Analysis of the L116K Variant of CooA, the Heme-containing CO Sensor, Suggests the Presence of an Unusual Heme Ligand Resulting in Novel Activity*

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CooA is the CO-sensing transcriptional activator from *Rhodospirillum rubrum*, in which CO binding to its heme prosthetic group triggers a conformational change of CooA that allows the protein to bind its cognate target DNA sequence. By a powerful in vivo screening method following the simultaneous randomization of the codons for two C-helix residues, 113 and 116, near the distal heme pocket of CooA, we have isolated a series of novel CooA variants. In *vivo*, these show very high CO-independent activities (comparable with that of wild-type CooA in the presence of CO) and diminished CO-dependent activities. Sequence analysis showed that this group of variants commonly contains lysine at position 116 with a variety of residues at position 113. DNA-binding analysis of a representative purified variant, L116K CooA, revealed that this protein is competent to bind target DNA with *Kₐ* values of 56 nM for Fe(III), 36 nM for Fe(II), and 121 nM for Fe(II)-CO CooA forms. Electron paramagnetic resonance and electronic absorption spectroscopies, combined with additional mutagenic studies, showed that L116K CooA has a new ligand replacing Pro² in both Fe(III) and Fe(II) states. The most plausible replacement ligand is the substituted lysine at position 116, so that the ligands of Fe(III) L116K CooA are Cys²⁷ and Lys¹¹⁶ and those in the Fe(II) form are His⁷⁷ and Lys¹¹⁶. A possible explanation for CO-independent activity in L116K CooA is that ligation of Lys¹¹⁶ results in a repositioning of the C-helices at the CooA dimer interface. This result is consistent with that repositioning being an important aspect of the activation of wild-type CooA by CO.

*Rhodospirillum rubrum*, a photosynthetic bacterium, can grow with CO as a sole energy source. The presence of CO is sensed by CooA, and CO-bound CooA activates the transcription of a series of genes encoding the CO oxidation system in *R. rubrum* (1). CooA contains a heme prosthetic group, as do other sensors for gaseous molecules such as soluble guanlylcyclase (sGC),¹ FixL, DOS, and HemAT (2–5). The heme moieties are directly involved in binding their respective effector molecules and thereby regulate the activity of the sensor proteins. In CooA, the signal of CO binding to the heme is transmitted to the DNA-binding domain, the consequence of which is a conformational change allowing CooA to bind its target DNA sequence.

The crystal structure of Fe(II) CooA revealed a novel subunit-swapped N-terminal Pro² as a heme ligand trans to His⁷⁷ in the homodimer (6). Because the Fe(II) form of CooA is 6-coordinate and low spin, the CO-sensing mechanism of CooA necessarily involves the displacement of one of these two ligands. Nuclear magnetic resonance and time-resolved resonance Ramans studies identified Pro² and His⁷⁷ as the displaced and retained ligands, respectively, in the Fe(II)-CO form of CooA (7, 8). CooA is a redox sensor as well as a CO sensor because only the Fe(II) form of CooA is competent to bind the physiological effector, CO. Interestingly, CooA undergoes a redox-dependent ligand switch, in which Cys²⁷ replaces His⁷⁷ in the Fe(III) form (9, 10). Although the physiological role and exact mechanism of the ligand switch is still elusive, this switch indicates that the position of the heme prosthetic group of CooA is highly flexible relative to surrounding protein matrix.

CooA belongs to the family of transcriptional activators containing the cAMP receptor protein (CRP) (11). In addition to similar target DNA sequences, CooA and CRP exhibit similar overall topologies, in which each protein is a dimer and each monomer contains two functionally distinct domains (Fig. 1). The effector-binding domain of each protein binds its respective small molecule (CO for CooA, cAMP for CRP). The end result of effector binding to this domain is a precise repositioning of the DNA-binding domain, which allows each protein to bind its cognate target DNA sequence. The two domains are connected through a long α-helix (designated as C-helix) that also serves as a subunit dimerization interface (6). Importantly, the comparison of the structure of effector (CO)-free CooA with that of effector (cAMP)-bound CRP (6) reveals a repositioning of these two C-helices with respect to each other, suggesting a role of this repositioning in the activation of these proteins in response to their respective effectors. Consistent with this concept, alterations of particular amino acids within the C-helices exert a variety of effects on the activity in CRP (12, 13) and in the fumarate and nitrate reductase activator protein (14), another member of this transcriptional activator family. It is therefore

¹ The abbreviations used are: sGC, soluble guanlylcyclase; CRP, cAMP receptor protein; EPR, electron paramagnetic resonance; WT, wild-type; MOPS, 3-(N-morpholino)propanesulfonic acid; PDB, Protein Data Bank.

33616 This paper is available on line at http://www.jbc.org
in the absence of CO, which has led to the hypothesis that the effector (CO) binds and reorganizes DNA by causing a perturbation of the DNA-binding pocket (9). The role of Pro2 appears to be to keep CooA inactive in the absence of CO, which has led to the hypothesis that the release of the heme from Pro2 might allow a sterical interaction between the heme and the C-helices, resulting in their repositioning. Consistent with this view, Gly117 on the C-helix is near the distal heme pocket of CooA. In this report, we address a novel CooA variant that, in the absence of effector (CO), effectively mimics the active conformation of wild-type (WT) CooA in the presence of CO. A possible underlying mechanism behind such a novel phenotype is discussed from a structural viewpoint.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and in Vivo Assays**—The construction of strains overexpressing WT CooA and CooA variants in an *Escherichia coli* background having a β-galactosidase reporter system in the chromosome was described previously, and in vivo activities were quantitated using the standard protocol (18). All the site-directed and region-randomized cooA mutations were constructed in a pET22-based expression plasmid, which provides tight control of CooA expression (15).

**Creation of cooA Mutations**—Site-directed mutagenesis involved polymerase chain reaction amplification of cooA with primers designed to incorporate the desired nucleotide change, as described elsewhere (19). The method used for dual randomization was essentially identical to the method used for site-directed mutagenesis, except that the primers contained randomized codons for both 113- and 116-positions. Screening of CooA variants involved the analysis of their ability to cause β-galactosidase accumulation on agar plates incubated under different growth conditions as described previously (15). Based on colony color, CooA variants could be classified as active, weakly active, or inactive.

Selected variants were examined quantitatively in an *in vivo* β-galactosidase assay, after which the cooA genes were sequenced to determine the causative residue changes.

**Purification of WT CooA and CooA Variants**—The purification of WT CooA and the ΔP3R4 CooA variant (>95% homogeneity) was performed as described previously (9). The purification of L116K and L116K ΔP3R4 CooA involves the published method through the ammonium sulfate precipitation following the polyethyleneimine precipitation (9). However, preliminary work indicated that CooA variants with the L116K substitution had lower stability than did WT CooA at normal salt levels but were substantially stabilized with 0.5 M NaCl. Therefore, the resulting ammonium sulfate (final 50%) pellet was dissolved in 25 mM MOPS, pH 7.4, 0.5 M NaCl, 5% glycerol and eluted with a linear gradient of 10–100 mM potassium phosphate in 25 mM MOPS, pH 7.4, 0.5 M NaCl, 5% glycerol. The resulting CooA-containing fractions were precipitated with 50% ammonium sulfate, and the samples were applied to Sephacryl S-100 (Amersham Biosciences) size exclusion column in 25 mM MOPS, pH 7.4, 1 M NaCl, 5% glycerol. Again, the resulting CooA fractions were precipitated with ammonium sulfate and resuspended in 25 mM MOPS, pH 7.4, 0.5 M NaCl. The residual ammonium sulfate was removed on a Sephadex G-25 fine (Amersham Biosciences) column equilibrated in 25 mM MOPS, pH 7.4, 0.5 M NaCl, and the final sample was stored at −80 °C until use. The heme content of CooA preparations was estimated using the extinction coefficient of WT CooA (20) or by a modified pyridine-hemochromogen method (20), and protein concentration was measured using the BCA assay (Pierce).

**Preparation of Hydroxyapatite Batch-treated CooA Samples**—Preparation of hydroxyapatite batch-treated CooA samples was carried out using the procedure described previously (16). By this method, heme-containing CooA accounted for ~10% of total protein, in the case of WT CooA. These samples were used for the comparison of electronic absorption spectra and heme accumulation of CooA variants.

**Electronic Absorption Spectroscopy**—Electronic absorption spectroscopy of CooA samples was performed using a Shimadzu UV-2401PC spectrophotometer. In the region of absorption, each sample was diluted with 0.5 M NaCl to final concentration. Spectra were recorded at room temperature; each spectrum was performed with 0.5 M NaCl at pH 7.4, 0.5 M NaCl, and the final sample was stored at −80 °C until use. For the spectra taken at pH 4, the protein samples were dissolved in 0.1 M sodium acetate buffer, pH 4, 0.1 M NaCl.

**EPR Spectroscopy**—Fe(III) CooA samples, purged with argon, were frozen and stored at 77 K. EPR spectra were recorded at room temperature on the Varian E100 spectrometer. The samples were excited with a flash lamp and relaxed for 1 min before EPR readings. EPR spectra were recorded at X-band and 10 K; each recorded spectrum represents the average of 4 (WT CooA) or 16 (L116K and L116K ΔP3R4 CooA) scans, each comprising 1024 points. The only EPR signals that were observed between 0 and 4000 gauss are those reported. Specific conditions for the recording of each spectrum are given in the legend to Fig. 7.

**In Vitro DNA-binding Assays**—*In vitro* DNA-binding assays of WT CooA and CooA variants were performed using the fluorescence polarization technique with a Beacon 2000 fluorescence polarization detector (PanVera Corp., Madison, WI) as described previously (15). As a fluorescence probe, a 26-base pair target DNA containing T20 was labeled with Texas Red on one end of the duplex and used at the concentration of 6.4 μM. Binding assays were performed in 40 mM Tris-HCl, pH 8.0, 6 mM CaCl2, 50 mM KCl, 5% (v/v) glycerol, and 1 mM dithiothreitol. Salmon sperm DNA was used as the nonspecific DNA competitor. Dissociation constants (Kd) were calculated by fitting of the binding data to a nonlinear equation with correction of the fluorescence quenching as described elsewhere (21).

**RESULTS AND DISCUSSION**

**Unique Functional Properties of L116K CooA**—As described in the Introduction, it is a reasonable hypothesis that CO binding to the heme of CooA causes a perturbation of the C-helices. Under such a scenario, one would expect that residues on the surface of the C-helices that are in the vicinity of the heme would be important for that process. We therefore...
examined the effects of the perturbation of C-helix residues near the heme. Ile\textsuperscript{113} and Leu\textsuperscript{116} together with Gly\textsuperscript{117} are the C-helix residues within 7 Å of the heme iron of CooA. These residues lie near the heme on Pro\textsuperscript{2} side, the ligand that is displaced by CO (Fig. 2). To investigate the functional importance of these hydrophobic residues in the CooA activation mechanism, we completely randomized the codons for both Ile\textsuperscript{113} and Leu\textsuperscript{116} and screened the resulting library of ~6,000 clones for functionally interesting variants. The screening involved the detection of β-galactosidase activity in colonies of an E. coli strain in which CooA regulates lacZ expression (15). One class included CO-responsive variants, a behavior rather like that of WT CooA. This class of CooA variant will be described in another report. Of relevance to this report, ~1.6% of the clones displayed significant activity under anaerobic conditions without CO, a behavior distinctly different from that of WT CooA (Table I). These CO-independent CooA variants were then reanalyzed in a quantitative liquid assay of in vivo β-galactosidase activity, and a number of them were sequenced to reveal the causative substitutions.

As seen in Table I, several patterns are apparent. As expected, all the selected variants showed high in vivo activity (comparable with that of WT CooA in the presence of CO) under anaerobic conditions (when CooA is in the Fe(II) state), the same conditions used in the initial screen. The variants also displayed substantial in vivo activity under “aerobic” and “anaerobic + CO” conditions, but these activities were dependent upon the residue at position 113. Although the activities under “anaerobic + CO” are generally lower than the anaerobic activities, they showed nearly identical activities when there was a large hydrophobic residue at position 113. Because both oxidation/reduction and CO binding occur at the heme moiety in CooA, this activity modulation suggests that local conformational change of the heme moiety may be the molecular mechanism behind this unusual phenotype. The loss in activity upon the introduction of CO is particularly striking in this group of variants and is clearly different from the behavior of WT CooA. Such a phenotype has not been found previously for CooA variants despite extensive studies of a variety of variants altered in the heme vicinity on either side including those altered at the heme ligands (Pro\textsuperscript{2}, Cys\textsuperscript{75}, and His\textsuperscript{77} variants) (9) or in the distal heme pocket (Gly\textsuperscript{117} variants) (16) (Fig. 3). This novel behavior suggests that the mechanism of activation in these variants might be rather different from that of WT CooA.

A second obvious pattern among the CO-independent variants is the presence of Lys at position 116, although a variety of residues were acceptable at position 113. Moreover, the activity of this group of variants under anaerobic conditions is almost invariant with respect to the residue at position 113, although the activities under “aerobic” and “anaerobic + CO” conditions are somewhat modulated by residues at that position. The basis of this modulation is unknown, but might reflect differential accumulation of heme-containing CooA in different position 113 variants. There is clearly an effect under aerobic conditions in rich medium (Table I), but other conditions have not been examined. The failure to detect variants of this phenotype with Arg at position 116 suggested that the behavior was not merely the result of the introduction of positive charge in the distal heme pocket of CooA. This view was confirmed by the construction of L116R CooA and L113K CooA. Consistent with the results of randomization, neither L116K nor II13K resulted in CO-independent CooA activation; L116R CooA was inactive (0.5, 0.7, and 0.8% for “aerobic,” “anaerobic,” and “anaerobic + CO” conditions) and L113K CooA was CO-responsive, though to a lower level than WT CooA (1, 4, and 58% for aerobic, anaerobic, and anaerobic + CO conditions). These results strongly imply that Lys\textsuperscript{116} is central to the CO-independent activity in these CooA variants.

**Purification of L116K CooA**—To understand the biochemical basis for the activity in the absence of CO, we isolated a representative variant, L116K CooA. With the procedures described under “Experimental Procedures,” the isolated material was ~90% pure based on SDS-PAGE. For spectral comparisons, we also isolated L116K ΔP3R4 CooA to a purity of ~70%. The column purification step of these CooA variants always contained high levels of salt (0.5-1 M NaCl) because such conditions stabilized the protein (data not shown); for the same reason, all the analyses were carried out in the presence of 0.5 M NaCl if not otherwise specified. However, the use of high salt did not completely eliminate the heme loss observed during purification. The final preparation contained only 50% of the heme expected for that amount of CooA. Because the DNA binding activity of L116K CooA is modulated by the oxidation or CO binding to the heme moiety (see below), heme-containing L116K CooA is responsible for the activity. Therefore, all the CooA concentrations used here were based on the concentration of heme rather than protein.

**In Vitro DNA-binding Analysis of L116K CooA**—Substantial in vivo activity of a CooA variant does not always mean that it has high DNA binding activity. A specific group of variants, with changes in the “activating regions” that interact with RNA polymerase, display increased in vivo activity but without an increase in the DNA-binding affinity (22). These CooA variants have a higher affinity for RNA polymerase, which has the effect of trapping the small population of CooA in the active form without effectors at the appropriate promoter. To determine whether the high in vivo activity of Fe(III) and Fe(II)
TABLE I

| CooA variants | Sequences | β-Galactosidase activity a | Accumulation b |
|---------------|-----------|---------------------------|---------------|
|               |           | Oxidation c | Reduction d | Reduction + CO |               |
| pEXT-20 c     | Ile       | 1           | 1           | 1              | 0             |
| WT            | Leu       | 1           | 2           | 100            | 100           |
| Isolate-1 (L116K) | Leu | 14         | 98          | 66             | 22            |
| Isolate-2     | Leu       | 25          | 113         | 91             | 63            |
| Isolate-3     | Val        | 9           | 101         | 37             | 70            |
| Isolate-4     | Gly        | 5           | 122         | 14             | 9             |
| Isolate-5     | Trp        | 9           | 114         | 69             | 50            |
| Isolate-6     | Phe        | 24          | 127         | 111            | 35            |
| Isolate-7     | Tyr        | 18          | 99          | 103            | 25            |
| Isolate-8     | Asn        | 6           | 107         | 17             | 20            |
| Isolate-9     | Ser        | 7           | 127         | 18             | 12            |
| Isolate-10    | Pro        | 3           | 122         | 7              | 16            |
| Isolate-11    | Ala        | 17          | 118         | 71             | 25            |

a Percentage of activity indicates the mean value of multiple measurements of activity relative to that of WT CooA expressed in the presence of CO, and showed variability <10%.

b Percentage of accumulation indicates the heme accumulation of variant CooA in cell-free extracts (of strains grown aerobically in rich medium) relative to that of WT CooA, based on the spectra of the Fe(III) form normalized for protein.

c Aerobically grown cells were used for the activity.

d Anaerobically grown cells were used for the activity.

e pEXT-20 indicates control strain containing only vector.

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L116K CooA is correlated with increased DNA binding activity, we tested the DNA binding of L116K CooA directly with fluorescence anisotropy using WT CooA as control. To eliminate possible nonspecific DNA binding of L116K CooA, 100 times excess of salmon sperm DNA was used for this assay. When the Texas-Red-labeled target DNA was titrated with increasing amounts of purified L116K CooA, all three states of the protein (Fe(III), Fe(II), and Fe(II)-CO L116K) showed an increase in anisotropy values reflecting $K_w$ values of 56, 36, and 121 ns, respectively (Fig. 4). In contrast, WT CooA showed DNA binding activity corresponding to a $K_w$ of 12 ns only in the presence of CO (Fig. 4); Fe(III) or Fe(II) WT CooA did not show any DNA binding up to 500 ns. These results indicate that, unlike WT CooA, L116K CooA in all three forms has an easily detectable population in a reasonable conformation for DNA binding. The ability of L116K CooA to bind PcooF DNA with approximately the same affinity as WT CooA, as well as its ability to induce in vivo activity, demonstrates that DNA binding by L116K CooA is substantially normal. This implies that L116K CooA recognizes DNA through the same mechanism as does WT CooA, which requires significant repositioning of F-helices containing DNA-binding domains. We were concerned that the Lys substitutions at position 116 might affect CooA dimerization, and therefore DNA binding, because Leu$^{117}$ lies on the C-helices that form the dimer interface. However, gel filtration analysis of Fe(III) L116K CooA showed that it migrated as a single peak at the position of dimeric WT CooA (data not shown). For technical reasons, it has not been possible to show that the DNA-bound form of CooA is a dimer, although the DNA affinities and dimerization of the DNA-free forms make that the most likely possibility. Nevertheless, it is a formal possibility that the poorer affinity DNA affinity of Fe(II)-CO L116K CooA might result from a modest shift to the monomeric form. Although all three forms of L116K CooA show substantial DNA binding in vitro, there are some modest differences between these results and those obtained in vivo. Most strikingly, the Fe(III) form has a greater affinity for DNA than does the Fe(II)-CO form (Fig. 4), yet displays lower activity in vivo (Table I). We assume that some of these differences reflect differences in the precise positioning of the activating regions, as noted above. Specifically, we assume that the DNA-binding domains are properly positioned to bind DNA in Fe(III) form of L116K CooA, but that the mis-positioning of the heme in Fe(II)-CO L116K CooA (relative to that typically seen in Fe(II)-CO WT CooA, for example) improperly positions the activating regions and therefore results in lower in vivo activity. Moreover, we also note that there are differences in the levels of accumulation of heme-containing CooA under these three conditions (data not shown), which might also affect the activities detected in vivo.

A Different Endogenous Ligand Replaces Pro$^a$ in Fe(III) L116K CooA—Given the close proximity of the position 116 residue to the heme and the unusually high aerobic DNA binding activity of L116K CooA, we expected that Fe(III) L116K would be structurally perturbed around the heme center and that this perturbation might be revealed by electronic absorption spectroscopy. Because of increased stability of L116K CooA in the presence of 0.5 M NaCl, all the spectra were taken at this salt concentration. As shown in Fig. 5, the electronic absorption spectrum of Fe(III) L116K CooA showed a slightly blue-shifted Soret maximum of 422 nm compared with Fe(III) WT CooA (Table II), but was consistent with a typical 6-coordinate low spin heme. The electronic absorption spectrum of Fe(III) WT CooA in 0.5 M NaCl gave the same 6-coor-
studied so far have shown significant perturbation of Pro2, surprising because all the aerobically active CooA variants.

The DNA binding activities were determined by the method of fluorescence polarization. Texas Red-labeled target DNA (6.4 nM) was used as probe, and 100× salmon sperm DNA was used as nonspecific competitor DNA in all samples. Open diamonds, closed circles, and closed triangles indicate Fe(III), Fe(II), and Fe(II)-CO forms, respectively, of CooA samples. Solid lines show the best fit of the data to an equation described by Lundblad et al. (21).

Moreover, the close spectral similarity between Fe(III) L116K ∆P3R4 CooA and Fe(III) L116K CooA (Fig. 5 and Table II) is consistent with the hypothesis that both CooA variants have the same ligand replacing Pro2. Because of the proximity of Lys116 to the heme and other arguments listed below, we suggest that the new ligand is actually the introduced Lys116 in both Fe(III) L116K ∆P3R4 CooA and Fe(III) L116K CooA.

The comparison of the electronic absorption spectra of the CooA variants at pH 4 gives a much clearer picture about the ligation structure of Fe(III) L116K CooA (at this pH, 0.5 M NaCl leads to the precipitation of Fe(III) L116K CooA, so 0.1 M NaCl was used for all samples). Under low pH conditions, both Fe(III) L116K and L116K ∆P3R4 CooA displayed the spectral features of 6-coordinate low spin hemes (Fig. 6), whereas Fe(III) WT has the features of a 5-coordinate high spin heme (the pK_a of the transition from 6- to 5-coordinate in Fe(III) WT CooA was previously reported to be −5.5; Ref. 16). This result strengthens the above suggestion that Fe(III) L116K CooA and Fe(III) L116K ∆P3R4 CooA share a common ligation structure, which is clearly different from that of Fe(III) WT CooA or of Fe(III) ∆P3R4 CooA. The inset in Fig. 6D shows the pH-dependent spectral change of Fe(III) L116K CooA, giving a pK_a of 3 for the 6- to 5-coordinate transition of the protein. Although it is not known if this pK_a value is of the suggested Lys116 or the trans-ligand, it has been reported that the pK_a of Lys could be lowered by as much as 7 pH units when it is a heme ligand (24).

Electron paramagnetic resonance (EPR) spectroscopy of Fe(III) L116K CooA confirms that the heme is low spin and reveals the presence of a cysteine thiolate ligand. The EPR spectrum of L116K CooA (Fig. 7) exhibits two overlapping sets of rhombic signals, with g values of 2.47, 2.24, and 1.89, and 2.42, 2.24, and 1.91, both characteristic of low spin Fe(III) heme. The g anisotropies are clearly indicative of the presence of a thiolate ligand; the rhombicity and tetragonality parameters place both signals in the P region of a Blumberg-Peisach plot (25). To verify that the thiolate ligand in the L116K variant is Cys75, a C75A substitution was introduced into L116K CooA. The electron absorption spectrum of Fe(III) L116K C75A CooA was distinct from that of Fe(III) WT CooA or that of Fe(III) L116K CooA, with a blue-shifted Soret maximum (Table II). The altered spectrum in the double variant suggests that Cys75 is indeed a ligand to the heme in Fe(III) L116K CooA. Furthermore, the specific ligand environment present in L116K CooA, including Cys75, is necessary for substantial in vivo activity in the Fe(III) form. In contrast to L116K CooA, the double variant, Fe(III) L116K C75A CooA, shows little in vivo activity (Table II).

The heme coordination environment of Fe(III) L116K CooA is distinct from that of WT CooA, as evidenced by the unique
A Protein Ligand-based CooA Activation

Spectral and functional comparison of L116K CooA with WT and related CooA variants

| CooA          | Fe(III) | Fe(II) | Fe(II)-CO | $A_{P(111)}/A_{P(110)}$ | $A_{P(111)-CO}/A_{P(111)}$ | $A_{P(111)-CO}/A_{P(110)}$ | Oxidation $^a$ | Reduction $^d$ | Reduction in CO | Accumulation $^b$ |
|---------------|---------|--------|-----------|-------------------------|---------------------------|---------------------------|----------------|----------------|-----------------|-----------------|
| pINT-20       |         |        |           |                         |                           |                           | 0.5            | 0.5            | 1               | 0               |
| WT$^c$        | 423.5   | 425.0  | 422.0     | 1.94                    | 2.03                      | 1.05                      | 1              | 2              | 100             | 100             |
| L116K$^c$     | 422.0   | 425.0  | 418.0     | 1.68                    | 1.68                      | 1.00                      | 14             | 98             | 66              | 22              |
| L116K ΔP3R4$^e$ | 421.0  | 424.5  | 418.0     | 1.72                    | 1.71                      | 0.99                      | 4              | 156            | 105             | 14              |
| L116K C75A$^f$| 386.5   | 425.0  | 423.0     | 1.04$^c$                | 1.21$^c$                  | 1.16                      | 1              | 2              | 114             | 10              |
| L116K H77A$^f$| 416.0   | 424.0  | 418.0     | NT$^g$                  | NT                        | NT                        | 2              | 105            | 96              | 10              |
| L116K C75A$^f$| 412.0   | 420.5  | 418.0     | NT$^g$                  | NT                        | NT                        | 16             | 49             | 19              | 10              |

$^a$ Percentage of activity indicates the mean value of multiple measurements of activity relative to that of WT CooA expressed in the presence of CO, and showed variability <10%.  
$^b$ Percentage of accumulation indicates the heme accumulation of variant CooA in hydroxylapatite batch-treated samples relative to that of WT CooA, based on the spectra of CO-bound form.  
$^c$ Aerobically grown cells were used for the activity.  
$^d$ Anaerobically grown cells were used for the activity.  
$^e$ Purified sample was used for measurement of Soret maximum.  
$^f$ Hydroxylapatite batch-enriched CooA sample was used for Soret maximum.  
$^g$ For comparison, low-spin, 6-coordinate absorbance ($A_{P(111)}$) was estimated for ΔP3R4 CooA from the comparison of the extinction coefficients; ε$^{422,0}_{nm}$ = 105 mm$^{-1}$ cm$^{-1}$ for 6-coordinate heme of CooA (20), ε$^{386.5}_{nm}$ = 94.5 mm$^{-1}$ cm$^{-1}$ for 5-coordinate heme of CooA was measured with reduced pyridine hemochrome method (20).  
$^h$ Not tested.

FIG. 6. Electronic absorption spectra of CooA variants at pH 4. L116K CooA (panel A), WT (panel B), and L116K ΔP3R4 (panel C) are shown with the thick solid line of each panel indicating the Fe(III) form and the dotted line indicating the Fe(II) form. All the spectra were measured in 100 mM sodium acetate, pH 4.0, 0.1 M NaCl. In panel D, the electronic absorption spectra of Fe(III) L116K CooA were measured as a function of pH. Arrows indicate the changes in the Soret peak corresponding to a decrease of pH. Inset of panel D shows the plot of absorbance at 421 nm versus pH in which solid line represents the best fit of the data to the Henderson-Hasselbalch equation.

An Endogenous Ligand Replaces Pro$^2$ in Fe(II) L116K CooA—Because L116K CooA has functionally novel properties in the Fe(II) form, it was also important to determine the heme ligands in that form. It has been demonstrated that WT CooA undergoes a ligand switch upon reduction, with His$^{77}$ replacing Cys$^{75}$ (9, 10). The electronic absorption spectrum of Fe(II) L116K CooA showed a 6-coordinate low spin heme, but lacked the Soret maximum near 450 nm, a diagnostic feature of thiolate-ligated Fe(II) heme proteins (Fig. 5). This result suggests that Fe(II) L116K CooA lacks a cysteinate ligand and that the ligand switch also occurs in this variant. This notion is further supported by the observation that the introduction of the C75A substitution fails to perturb the spectrum of Fe(II) L116K CooA (Table II). Finally, we constructed a strain with L116K H77A CooA and measured the Soret maximum Soret peak ratio of each panel indicating the Fe(III) form. All the spectra were measured in 100 mM sodium acetate, pH 4.0, 0.1 M NaCl. In panel D, the electronic absorption spectra of Fe(III) L116K CooA were measured as a function of pH. Arrows indicate the changes in the Soret peak corresponding to a decrease of pH. Inset of panel D shows the plot of absorbance at 421 nm versus pH in which solid line represents the best fit of the data to the Henderson-Hasselbalch equation.

Table II: Spectral and functional comparison of L116K CooA with WT and related CooA variants

| CooA          | Soret maximum | Soret peak ratio | β-Galactosidase activity$^a$ | Oxidation$^d$ | Reduction$^d$ | Reduction in CO | Accumulation$^b$ |
|---------------|---------------|------------------|------------------------------|----------------|----------------|-----------------|-----------------|
|               | Fe(III) | Fe(II) | Fe(II)-CO | $A_{P(111)}/A_{P(110)}$ | $A_{P(111)-CO}/A_{P(111)}$ | $A_{P(111)-CO}/A_{P(110)}$ | Oxidation% | Reduction% | Reduction in CO% | Accumulation% |
| pINT-20       |         |        |           |                         |                           |                           | 0.5            | 0.5            | 1               | 0               |
| WT$^c$        | 423.5   | 425.0  | 422.0     | 1.94                    | 2.03                      | 1.05                      | 1              | 2              | 100             | 100             |
| L116K$^c$     | 422.0   | 425.0  | 418.0     | 1.68                    | 1.68                      | 1.00                      | 14             | 98             | 66              | 22              |
| L116K ΔP3R4$^e$ | 421.0  | 424.5  | 418.0     | 1.72                    | 1.71                      | 0.99                      | 4              | 156            | 105             | 14              |
| L116K C75A$^f$| 386.5   | 425.0  | 423.0     | 1.04$^c$                | 1.21$^c$                  | 1.16                      | 1              | 2              | 114             | 10              |
| L116K H77A$^f$| 416.0   | 424.0  | 418.0     | NT$^g$                  | NT                        | NT                        | 2              | 105            | 96              | 10              |
| L116K C75A$^f$| 412.0   | 420.5  | 418.0     | NT$^g$                  | NT                        | NT                        | 16             | 49             | 19              | 10              |

$^a$ Percentage of activity indicates the mean value of multiple measurements of activity relative to that of WT CooA expressed in the presence of CO, and showed variability <10%.  
$^b$ Percentage of accumulation indicates the heme accumulation of variant CooA in hydroxylapatite batch-treated samples relative to that of WT CooA, based on the spectra of CO-bound form.  
$^c$ Aerobically grown cells were used for the activity.  
$^d$ Anaerobically grown cells were used for the activity.  
$^e$ Purified sample was used for measurement of Soret maximum.  
$^f$ Hydroxylapatite batch-enriched CooA sample was used for Soret maximum.  
$^g$ For comparison, low-spin, 6-coordinate absorbance ($A_{P(111)}$) was estimated for ΔP3R4 CooA from the comparison of the extinction coefficients; ε$^{422,0}_{nm}$ = 105 mm$^{-1}$ cm$^{-1}$ for 6-coordinate heme of CooA (20), ε$^{386.5}_{nm}$ = 94.5 mm$^{-1}$ cm$^{-1}$ for 5-coordinate heme of CooA was measured with reduced pyridine hemochrome method (20).  
$^h$ Not tested.
CooA was observed in all the variants containing the L116K substitution, although it is unclear whether CO binding displaces His77 or Lys 116 in these proteins. As noted previously, the activity of the Fe(II)-CO form of L116K CooA is lower than that of the Fe(II) form. The less effective DNA binding activity of Fe(II)-CO L116K CooA could be rationalized by either binding to the “wrong side” of the heme or by perturbation of the heme position when CO was bound on the “Pro2 side,” given the fact that Fe(II)-CO L116K CooA effectively mimics the active conformation of Fe(II)-CO WT CooA.

Although Fe(II)-CO L116K CooA was stable, a small portion of the CooA (∼5%) was irreversibly precipitated upon the initial addition of CO to the Fe(II) form, as reflected in a reduced ratio of $A_{Fe(II)-CO}/A_{Fe(II)}$ compared with WT CooA (Table II). This small amount of precipitation cannot be responsible for the 4-fold increase of $K_D$ value for DNA binding, so we do not believe that it significantly perturbs the reported results, although the cause of the phenomenon is unknown.

**Working Hypothesis**—The data presented here show that Pro2 has been replaced by another ligand in the Fe(III) and Fe(II) states of L116K CooA and Lys116 is an excellent candidate for the replacement ligand. Fig. 8 shows a representation of the position of Lys116 relative to the known Fe(II) WT CooA structure as viewed with the SwissPro program. Among 16 possible conformers, we chose the Lys116 conformer that gives the shortest distance from the N-ε of Lys116 to the nitrogen ligand of His77. The distance from heme iron to the nitrogen ligand of His77 is 2.12 Å in the known Fe(II) WT CooA and the distance between the nitrogen ligand of His77 and the N-ε of Lys116 was calculated to be 5.39 Å for the Lys116 conformer (Fig. 8), if the protein backbones remained unchanged. The difference of 3.27 Å is apparently too far for a Lys116—Fe ligation, considering that the only structurally known ligation of this type is the 2.1-Å Lys—Fe bond length in cytochrome c nitrite reductase (17). Lys116 ligation therefore would require a significant change in the relative positions of the heme and the C-helix region compared with their positions in the Fe(II) WT CooA structure. Because Lys116 ligation suggests that the
heme is attached to His\textsuperscript{77} and Lys\textsuperscript{116}, it would also alter the relative position of the C-helix of one subunit with respect to its own effector-binding domain of the same subunit (intrasubunit reorientation). However, accepting the uncertainty in the precise ligand arrangement in L116K, it is still appropriate to ask how this ligation might result in the novel DNA binding activities of the protein. Structural comparison between effector-free CooA and effector-bound CRP (6) showed that there is a difference in the relative conformations of the long C-helices in these two proteins and led to the hypothesis that this conformational change might serve as the signal transduction link between the CO binding to the heme and the movement of the DNA-binding regions. Consistent with this, we have found that altering the relative position of the C-helices in the 121–126 region by improving its quality as a leucine zipper leads to altering the relative position of the C-helix of one subunit with respect to its heme environment change of WT CooA upon CO binding by a mechanism involving a spatial reorientation of the heme moiety relative to the C-helix.

Conclusion—The major conclusions of this paper are as follows. (i) The fact that L116K CooA actually loses activity upon CO binding is interesting and thus far novel among CooA variants. The variant has perturbed the ligation of Pro\textsuperscript{2}, and the ligand that replaces it is apparently Lys\textsuperscript{116}, a ligand that is relatively unusual among heme proteins. This conclusion is supported by a variety of spectral and mutational results. (ii) A reasonable hypothesis for the CO-independent activity in L116K involves a repositioning of the C-helices, which is consistent with the current view of CO activation of WT CooA, though the precise mechanism for the repositioning in the two proteins must be different.

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Analysis of the L116K Variant of CooA, the Heme-containing CO Sensor, Suggests the Presence of an Unusual Heme Ligand Resulting in Novel Activity
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