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Biochemical and Biophysical Properties of the CO-Sensing Transcriptional Activator CooA

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ABSTRACT
Studies of heme-containing gas sensor proteins have revealed a novel function for heme, which acts as an active site for sensing the corresponding gas molecule of a physiological effector. Heme-based O_2, NO, and CO sensor proteins have now been described in which these gas molecules act as a signaling factor that regulates the functional activity of the sensor proteins. CooA is a CO-sensing transcriptional activator found in the photosynthetic bacterium *Rhodospirillum rubrum*. The binding of CO to the heme group stimulates the transcriptional activator activity of CooA. The mechanisms of CO sensing by CooA and CO-dependent activation of CooA have now been analyzed by both molecular biological and spectroscopic studies and are discussed in this Account.

Introduction

A variety of gas molecules serve as substrates and/or reaction products in many enzymatic reactions including oxygen respiration, denitrification, nitrogen fixation, and methanogenesis. Recently, a novel physiological function of gas molecules that act as signaling molecules has been elucidated and studied extensively.[1−4] As shown in Table 1, ethylene, O_2, NO, and CO are known to act as physiological effectors in the control of various biological functions. The corresponding receptor (sensor) proteins are required for these gas molecules to act in this way. Figure 1 shows a general scheme for the functional mechanism of these gas sensor proteins. A gas molecule that plays the role of the input signal is sensed by an active site of a sensor domain. An intramolecular signal transduction is induced following the detection of the gas molecule, which controls the functional activity in response to the input signal. Generally, a conformational change in the sensor protein facilitates this intramolecular signal transduction.

All of the gas sensor proteins so far reported have a metal-containing prosthetic group at the sensor active site. Specific interactions between protein and gas, for which the metal-containing prosthetic group is used, are required for the gas sensor protein to detect its physiological effector gas. Heme groups have been widely found at the active sites of the O_2, NO, and CO gas sensor proteins, as shown in Table 1. This is not surprising as heme can bind O_2, NO, and CO reversibly.

Table 1. Typical Examples of Gas Molecule Sensor Proteins

sensor protein	effector	prosthetic group	function
FixL	O_2	heme	sensor kinase in FixL/FixJ two-component system
HemAT	O_2	heme	signal transducer for aerotaxis
DOS	O_2	heme	phosphodiesterase
AxPDEA1	O_2	heme	phosphodiesterase, a regulator for cellulose synthesis
sGC	NO	heme	guanylate cyclase
CooA	CO	heme	transcriptional activator
NPAS2	CO	heme	transcriptional factor
ETR1	C_2H_4	Cu^{+} [a]	ethylene receptor in plants

[a] The structure of the active site is not known.

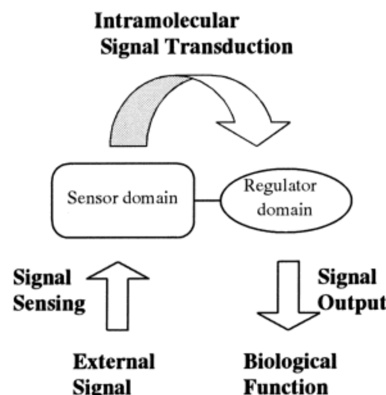

FIGURE 1. Functional scheme for gas sensor proteins.

FixL is the sensor kinase in the FixL/FixJ two-component signal transduction system, which regulates nitrogen fixation gene expression in Rhizobia in response to O_2.[5,6] The kinase activity of FixL is repressed in the presence of O_2, during which time O_2 is bound to the heme in FixL. HemAT is an oxygen-sensing signal transducer protein responsible for aerotaxis control.[7] A myoglobin-like heme domain exists in the amino-terminal region of HemAT, which is responsible for the O_2 response.[7−9] DOS and AxPDEA1 are new members of the heme-based O_2 sensor proteins and consist of PAS and phosphodiesterase domains, respectively.[10,11] With the exception of FixL, the precise mechanisms for sensing O_2 and the concomitant signal transduction pathways that are activated via these O_2 sensor proteins have not yet been elucidated.

Soluble guanylate cyclase (sGC), which catalyzes the conversion of GTP into cyclic GMP that acts as a second messenger, is the only known example of a heme-based NO sensor protein.[1] The catalytic activity of sGC increases by several hundredfold upon NO binding to the heme group. The heme in sGC is 5-coordinate with a histidine as the proximal heme ligand. The Fe−His bond is cleaved to form a 5-coordinate nitrosyl heme complex upon NO binding to the heme in sGC, which induces an activating conformational change in sGC.

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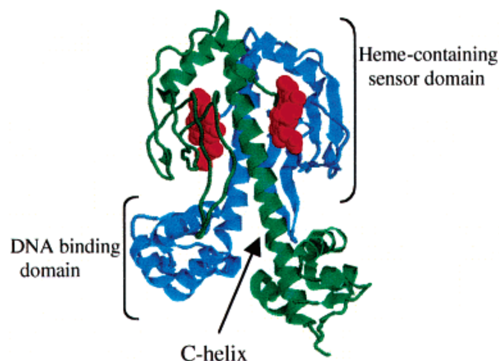


FIGURE 2. Structure of CooA dimer. This figure is drawn based on the coordinates in PDB accession code 1FT9. Monomer A and monomer B are colored in blue and green, respectively. The heme groups in CooA are represented by red space-filling models.

CooA is the first example of a CO sensor protein.[12,13] Recently, it has been reported that the neuronal PAS domain protein 2 (NPAS2), which is a mammalian transcription factor responsible for the regulation of circadian rhythm, is a heme-based CO sensor protein.[14] The most interesting feature of these two CO sensor proteins is that CO plays a physiological role in regulating the function of both. In this Account, I discuss the structure and function of the CO sensor protein CooA.

General Properties of CooA

CooA is a transcriptional activator found in a purple nonsulfur photosynthetic bacterium, *Rhodospirillum rubrum*.[12,13] *R. rubrum* can grow on CO as the sole energy source under anaerobic conditions.[15] CO dehydrogenase and hydrogenase that are encoded by the *coo* operons are expressed under these conditions and are the key enzymes that facilitate growth in a CO environment.[16] CooA is responsible for the transcriptional regulation of the expression of the *coo* operons in response to CO.

The structure of reduced CooA was determined by Lanzilott et al. [17] and is shown in Figure 2. CooA is a homodimer in which each subunit consists of 221 amino acid residues. The first methionine is removed by post-translational modification.[12] Each subunit contains one protoheme that acts as a CO sensor. On the basis of the amino acid sequence homology, CooA was thought to be a member of the CRP/FNR transcriptional regulators, and this was confirmed by X-ray crystallographic analysis.[17,18] The molecular folding of the reduced CooA resembles that of CRP, a global transcriptional regulator in *E. coli*. The CooA monomer consists of two domains, a heme-containing sensor (effector-binding) domain and a DNA-binding domain, in its amino- and carboxy-terminal regions, respectively.[19,20] The heme-containing sensor domain exhibits a β-roll architecture consisting of eight antiparallel β-strands and three helical segments.[17] The sensor and DNA binding domains are linked with a hinge region (residues 130−140), and the dimer interface is composed of a long α-helix, which is designated as the C-helix.[17]

The transcriptional activator activity of CooA is controlled by whether or not CooA binds to its target DNA.[19−22] Only CO-bound CooA can bind to its target DNA, indicating that CO binding to the heme group activates CooA as a transcriptional activator. A helix−turn−helix (HTH) motif, which is a common DNA binding motif in prokaryotes, in the DNA-binding domain is responsible for the specific recognition and binding of target DNA.[23]

Structure of the Active Site for Sensing CO

As the heme in CooA plays a central role in both sensing CO and for regulating CooA activity in response to CO, elucidating the heme environmental structure, including the coordination structure of the heme, is very important for understanding the structure−function relationships of CooA. X-ray crystallographic analysis by Lanzilotta et al. has revealed a very unique coordination structure of the heme in ferrous CooA.[17] They show that the nitrogen atom of the amino-terminal proline residue of one subunit is coordinated to the heme in another subunit. CooA is the first and only example of a metalloprotein in which a proline residue serves as a ligand of a metal ion in a protein matrix. The ferrous heme in CooA is 6-coordinate with Pro^2 and His^{77} as the axial ligands. The crystal structures of the ferric and CO-bound CooA have not been determined yet. We have elucidated the coordination structure of the heme in CooA by mutagenesis and spectroscopic studies. The proposed model of the coordination structure of the heme in CooA is shown in Figure 3.

The ferric, ferrous, and CO-bound hemes in CooA are 6-coordinate with Pro^2 and Cys^{75}, Pro^2 and His^{77}, and His^{77} and CO, respectively, as the axial ligands. The bond distances between the heme iron and the axial ligands are shown in Figure 3. These distances were determined by EXAFS analyses.[24] CooA shows some unique properties of the coordination structure of the heme. First, a proline residue is coordinated to the ferric and ferrous hemes as an axial ligand.[25] Second, ferrous CooA reacts with CO under physiological conditions generating the CO-bound form of the protein, though the ferrous heme is coordinately saturated with two endogenous axial ligands. Third, the ligand exchange between Cys^{75} and His^{77} proceeds during the change in the oxidation state of the heme iron. This ligand exchange is fully reversible. In the following sections, I discuss how these unique properties are related to the function of CooA.

The Mechanism by Which CO Activates CooA

Only CO-bound CooA functions as a transcriptional activator, indicating that CO binding to heme activates CooA. One of the two axial ligands of the ferrous heme in CooA is replaced by CO upon binding, which is an activating trigger of CooA. A crucial question therefore is which of the two axial ligands of the ferrous heme is replaced by CO?

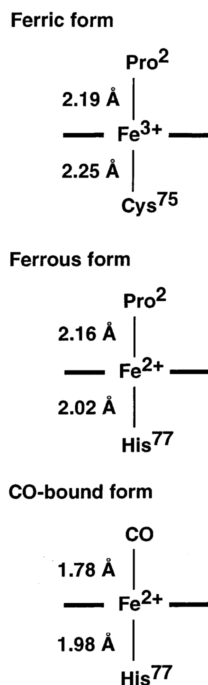


FIGURE 3. Coordination structure of the heme in CooA. The bond lengths elucidated by EXAFS analyses are shown in angstrom units.

Picosecond time-resolved resonance Raman spectroscopy reveals that a transient Raman band due to the Fe–His stretching mode (ν(Fe–His)) is observed at 211 cm^{-1} upon photolysis of CO-bound CooA.[26] The ν(Fe–His) band is observable in the 200–250 cm^{-1} range only for 5-coordinate reduced hemes. Upon photolysis of CO-bound CooA, a transient Raman band, due to a ν4 band at 1355 cm^{-1}, is also observed.[26] The frequency of the ν4 band at 1355 cm^{-1} is typical for 5-coordinate hemes with a nitrogenous axial ligand. The decay kinetics for the intensity of the signal at 1355 cm^{-1} is similar to that for the transient ν(Fe–His) band at 211 cm^{-1}. These show that the transient Raman band at 211 cm^{-1} is indeed due to a 5-coordinate heme produced by photolysis of CO-bound CooA. No transient signal due to the ν(Fe–His) band is observed when CO-bound H77Y CooA, in which His^{77} is replaced by Tyr, is used in place of wild-type CooA.[26]

NMR spectroscopy reveals that ferrous CooA shows ring current shifted ^{1}H NMR signals at −4.5, −3.6, and −2.8 ppm due to δ1-, α-, and δ2-protons of Pro^2, respectively.[27] Here, the proton located near the heme at δ-position of Pro^2 is referred to as δ1-proton and another proton at δ-position of Pro^2 is referred to as δ2-proton. These ring current shifted signals which are due to the protons of Pro^2 disappear upon CO binding to the heme in CooA. These results using time-resolved resonance Raman and NMR spectroscopies show clearly that Pro^2 is replaced by CO when CO-bound CooA is formed and that His^{77} is retained as the proximal ligand of the CO-bound heme in CooA.

Pro^2 is the amino-terminal residue of CooA because Met^1 is removed by posttranslational modification.[12,27] Therefore, the dissociation of Pro^2 from the heme iron upon CO binding would cause a conformational change of the polypeptide main chain in the amino-terminal region including Pro^2. A simple but plausible hypothesis for the mechanism of CO-dependent activation of CooA could therefore have been that the activation of CooA by CO is caused by a conformational change of the amino-terminal region induced by the dissociation of Pro^2 upon CO binding. However, this turns out not to be the case, as described below.

Table 2. Transcriptional Activity of CooAΔN5 and CooAΔN14

	specific activity[a]	
	+CO	−CO
wild-type CooA	15.7	0.23
CooAΔN5	13.2	0.29
CooAΔN14	6.1	0.26

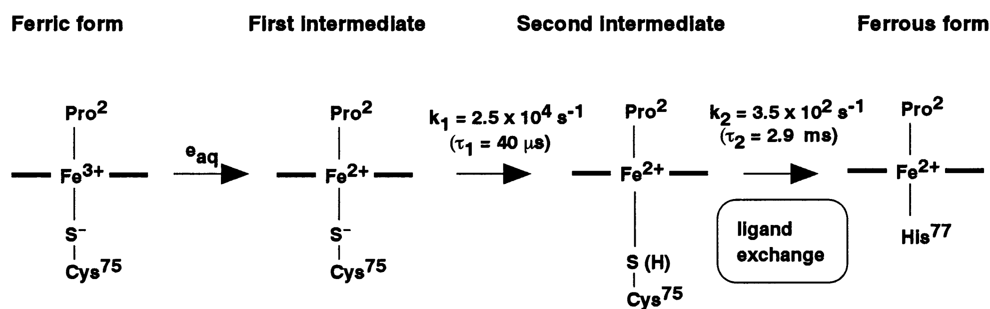
[a] Transcriptional activator activity of wild-type and mutant CooA proteins with (+) and without (−) CO are measured by using an in vivo reporter system as previously described.[19]

Two truncated mutants of CooA in which several amino acid residues are removed from the amino terminus, CooAΔN5 and CooAΔN14, were generated to investigate the functional role of the amino-terminal region. Four and 13 amino acid residues are removed from the amino terminus in CooAΔN5 and CooAΔN14, respectively. CooAΔN5 and CooAΔN14 also show CO-dependent transcriptional activator activity as does wild-type CooA[24] (Table 2). A similar result was also reported for other Pro^2 and amino terminal region mutants.[28] These results show that proline binding to the ferrous heme is not essential for the function of CooA, particularly in the case of CO-dependent activation of CooA. Although it is clear that the dissociation of Pro^2 from the heme upon CO binding is a trigger of CO-dependent activation, the molecular mechanism of this activation process is not yet known. Lanzilotta et al. show that bending and rotation of the C-helix about the dimmer interface will take place when CooA is activated by CO binding and propose that the movement of the entire heme and amino terminus upon CO binding causes a reorientation of the C-helix and a conformational change of the DNA-binding domain.[17,29–31]

Redox-Controlled Ligand Exchange between Cys^{75} and His^{77}

A redox-controlled ligand exchange occurs between Cys^{75} and His^{77} during the change in the oxidation state of the heme in CooA.[32] Cys^{75} that is coordinated to the ferric heme in CooA is replaced by His^{77} upon the reduction of the heme iron, and vice versa. Pulse radiolysis studies reveal the kinetics of the ligand exchange induced by the reduction of the heme in CooA, as shown in Scheme 1.[33] The ligand exchange from Cys^{75} to His^{77} proceeds via two reaction intermediates. The first intermediate shows a Soret peak at about 440 nm, indicating that a 6-coordinate ferrous heme with a thiolate axial ligand is formed as the first intermediate when CooA is reduced by pulse radiolysis. The first intermediate is converted into the second one with a time constant of 40 μs. In the second intermediate, the protonation of Cys^{75} thiolate and/or the

Scheme 1. Reaction Scheme for Ligand Exchange Induced by Pulse Radiolysis



elongation of the Fe−S bond seems to take place. The second intermediate is converted into the final product, the ferrous form of CooA, with a time constant of 2.9 ms. During the conversion of the second intermediate to the final product, a conformational relaxation of the polypeptide chain at the proximal side including Cys^{75} and His^{77} would take place to adjust the orientation of His^{77} to an appropriate position for the ligand exchange. The ligand exchange from Cys^{75} to His^{77} is thought to proceed concertedly with this conformational relaxation.

H77G CooA, in which His^{77} is replaced by Gly, shows kinetics similar to that of wild-type CooA for ligand exchange induced by pulse radiolysis; i.e., a similar biphasic kinetics with two reaction intermediates is observed. Additionally, little difference is seen between the transient absorption spectra of the reaction intermediates and the time constant for the conversion of the first intermediate to the second one (40 and 44 μs for wild-type and H77G CooA, respectively) in the case of both wild-type and H77G CooA. These results indicate that the same reaction intermediates are formed in the case of H77G CooA as is the case of wild-type CooA, although the ligand exchange does not take place in H77G CooA due to the lack of His^{77}. The second intermediate in the case of H77G CooA is converted into the final product with a time constant of 1.1 ms. This process, in the case of H77G CooA, corresponds to a conformational relaxation at the proximal side without ligand exchange. The fact that the ligand exchange proceeds with a conformational relaxation at the proximal side reveals a flexibility of the polypeptide around the heme in CooA.

The number of hydrogen bonds around the proximal heme pocket will reflect the flexibility of the polypeptide around the heme in CooA. Only one hydrogen bond (the hydrogen bond between the oxygen of Gly^{43} and the nitrogen of Met^{76}) exists within 10 Å from the heme iron in the proximal heme pocket for CooA, whereas 6−10 hydrogen bonds exist within 10 Å from the heme iron in the proximal heme pocket of other heme proteins.[33] Increasing the number of hydrogen bonds may cause polypeptide chains to become rigid. Therefore, a smaller number of hydrogen bonds in the proximal heme pocket in CooA compared to other heme proteins indicates flexibility of the polypeptide chain in the proximal heme pocket. Indeed, crystallographic *B* factors of residues 77−86 are in fact larger than the average *B* factors of solvents in CooA crystals, suggesting flexibility of the polypeptide around the heme group.[17] Lanzilotta et al. propose that disruption and loss of some helical structure in the switch region (residues 130−140) takes place upon CO binding to the heme, which causes bending of the DNA binding domain.[17] These results indicate that the flexibility of the peptide main chain in CooA plays an important role in the functional regulation of CooA.

Redox Properties of CooA

CooA shows a hysteresis with the apparent oxidation and reduction potentials of −260 and −320 mV (vs NHE), respectively, in electrochemical redox titrations.[24,34] A similar hysteresis in the redox titrations is observed for cytochrome cd1 from *Paracoccus pantotrophus*.[35] Both CooA and *Paracoccus pantotrophus* cytochrome cd1 show an axial ligand exchange induced by the change in the oxidation state of the heme iron, which is related to the hysteresis in the redox titrations.

The low oxidation potential of CooA may be important for the regulation of CooA activation. CooA is required for CO-dependent expression of the *coo* operons which encode a CO-oxidizing system that allows *R. rubrum* to grow on CO as the sole energy source. CooA is activated only in the presence of CO under anaerobic conditions that induce the expression of the *coo* operons. Even in the presence of CO, CooA must not be activated under aerobic conditions. The low oxidation potential of CooA would facilitate the oxidation of the heme and thus prevent CooA from being activated in vivo, once oxygen is present in the cells.

Discrimination of CO from Other Gas Molecules

Gas molecule sensor proteins selectively sense their physiological effector gas. CooA should distinguish CO from other gas molecules and therefore be activated only by CO. A stable O_2-bound CooA is not formed because autoxidation takes place to form ferric CooA when ferrous CooA reacts with O_2.[12] Ferric CooA cannot bind to target DNA and is not active as a transcriptional activator. These results indicate that O_2 cannot activate CooA because autoxidation takes place following the reaction of CooA with O_2. Thus, discriminating O_2 from CO by CooA is achieved by the 6-coordinate heme that can bind CO, but not O_2. Generally, a 5-coordinate heme can bind O_2 at its

vacant distal site, and this bound O_2 is stabilized by hydrogen bonding to a distal amino acid residue such as a histidine. Recently, it has been reported that 6-coordinate heme proteins with two endogenous axial ligands such as hexacoordinate hemoglobins, neuroglobin, and cytoglobin form a stable O_2-bound form, though the deoxy form of these heme proteins is 6-coordinate with two endogenous axial ligands.[36−38] In these cases, the heme-bound oxygen is stabilized by hydrogen bonding to a distal amino acid residue when O_2 is bound to the heme. In the case of CooA, a stable O_2-bound heme is not formed, probably because there is no distal amino acid residue stabilizing the bound O_2 via hydrogen bonding.

As the heme in CooA is 6-coordinate and coordinately saturated, a very limited number of external ligands could bind. Consequently, among gas molecules, only CO and NO can bind to the heme in CooA. Reductive nitrosylation proceeds when ferric CooA reacts with NO, which is revealed by electronic absorption and EPR spectroscopies.[39] The Soret peak of ferric CooA shifts from 423 to 398 nm upon reaction with NO. The reaction product of ferric CooA with NO shows a typical EPR spectrum for a 5-coordinate nitrosyl−Fe(II) heme. Although a weak signal at $g = 2.03$ due to a 6-coordinate nitrosyl−Fe(II) heme is observed, a 5-coordinate nitrosyl−Fe(II) heme is predominant.[47] Similar electron absorption and EPR spectra are obtained when ferrous CooA reacts with NO, indicating that a 5-coordinate nitrosyl−Fe(II) heme similar to that formed in the case of ferric CooA is generated.

Although NO can bind to the heme in CooA, NO-bound CooA is not active as a transcriptional activator.[39] The reason for this is because the coordination structure of the heme is different between the CO-bound and NO-bound forms. While the CO-bound heme in CooA is 6-coordinate with His[77] and CO as the axial ligands, the NO-bound form is 5-coordinate with NO as an axial ligand. Both of the endogenous axial ligands are dissociated from the heme iron upon NO binding, which will cause a very different conformational change around the heme from that induced by CO binding. These results show that CooA discriminates between CO and NO using a different mechanism from that used for discriminating between CO and O_2.

Recognition of the Target DNA and Transcriptional Activation by CooA

Only CO-bound CooA can recognize and bind to the target DNA, indicating that CooA becomes the on state by binding CO. Crystallographic analyses of ferrous CooA give some useful information on the mechanism of stabilization of the on state as described below. Disruption and loss of some helical structure in the C-helix takes place in going from the off to on states.[17] Leu[130] in the hinge region (residues 130−140) plays a role as a fulcrum in going from the off to on states.[17] The interaction of the symmetry-related Leu residues is not changed between the CooA (off state) and CRP (on state) structures (see Figure 2 in ref 17),[17] which supports the function of Leu[130] as a fulcrum.

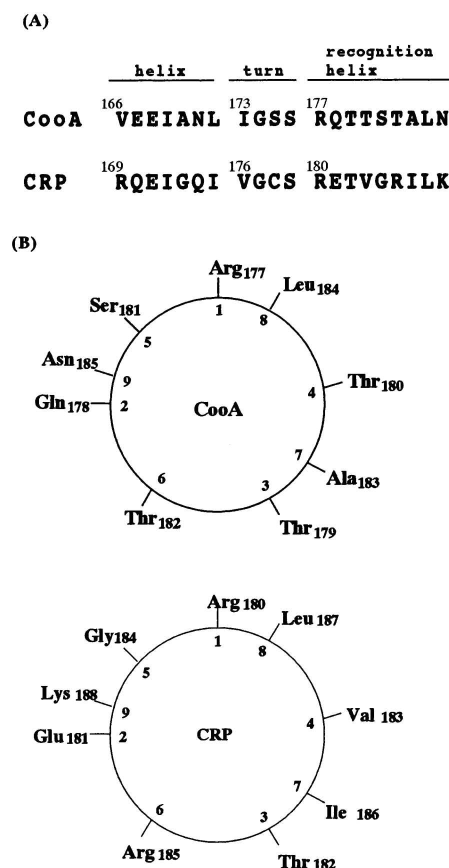


FIGURE 4. Amino acid sequences of the helix−turn−helix motif (A) and helical wheel models of the recognition helix (B) in CooA and CRP.

In the on state of CRP, Phe[136] of one subunit contacts the hairpin turn consisting of residues 52−57 of another subunit, and vice versa.[17] However, Phe[132] in CooA monomer B (fully off state), the position of which corresponds to Phe[136] in CRP, contacts the recognition helix centered at Thr[182].[17] The sequence for CRP in residues 52−57 (KDEEGK) is similar to that for CooA in residues 57−63 (VGEERES), and the hairpin structure is conserved.[17] A similar interaction between Phe[132] and the hairpin region would exist in the on state of CooA, suggesting that a swapping of interacting partner takes place upon CO binding.[17] Another swapping of interacting partner would occur in going from the off to on states; i.e., the salt bridge between Arg[138] in monomer A and Glu[59] in monomer B ruptures and is replaced by an interaction between the conserved Arg and the DNA phosphate backbone.[17] The recognition helices of the HTH motif in each subunit adopt a proper orientation to fit into a major groove of the target DNA in the on state.

The amino acid sequence and a helical wheel model of the recognition helix of the HTH motif in CooA are shown with those for CRP in Figure 4. CooA and CRP belong to the same family of transcriptional regulators, and show amino acid sequence homology.[18] In the case of CRP, Arg[180] and Gln[181] within the recognition helix in the HTH motif are directly hydrogen bonded to the base

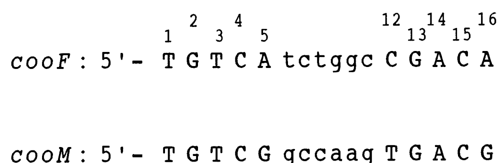


FIGURE 5. DNA sequences of CooA-dependent promoters. Only the sequences of the sense strands are shown with the numbering of the base pairs.

pairs in the major groove of the target DNA and are responsible for the specific binding of CRP to the target DNA.[40] Site-directed mutagenesis and in vivo reporter assay studies reveal that Arg^{177}, Gln^{178}, and Ser^{181} are responsible for the recognition of the target DNA by CooA.[23] The positions of Arg^{177} and Gln^{178} in CooA correspond to those of Arg^{180} and Glu^{181} in CRP, as shown in Figure 4.

The mutation of Ala^{183} to Ile or Thr results in a unique phenotype that shows constitutive activity regardless of the presence or absence of CO, though CooA loses its transcriptional activator activity following mutation at residue positions 177, 178, and 181.[23] These results suggest that the replacement of Ala^{183} by either Ile or Thr causes a conformational change similar to that induced by CO binding to the heme in wild-type CooA. Kerby et al. have obtained CO-independent and constitutively active mutants by random mutation of a six-residue portion of the C-helix constituting the dimer interface, and proposed that the repositioning of the dimer interface is crucial for the activation of CooA by CO.[41]

DNA sequences of CooA-dependent promoters are shown in Figure 5. In vivo reporter assay studies with mutant promoters reveal that the base pairs at positions 2/15, 3/14, and 4/13 (see Figure 5 for numbering), which are conserved between the *cooF* and *cooM* promoters,[42,43] are responsible for the specific interaction with CooA.[23] The following model is proposed for the hydrogen bonding responsible for the specific interaction between CooA and its target DNA: Arg^{177} and Gln^{178} are hydrogen bonded to the guanine at positions 4/13 and to the adenine at positions 3/14, respectively.[23] Ser^{183} forms a hydrogen bond with the N7 of the guanine at positions 2/15.[23]

The mechanism by which CooA activates the transcription of the *coo* operons is thought to be similar to that for CRP at class II CRP-dependent promoters.[23,44,45] One of the mechanistic components of transcriptional activation at class II CRP-dependent promoters is "direct activation" mediated by protein–protein interaction between amino acid residues at positions 19, 21, 96, and 101 (activation region 2) of the downstream subunit of CRP and amino acid residues from 162 to 165 within the RNA polymerase α-subunit amino-terminal domain. The CRP/CooA chimera that consists of the amino-terminal region from Val^{1} to Pro^{154} of CRP and the carboxy-terminal region from Gly^{151} to Asp^{222} of CooA shows the same phenotype as CPR, when one of the amino acid residues in activation region 2 mentioned above is mutated.[44] Leduc et al. have reported the existence of activation region 2 also in CooA as is the case of CRP.[46]

Concluding Remarks

CooA is a novel and interesting protein from several points of view. First, it is the first known biological system in which CO plays a physiological role. Though CO is well-known to be a poison for oxygen respiration, its positive physiological functions were not reported. CooA is the first CO sensor protein identified and characterized at the molecular level. Second, CooA is the first example of a transcriptional regulator containing a heme as a prosthetic group. The heme in CooA, which acts as the active site for sensing CO, shows unique properties compared with those of other heme proteins, as described in this Account. These unique properties are responsible for the selective sensing of CO, the CO-dependent activation of CooA, and the tuning of the redox potential. Though CooA has been extensively studied by biochemical and biophysical techniques, its structure–function relationships are not yet fully understood. Further studies are required to elucidate the molecular mechanisms of CO sensing, CO-dependent activation, and transcriptional activation.

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