO (spectrum B) adducts of CooA. A line at 487 cm$^{-1}$ can be assigned to the stretching mode of Fe-CO, $\nu$(Fe-CO), on account of its 4 cm$^{-1}$ low frequency shift upon the 13CO substitution. The C-O stretching frequency, $\nu$(C-O), appeared at 1969 cm$^{-1}$ for natural abundance CO and at 1927 cm$^{-1}$ for 13CO. There is a well-known inverse correlation between the frequencies of the Fe-CO and C-O stretching modes (15, 31–32), which can be used to estimate the strength of the proximal ligand. The frequencies measured for CooA-CO lie on the line composed by heme proteins with neutral histidine as a proximal ligand (correlation plot not shown). This result, as well as the appearance of the Soret absorption maximum at 422 nm, suggests that a histidine ligates to the heme iron in CO-bound CooA, which can be compared with the recent mutagenesis studies demonstrating His-77 as a possible candidate for the ligand (2).4

The Fe-CO stretching mode, $\nu$(Fe-CO), displays a wide range of frequencies (450–550 cm$^{-1}$) reflecting the polarity of the distal heme pocket (16–18, 31–34). The isolated Fe-CO unit as is observed for model compounds and for Mb-CO in the acidic solution shows frequencies at ~490 and ~1965 cm$^{-1}$ for $\nu$(Fe-CO) and $\nu$(C-O), respectively, and is labeled as the A$_0$ conformer in Mb (34). It is considered that the distal His swings...
out of the heme pocket and influences no positive interaction on the Fe-CO unit of acidic Mb (17, 18, 34). The appearances of υ(Fe-CO) and υ(C=O) at 487 and 1969 cm⁻¹, respectively, for CO-bound CooA demonstrate that the Fe-CO unit has a conformation similar to the A₃ conformer of Mb. This observation indicates the absence of any significant interactions between the bound CO and the distal heme pocket, that is, the absence of the distal histidine or any positively and sterically interacting residues around the iron-bound CO. Since ferrous CooA has a six-coordinated iron, CO should replace one of the axial ligands to form CO-bound CooA. The replaced ligand, whose identity has not been specified, moves away and affords no interaction with the bound CO. The absence of the distal amino acid residues that interact with CO is consistent with the interaction with the bound CO. The absence of the distal amino acid residues that interact with CO is consistent with the observation that stable oxygen-bound CooA is not formed (1), because the interaction with the positively charged residue would stabilize the iron-bound oxygen. In spectrum A of Fig. 2, the Fe-C-O bending mode, υ(Fe-C-O), was not observed. The low intensity of υ(Fe-C-O) is characteristic of heme proteins with an unconstrained heme pocket (15), further supporting the open conformer of CO-bound CooA.

Fig. 3 depicts the resonance Raman spectra of natural abundance (spectrum B) and ¹³C¹⁸O-labeled (spectrum C) CO-bound CooA in the presence of the target DNA. Comparing with the spectrum obtained in the absence of DNA (spectrum A), addition of 20 eq of the target DNA per monomeric CooA results in the narrowing of the major υ(Fe-CO) line at 486 cm⁻¹ from 20 to 15 cm⁻¹ measured by Gaussian line fitting concomitant with the appearance of a weak line at 519 cm⁻¹. Both of the lines can be assigned to Fe-CO stretching modes, because the isotopic shifts for both lines were observed (spectrum D).⁵ The frequency at 519 cm⁻¹ corresponds to the A₃ conformer of Mb-CO (34). These observations show that the specific DNA binding causes the structural change around the distal heme environment, which shifts the equilibrium between A₀ and A₃ conformers to the A₃ site and the narrowing of the A₀ mode. Although some controversy exists on the interpretation of the A₃ conformer of Mb (18, 32, 33), it is considered that a strong hydrogen bonding would increase the back-donation and enhance the Fe-C stretching frequency to ~520 cm⁻¹ (35). A side chain of the replaced axial ligand is a possible candidate that exerts hydrogen-bonding interaction to the iron-bound CO. This result demonstrates that the distal heme site and the DNA binding domain of CooA are structurally connected to each other. Although the observed effects of the DNA binding are relatively minor, we consider it reasonable since the CO-bound protein is in the DNA-binding form either in the presence and the absence of the target DNA.

The kinetics of CO recombination to ferrous CooA after the photolysis of CO in the absence of the target DNA is illustrated as trace A of Fig. 4. The observed kinetics consists of bimolecular and geminate processes (geminate data not shown). We could not resolve the geminate process well due to its fastness that is comparable to the response time of our system (about 20 ns).⁶ The bimolecular process can be separated into three exponentials. The linear dependence of the decay rate constants (k₃obs) of the exponentials on CO concentrations from 0.2 to 1.0 mM ascertains that they are the bimolecular rebinding reactions. The calculated association rate constants (k₃on) and the relative populations are 32, 6.8, and 1.2 μM⁻¹ s⁻¹, and 53, 31, and 16%, respectively (Table II). The relative populations are independent of the CO concentration. The kinetic difference spectra between the transient and the initial CO-bound species show that the CO rebinds to the five-coordinated heme before the coordination of the internal ligand (data not shown). The observed heterogeneity, hence, suggests the presence of conformational transitions that compete with the CO rebinding within the five-coordinated protein. The transitions between the activated and inactivated forms and/or the allosteric interaction between the two monomers are possibly responsible for the observed heterogeneity. The addition of the target DNA decelerated the CO association (trace B), while the kinetic trace was insensitive to the addition of nonsense DNA (trace C). The DNA titration experiment shows that the change reached a maximum with 10 eq of the target DNA per monomeric CooA without changing the quantum yield of the photodissociation (data not shown). The fitting calculation of trace B gives rate constants of 24 (43%), 6.4 (40%), and 1.2 (17%) μM⁻¹ s⁻¹ and shows that the fastest phase is primarily decelerated. The slow CO association demonstrates that the CO binding site is more crowded in the DNA-bound conformer, which is consistent with the observed conformational transition of the Fe-CO unit.

The dissociation rate constants (k₄off) of CO from CO-bound CooA were determined by the oxidation method and are summarized in Table II (20, 21). The kinetic traces could be fitted with a single exponential within the noise level of the traces. The obtained rate constants are 0.021 ± 0.003 s⁻¹, 0.021 ± 0.004 s⁻¹, and 0.023 ± 0.003 s⁻¹ for the samples without DNA, in the presence of nonsense DNA, and in the presence of the target DNA, respectively. All values are identical within our experimental uncertainty, and are similar to those of myoglobin and each subunit of hemoglobin possessing a neutral histidine as a trans ligand (36). If we estimate the change in the free energy between the CO-bound and unbound (five-coordinated) states using the fastest k₃off rate (ΔG = −RTln(k₃off[C]/k₃on)), the largest difference caused by the DNA binding (ΔΔG = ΔG₅DNA − ΔG₀DNA) is not more than 1 kJ mol⁻¹. This is in accordance with the cAMP binding to CRP at low salt concentration, whose free energy difference is about 2 kJ mol⁻¹ (37).

It is proposed that the activation of CooA is induced by the displacement of the distal ligand by CO, which in turn modifies the conformation of the DNA binding domain (1, 2). The present resonance Raman and kinetic data afford the first experimental evidence that supports the proposed mechanism. First, the open conformation of the Fe-CO unit for CO-bound CooA in the absence of DNA implicates that the distal ligand in the ferrous form is replaced by CO with a rather large conformational transition. Second, the structure of the heme distal site of CooA-CO is susceptible to the conformational transition at the DNA binding domain. This suggests that the structural alterations around the heme distal site can be efficiently trans-

### Table II

| Protein | k₃off | k₃on | k₄off | k₃off | k₄off |
|---------|-------|------|-------|-------|-------|
| CooA − DNA (%) | 32 ± 1 (53 ± 5) | 6.8 ± 0.5 (21 ± 2) | 1.2 ± 0.1 (16 ± 3) | 0.021 ± 0.003 |
| CooA + DNA (%) | 24 ± 1 (43 ± 5) | 6.4 ± 0.1 (40 ± 2) | 1.2 ± 0.1 (17 ± 4) | 0.023 ± 0.003 |

⁵ We could not detect the C=O stretching Raman line in the presence of the target DNA due to the strong fluorescence background originating from the DNA. Detection of the line using infrared spectroscopy was also found impossible, because of its necessity to use concentrated samples (>1 mM), which caused aggregation of the target DNA.

⁶ The dissociation rate constants (k₄off) of CO from CO-bound CooA were determined by the oxidation method and are summarized in Table II (20, 21). The kinetic traces could be fitted with a single exponential within the noise level of the traces. The obtained rate constants are 0.021 ± 0.003 s⁻¹, 0.021 ± 0.004 s⁻¹, and 0.023 ± 0.003 s⁻¹ for the samples without DNA, in the presence of nonsense DNA, and in the presence of the target DNA, respectively. All values are identical within our experimental uncertainty, and are similar to those of myoglobin and each subunit of hemoglobin possessing a neutral histidine as a trans ligand (36). If we estimate the change in the free energy between the CO-bound and unbound (five-coordinated) states using the fastest k₃off rate (ΔG = −RTln(k₃off[C]/k₃on)), the largest difference caused by the DNA binding (ΔΔG = ΔG₅DNA − ΔG₀DNA) is not more than 1 kJ mol⁻¹. This is in accordance with the cAMP binding to CRP at low salt concentration, whose free energy difference is about 2 kJ mol⁻¹ (37).

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mitted to the DNA-binding domain of CooA. Further spectroscopic and kinetic investigations combined with the site-directed mutagenesis are important to clarify the heme coordination structure as well as the gene-activation mechanism of CooA.

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