Binding of CO at the Pro² Side Is Crucial for the Activation of CO-sensing Transcriptional Activator CooA

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CooA is a heme-containing transcriptional activator that anaerobically binds to DNA at CO atmosphere. To obtain information on the conformational transition of CooA induced by CO binding to the heme, we assigned ring current-shifted ¹H NMR signals of CooA using two mutants whose other axial ligands of the heme were replaced. In the absence of CO, the NMR spectral pattern of H77Y CooA, in which the other axial ligand of the heme is confirmed to be His 77 as has been demonstrated in our previous studies (9, 11). It is further shown that the overall fold of the protein is indeed preserved even though the protein forms CO-bound heme (9, 11). The gene products encoded in these operons constitute a CO oxidizing system that enables the bacterium to utilize CO as the sole energy source under CO atmosphere (4, 5). Interestingly, the Pro² signals were undetectable in the upfield region of the spectrum of the CO-bound state, which confirms that CO displaces Pro². Interestingly, the Pro² signals were observed for CO-free-bound H77Y CooA, implying that CO binds to the trans position of Pro² in H77Y CooA. The abolished CO-dependent transcriptional activity of H77Y CooA is therefore the consequence of Pro² ligation. These observations are consistent with the view that the movement of the NH₂ terminus triggers the conformational transition to the DNA binding form.

The photosynthetic bacterium Rhodospirillum rubrum has a heme-based CO sensor, CooA, which activates the expression of cooMKLXUH and cooFSCTJ operons in response to CO (1–3). The gene products encoded in these operons constitute a CO oxidizing system that enables the bacterium to utilize CO as the sole energy source under CO atmosphere (4, 5). CooA belongs to a family of transcriptional activators that includes the cAMP receptor protein and the fumarate-nitrate reductase activator protein (1). The investigation on CooA is therefore important to understand the general activation mechanism of this family of transcriptional activators (1–4).

CooA is an ~50-kDa homodimeric protein, with each monomer possessing a low spin heme in the ferrous state (6–8, 16). In the absence of CO, CooA possesses the six-coordinated heme with two endogenous ligands and is in the inactivated state that does not bind to DNA (the CO-free state or the low affinity state). The CO-free state denotes hereafter CooA with the ferrous heme in the absence of CO. In the presence of CO, one of the axial ligands of the ferrous heme is displaced with CO, which leads to the capability of CooA to bind to a specific sequence of DNA (the CO-bound state or the high affinity state) (7). Thus, the molecular mechanism of the conformational transition triggered by the CO binding is of essential importance to understand the function of CooA (7–9).

A significant advance in the understanding of the function of CooA has been presented in the recent x-ray crystal structure (10). It is confirmed that the overall fold of the protein is indeed similar to cAMP receptor protein that forms dimeric structure interfaced by long helices (C-helix). The most prominent structural characteristic of CooA is the ligation of the NH₂-terminal Pro² of one subunit to the heme iron in the other subunit. The other axial ligand of the heme is confirmed to be His 77 as has been demonstrated in our previous studies (9, 11). It is further proposed that the straight C-helix in the low affinity state becomes bent in the high affinity state upon the CO binding. However, since the x-ray structure of CooA is presented only for the CO-free state, the molecular mechanism that triggers the proposed conformational transition remains to be elucidated.

It was suggested from the resonance Raman spectroscopy that the axial ligand of the CO-bound form is likely to be His 77, and the release of the other ligand, identified as Pro² later, triggers the conformational transition at the DNA binding domain (12). However, Roberts and his co-workers (13, 14) proposed that CO displaces His 77 instead of Pro² and the changes in the His 77 ligation triggers the DNA binding. While our recent investigations using time-resolved resonance Raman and extended x-ray absorption fine structure spectroscopies have unequivocally indicated the ligation of His 77 in the CO-bound CooA (12, 15), the site-directed mutagenesis of the axial ligands seems inconsistent with the movement of Pro² for the trigger of the DNA binding activity. For example, the deletion of NH₂-terminal residues (CooAΔN5) fully maintained the activity of CooA (15). Furthermore, the mutation of His 77 (H77Y CooA) abolished the CO-induced transcriptional activity of CooA even though the protein forms CO-bound heme (9, 11). Investigation on the detailed coordination structures of the

We confirmed that the NH₂-terminal methionine (Met²) is removed after the expression of wild-type CooA in the host cell.
heme in these mutants is necessary to clarify the functional significance of these axial ligands and their functional differences.

To characterize the structural changes induced by the CO binding to the heme in CooA and clarify the significance of the release of the NH2-terminal Pro2 ligand, we conducted 1H NMR investigation of CooA in the absence and presence of CO. As we reported previously, reduced CooA provides characteristic 1H NMR signals between −1 and −6 ppm that show drastic changes upon the CO binding (11). These ring-current-shifted signals are originating from residues around heme and therefore should provide us with the detailed information on the conformational transition triggered by the CO binding (11). However, due to the unprecedented coordination structure of the heme in CooA, the assignments of the upfield NMR signals have not been presented. We therefore compared the NMR spectra of CooA in the absence and presence of CO with those of the axial mutants to assign the upfield NMR signals. We confirmed that the proximal ligand of the CO-bound CooA is His77, as we have indicated in the previous studies (12). Furthermore, the changes in the NMR spectra induced by the axial mutations provide us with a simple and consistent picture on the triggering event for the conformational transition of CooA.

**EXPERIMENTAL PROCEDURES**

Procedures for the construction of the expression systems, the expression and purification for the axial mutants, were reported previously (6–8). NH2-terminal analysis for CooA expression and purification for the axial mutants, were reported previously (6–8).

CooA was prepared by flowing CO over the reduced protein for 10 min with mild stirring.

Proton NMR experiments were performed at 25 °C on a 500-MHz spectrometer (Bruker, AVANCE DRX500) equipped with an Indy work station (Siemens, Graphics) (17). A 1331-pulse sequence was used to minimize resonances inside the 0–10 ppm region (18). The proton shifts in D2O or at pH 7.4 containing 100% D2O. Protein concentrations were over 300 μM on the heme basis, respectively.

**RESULTS AND DISCUSSION**

**Assignments of the 1H NMR Peaks for the CO-free State**—Fig. 1A shows the 1H NMR spectrum for the CO-free state of CooA in H2O, in which several characteristic and well resolved resonances are observed in the upfield region (−6 to −1 ppm) (11). These ring-current-shifted signals can be assigned to protons of the amino acid residues nearby the porphyrin ring. As shown in Fig. 1D, three of the signals (b, d, and e) at −2.8, −3.6, and −4.5 ppm are nonexchangeable. Based on the intensity of an isolated resonance at 10.3 ppm from the NH of a single tryptophan at position 110 (spectrum not shown), the intensity of each signal (b, d, and e) corresponds to a single proton. Each of the exchangeable signals at −6.1 (a) and −4.0 (c) ppm is also estimated to possess a single proton intensity.

While upfield shifted NMR signals have been observed for several heme proteins, the spectral pattern of CooA is unique and indicates the distinct coordination structure. For example, the ferrous state of cytochrome c550 M100K mutant (19) indicates signals between −1 and −4 ppm that are assigned to protons of an axial ligand, Lys100. However, the upfield shifted signals of CooA were observed in the region below −4 ppm (−4.0, −4.5, and −6.1 ppm). In contrast, the ferrous cytochrome f,2 in which the NH2-terminal amino group of Tyr2 is coordinated to heme (21–23), indicates four nonexchangeable signals at −1.6, −3.3, −6.8, and −8.2 ppm (20). The x-ray structure of cytochrome f (23) suggests that these signals can be assigned to C6H of Tyr1, C5H of Tyr1, C4H of Pro2, or C3H of Phe6, all of which are located within 5 Å from the heme iron. While the signal pattern of cytochrome f is different from that of CooA, the spectrum exemplifies that the coordination of NH2-terminal amino group can shift signals below −4 ppm.

The coordination structure of CooA can consistently explain its NMR spectrum. Because Pro2 and His77 are the axial ligands of the CO-free heme (10, 15), the signals b, d, and e are likely derived from the nonexchangeable protons of the proline ring of Pro2 and/or imidazole ring of His77. The nonexchangeable protons of histidines bound to low spin ferrous hemes, however, are usually observed at around 1–2 ppm (24). Therefore, given that the signals b, d, and e originate from the protons in the axial ligands, they are likely due to the protons from Pro2. The x-ray structure of the CO-free CooA shows that the distances from the heme iron to protons attached to C6 and

2 The reported NMR spectrum for ferrous cytochrome f only showed the region up to −5 ppm (20). We re-examined the NMR spectrum of ferrous cytochrome f and found two additional signals at −6.8 and −8.2 ppm.

3 K. Yamamoto H. Ishikawa, H. Harada, K. Ishimori, and I. Morishima, unpublished results.
C₆ of Pro² are less than 4 Å (7) and suggests that signals b, d, and e are derived from one methyne proton at C₆ and two methylene protons at the C₅ position of Pro². Furthermore, we detected a significant cross-peak between the signals b and e in preliminary two-dimensional COSY measurements (spectra not shown). We therefore assigned signals b, d, and e to C₅-H, C₆-H, and C₅,₆-H of Pro², respectively.

The above assignments are supported by the NMR spectra of the axial ligand mutants, H77Y CooA and CooAΔN5. We constructed the CooAΔN5 mutant, in which four residues, Pro²-Pro⁵-Pro⁶-Arg-Phe⁶, are deleted from the amino terminus.⁵ While the optical absorption (15) and resonance Raman spectra (spectra not shown) of CooAΔN5 in the CO-free state are very similar to those of WT CooA, the CooAΔN5 mutant shows distinct NMR spectra from those of wild-type CooA as shown in Fig. 1, C and F. Nonexchangeable proton signals, signals b, d, and e, are completely missing in the mutant, confirming that these signals are derived from Pro².

In contrast to CooAΔN5, the spectral changes induced by the substitution of His⁷⁷ for Tyr (H77Y CooA) are less drastic, as shown in Fig. 1, B and E. The spectra of H77Y CooA² are almost identical to those of wild-type CooA, although an exchangeable proton signal at ~4.0 ppm (c) disappears and the spectral pattern between ~0.5 and ~2.0 ppm is perturbed. The minor spectral changes support the assignment that the signals b, d, and e originate from the Pro² side. While the assignments for the nonexchangeable signals are convincing, those of the exchangeable protons are still ambiguous. Since signal a is the most upfield shifted and is insensitive to the substitution of His⁷⁷ for Tyr (H77Y CooA) and is less drastic, as shown in Fig. 1, B and E. The spectra of H77Y CooA² are almost identical to those of wild-type CooA, although an exchangeable proton signal at ~4.0 ppm (c) disappears and the spectral pattern between ~0.5 and ~2.0 ppm is perturbed. The minor spectral changes support the assignment that the signals b, d, and e originate from the Pro² side.

The assignment of the signal c is also unclear. This signal disappears by the mutation at His⁷⁷ and likely belongs to a residue located in the His⁷⁷ side. Although NₓH of His⁷⁷ might be a candidate for the signal c, this is unlikely since the axial His NH proton of cytochrome b₃ possesses a signal at 1 ~2 ppm (24). Another candidate for the signal c would be the sulphydryl proton of Cys⁷⁵, which is one of the axial ligands for the ferrous heme and displaces from the ferrous hemeprotein (7, 9, 11, 25). The distance between the SH proton and the heme iron is less than 4 Å in the CO-free CooA (10), which is consistent with the prominent upfield shift. The NMR signals for most sulphydryl protons in proteins, however, are not observed due to the rapid exchange with solvent water (26). If the signal c is due to the SH proton from Cys⁷⁵, its exchange rate should be retarded, probably by a poor accessibility of solvent waters.

Assignments of the ¹H NMR Peaks for the CO-bound State—To obtain an insight into the activation mechanism of CooA, we measured the NMR spectra of CO-bound wild-type and mutant CooA and compared them with those in the absence of CO. As shown in Fig. 2A, the CO-bound form of wild-type CooA shows no NMR signals from the nonexchangeable protons of Pro² in the region between ~2 and ~6 ppm. The exchangeable signal observed at ~4.5 ppm is not affected by the deletion of the NH₂ terminus (Fig. 3, C and F). Therefore, the proton giving the signal at ~4.5 ppm should be located in the His⁷⁷ side and likely corresponds to the SH proton of Cys⁷⁵ that indicates signal c at ~4.0 ppm in the CO-free CooA (Fig. 1, B and E). The small shift of the peak upon the CO binding might indicate that the conformational transition of the His⁷⁷ side is rather small.

The assignment of Pro² signals for the CO-free CooA and no proton signals from the C₆ and C₅ positions of Pro² in the CO-bound form of the spectrum gives us clear evidence that Pro² displaces from the heme iron in the CO-bound form of CooA. These results further support our previous conclusion from the time-resolved resonance Raman and extended X-ray absorption fine structure spectroscopies (12, 15). Based on the decrease of the ring-current shift for the proton signals from Pro² upon CO binding, the ligand should be displaced from the original location by at least 4 Å in the CO-bound form (10). The large displacement of Pro² induced by the CO binding suggests the importance of the Pro² side as the first event of the conformational transition of CooA.

**CO-dependent Activation Mechanism of CooA—While the results presented above indicate the movement of Pro² as the trigger for the activation of CooA, the activities of CooAΔN5 and H77Y CooA might be interpreted inversely as suggesting the importance of the His⁷⁷ side. CooAΔN5 fully maintains the activity of CooA, while H77Y CooA completely abolishes the CO-induced transcriptional activator activity (9, 11, 15). Examinations of ¹H NMR spectra of these mutants in the CO-bound state, however, give us a clear explanation on their activities based on the movements of the Pro² side.
We will first examine the CooAΔN5 mutant that maintains almost the same transcriptional activity as wild-type CooA (15). Because the $^1$H NMR spectra for the CO-free state of CooAΔN5 do not indicate the hyperfine-shifted signals that are characteristic of the five-coordinated ferrous hemes (spectrum not shown), the ferrous heme of CooAΔN5 is in a low spin state with two axial ligands. We suggest that the two ligands are His$^{77}$ and NH$_2$-terminal amine, since no other ligands are available around the heme in the x-ray structure of wild-type CooA (10). The ligation of the truncated amino terminus is consistent with the $^1$H NMR spectrum of CooAΔN5 (Fig. 1, C and F) that indicates nonexchangable signals at $-2.8$, $-1.4$, and $-0.8$ ppm assignable to protons of C$_2$H and $\beta$-methylene of the N-terminal Met$^5$. Furthermore, the heme marker lines of the resonance Raman spectrum appear at frequencies suggesting the coordination of neutral nitrogen (spectrum not shown), since the frequencies of $v_2$, $v_3$, and $v_4$ for the CO-free state of CooAΔN5 are 1579, 1491, and 1361 cm$^{-1}$, respectively, which are similar to those of cytochrome $b_5$ at 1583, 1493, and 1361 cm$^{-1}$, respectively (25). These results suggest that the truncated NH$_2$-terminal region is flexible enough to coordinate to the heme iron.

As depicted in Fig. 2, C and F, the CO-bound form of CooAΔN5 showed almost the same NMR spectra in the upfield region as those of wild-type CooA. Furthermore, the resonance Raman spectrum for the CO-bound CooAΔN5 mutant can be superimposed on that of the wild-type CooA, including the heme marker lines and the Fe-C stretching line (spectrum not shown). The frequencies of $v_2$, $v_3$, $v_4$, and $\nu$(Fe-C) in the spectrum of CO-bound CooAΔN5 are 1580, 1498, 1370 and 487 cm$^{-1}$, respectively. These of CO-bound wild-type CooA are 1579, 1497, 1369, and 487 cm$^{-1}$, respectively. These results indicate that the CO-bound form of CooAΔN5 retains the His$^{77}$-Fe-CO coordination structure and explain the activity of the mutant that is similar to that of wild-type CooA (15).

We will next consider the H77Y mutant. Noticeably, both of the CO-free and CO-bound forms of H77Y CooA provide the characteristic three signals that are similar to those of wild-type CooA in the CO-free form (Figs. 2B and 1A). For example, the CO-bound state of H77Y CooA exhibits the triad of the nonexchangeable resonances at $-2.6$, $-3.5$, and $-4.3$ ppm corresponding to the signals for the CO-free state of wild-type CooA at $-2.8$, $-3.6$, and $-4.5$ ppm, respectively (Fig. 1A). The appearance of the Pro$^2$ signals implies that CO binds to the trans position to Pro$^2$ in H77Y CooA, resulting in the formation of the CO-Fe-Pro$^2$ complex as depicted in Fig. 3D. Since H77Y CooA is not activated by CO (9, 11), we conclude that the binding position of CO to the heme iron is crucial for the CooA function.

We summarized the coordination structures of wild-type H77Y CooA and CooAΔN5 in the CO-free and CO-bound states in Fig. 3. The binding of CO to the Pro$^2$ side (Fig. 3, B and F) can be a trigger to induce the conformational change to the high affinity form, whereas the binding of CO to the His$^{77}$ side and the maintenance of the Pro$^2$ ligation (Fig. 3D) cannot induce the conformational change for the activation. These results provide us with a simple and consistent picture on the activation mechanism of CooA; the movement of the NH$_2$-terminal loop upon the CO binding for more than 4 Å triggers the conformational transition of the whole protein to the high affinity form. As suggested in the x-ray results (10), the movement of the NH$_2$-terminal loop might modulate the C-helix, which forms the dimer interface and locates near the heme, to assume the bent conformation.

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