Roles of Arg-97 and Phe-113 in Regulation of Distal Ligand Binding to Heme in the Sensor Domain of Ec DOS Protein

RESONANCE RAMAN AND MUTATION STUDY

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The direct oxygen sensor protein isolated from Escherichia coli (Ec DOS) is a heme-based signal transducer protein responsible for phosphodiesterase (PDE) activity. Binding of O2, CO, or NO to a reduced heme significantly enhances the PDE activity toward 3',5'-cyclic diguanylic acid. We report stationary and time-resolved resonance Raman spectra of the wild-type and several mutants (Glu-93 → Ile, Met-95 → Ala, Arg-97 → Ile, Arg-97 → Ala, Arg-97 → Glu, Phe-113 → Leu, and Phe-113 → Thr) of the heme-containing domain of Ec DOS. For the CO- and NO-bound forms, both the hydrogen-bonded and non-hydrogen-bonded conformations were found, and in the former Arg-97 forms a hydrogen bond with the heme-bound external ligand. The resonance Raman results revealed significant interactions of Arg-97 and Phe-113 with a ligand bound to the sixth coordination site of the heme and profound structural changes in the heme propionates upon dissociation of CO. Mutation of Phe-97 and Phe-113 significantly influenced the transient binding of Met-95 to the heme upon photodissociation of CO. This suggests that the electrostatic interaction of Arg-97 and steric interaction of Phe-113 are crucial for regulating the competitive recombination of Met-95 and CO to the heme. Based on these results, we propose a model for the role of the heme propionates in communicating the heme structural changes to the protein moiety.

Heme-based sensors are a class of enzymes that regulate the enzymatic activities and DNA binding in response to the presence of diatomic gas molecules CO, NO, or O2. (1–6). The direct oxygen sensor from E. coli (Ec DOS) is a heme-based signal transducer protein responsible for phosphodiesterase (PDE) activity (7, 8). The Ec DOS is composed of a C-terminal PDE catalytic domain and the N-terminal heme-containing domain. The latter is a prototypical PAS domain, which is a ubiquitous protein sensory domain found in all kingdoms (9) and has a conserved α/β folds consisting of ~147 residues (10, 11). The Ec DOS protein has a PDE activity specific to 3',5'-cyclic diguanylic acid (12, 13), and the binding of O2, CO, or NO to the reduced heme significantly enhances the PDE activity (14, 15). Thus, Ec DOS is a novel gas-sensor enzyme that has unprecedented ability to be activated by different gas molecules. Because the CO and NO concentrations in the cells are very low (nanomolar), it is likely that Ec DOS is predominantly an oxygen sensor enzyme whereby catalysis is regulated in response to the micromolar O2 level (16). It is believed that the structural changes in the heme vicinity caused by ligand binding to the heme provide the initial event in the gas sensing, followed by intramolecular signal transduction from the heme to the functional domain, and thus regulating the PDE activity.

Fig. 1 illustrates the crystal structure of the truncated heme sensor domain (Ec DOSH) of Ec DOS protein (10). In the reduced form (Fig. 1A), Met-95 and His-77 are the heme axial ligands in the distal and proximal sides, respectively, and Met-95 forms a hydrogen bond with heme 7-propionate. Upon O2 binding to the reduced heme, Met-95 is replaced by O2 and heme 7-propionate forms a hydrogen bond with Arg-97 instead of Met-95. This stabilizes the heme-coordinated O2 (Fig. 1B). Thus, the replacement of a distal axial ligand from Met-95 to O2 perturbs the heme 7-propionate hydrogen bonding network, resulting in large conformational changes in the FG loop (10). On the other hand, the role of other distal residues (Fig. 1) and the heme 7-propionate hydrogen bonding network are not clear in the CO- or NO-sensing mechanism.

To understand the gas-sensing mechanism of Ec DOS, it is essential to determine the changes in the heme and surrounding structures caused by distal ligand binding/dissociation. Although the crystal structure provides information about the O2-free (reduced) and O2-bound forms (10), there is not...
enough information available about how the structural changes
are transmitted from the heme to the PDE catalytic domain. A
useful way to answer this question for heme proteins is to use
time-resolved studies, in which the ligand dissociation reaction
is initiated by photodissociation of heme-bound external
ligand.

In the present study, we have performed stationary and time-
resolved resonance Raman (TR³) investigations of wild-type
(WT) Ec DOS and several variants (E93I, M95A, R97I, R97A,
R97E, F113L, and F113T) and examined the enzymatic activi-
ties for the full-length Ec DOS. We found the simultaneous
presence of hydrogen-bonded and non-hydrogen-bonded
forms for CO and NO adducts, and Arg-97 forms a hydrogen
bond with the heme-bound external ligand. We also point out
the importance of Arg-97, Phe-113, and heme propionates in
the regulation of ligand binding at the distal side.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Cloning of the full-length Ec DOS and
the heme-containing sensor domain Ec DOSH, the construc-
tion of expression plasmids, and the purification procedure of
the WT and mutant proteins were performed essentially as
described previously (8, 15). Site-directed mutagenesis was per-
formed by using a PCR-based approach as implemented in the
QuickChange™ kit (Stratagene). The purities of

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formed by using a PCR-based approach as implemented in the
QuickChange™ kit (Stratagene). The purities of Ec DOS sam-
ple were confirmed to be >95% homogeneous by SDS-PAGE.

For RR measurements, Ec DOSH was further purified by gel
filtration through Superdex 75 (26/60 cm) pre-equilibrated
with 50 mM Tris-HCl buffer at pH 7.5. The concentration of
protein was adjusted to 100 µM in 50 mM Tris-HCl buffer (pH
7.5). The oxidized Ec DOSH was prepared by adding an excess
amount of potassium ferricyanide to the purified protein and
afterward potassium ferricyanide was removed by gel filtration
with Sephadex G-25. Reduced Ec DOSH was prepared by add-
ing a minimum amount of sodium dithionite solution (final
concentration, 0.5 mM) into the protein solution under nitro-

gen atmosphere. The NO-adduct of Ec DOSH was prepared by
incubating the dithionite-reduced Ec DOSH with NO-satu-
rated buffer.

For TR³ experiments, ~100 µl of 200 µM oxidized protein
(50 mM Tris-HCl buffer at pH 7.5), was transferred to an airtight
spinning cell, into which CO gas was incorporated to a pressure
of 1 atm (after evacuation of the internal pressure to 0.01
mmHg). The pump/fill procedure was repeated at least three
times, and finally a small amount of degassed dithionite solu-
tion (final concentration, 0.5 mM) was added for the reduction
of the oxidized protein.

**Resonance Raman Measurements**—RR spectra of Ec DOSH
proteins were obtained with a single polychromator
(SPEX750M, Jobin Yvon) equipped with a liquid nitrogen-
cooled charge-coupled device detector (Spec10:400BLN, Roper
Scientific). The excitation wavelength employed was 413.1 nm
from a krypton ion laser (BeamLok 2060, Spectra Physics).
The laser power at the sample point was adjusted to 1 milliwatt
for the oxidized, reduced, and NO-bound forms and to 0.1 milli-
watt for the CO-bound form to prevent photodissociation.
Raman shifts were calibrated with indene, acetone, and an

aqueous solution of ferrocyanide. The accuracy of the peak
positions of well defined Raman bands was ±1 cm⁻¹.

**Time-resolved Resonance Raman Measurements**—Nanosec-
cond TR³ experiments were performed as described previously
(17). TR³ spectra were obtained with two 10-ns pulsed lasers
operating at 30 Hz. The probe light (435.7 nm) was generated by the
H₂-first anti Stokes Raman shift of the second harmonic of an
Nd:YAG laser (Quanta-Ray, LAB-130), and its power was
made as low as possible (~80 µl) to avoid photodissociation by
the probe pulse. The pump light (532.0 nm) was the second
harmonic of an Nd:YAG laser (Quanta-Ray, LAB-130), and its
power was adjusted to 5 ml/pulse at the sample point. The delay
time (Δt₉) of the probe pulse from the pump pulse was con-
trolled through independent firing of two Q-switch lasers by a
pulse generator (Stanford Research, DG-535) for Δt₉ values
between ~0.5 µs and 1 ms and was monitored on an oscillo-
scope (Iwasaki, DS-4242) by photodiode detection. Scattered
light at right angle was collected and focused by two synthetic
quartz lenses onto an entrance slit of a single spectograph
(Spex, 500M) equipped with a non-blazed holographic grating
and a liquid nitrogen-cooled charge-coupled device detector
(Princeton Instruments, CDD-1100PB). Raman shifts were cal-
ibrated using indene, and the accuracy of frequencies are ±1
cm⁻¹ for well defined peaks.

**Phosphodiesterase Assay**—PDE activities of the full-length Ec
DOS were determined using a colorimetric assay for free phos-
phate as described previously (15). The measurements were
performed under anaerobic conditions (O₂ concentration
<10 ppm) in a glove box. Phe-113 mutants were fully reduced by
adding 10 mM dithiothreitol, and the formation of the reduced
form was confirmed by absorption spectroscopy. The CO- and
O₂-bound forms were prepared by diluting the sample in the
gas-saturated, reduced PDE solution containing 10 mM dithio-
threitol. The NO-bound form was prepared by adding 50–100
µM 6-(2-hydroxy-1-methyl-2-nitrosohydrazine)-N-methyl-1-

hexanamine to the sample of the reduced form. The PDE reac-
tion was performed at 25 °C in a mixture with 71.4 mM Tris-HCl
(pH 8.0), 71.4 mM NaCl, 7.14 mM MgCl₂, 2.14 units/µl calf
intestine alkaline phosphatase, 14.3 mM dithiothreitol, and 0.29
µM Ec DOS and initiated by the addition of 0.3 volume of 0.33
mM 3',5' cyclic diguanylic acid (purchased from BIOLOG, Bremen,
Germany). After the desired time, the reaction was termi-
nated by the addition of an equal volume of 1 M HCl. The solu-

tion was then centrifuged for 5 min at 15,000 x g to remove
denatured proteins as precipitates. The supernatant (100 µl)
was mixed with 200 µl of BIOMOL Green, and the mixture was
incubated at 25 °C for 30 min. The change in absorbance at 630
nm was measured. The initial rates of the reactions are averages
of at least three time course experiments.

**RESULTS**

The crystal structure of the O₂-bound form of Ec DOSH
showed that Arg-97 forms hydrogen bonds with the heme-co-
ordinated O₂ (Fig. 1B), while the interactions of the heme-
bound CO or NO with the surrounding residues are not known.
To investigate these interactions, we prepared several mutants
of the heme distal side residues (E93I, M95A, R97I, and F113L)
and measured their RR spectra. It is well established that the
heme of WT Ec DOSH adopts a six-coordinate low spin (6c-ls) state in the oxidized and reduced forms (18–20). The RR spectra of the reduced form for the mutants are similar to that of WT except for M95A mutant, in which the mutation of the distal axial ligand (Met-95 in Fig. 1A), produces a 5-coordinate high spin (5c-hs) structure (supplemental Fig. S1). In addition, the RR spectra of the oxidized form for all the mutants are similar to that of WT (supplemental Fig. S1). The spectral similarity of M95A to WT was expected, because hydroxy ion (or water molecule) is coordinated to the heme axial site in the oxidized form as revealed by the crystal structure (11). These results indicate that the mutations cause no significant change on the coordination- and spin-states of the oxidized heme of the mutants.

Binding of Different Ligands—Because of the sensitivity of the Fe–CO and the C–O stretching frequencies to the heme environment, the CO adducts of heme proteins provide a valuable probe of the heme pocket. The CO-isotope dependence in RR spectra of CO-bound form of WT Ec DOSH is illustrated in Fig. 2. Spectra a and b in Panel A represent the spectra of $^{12}$C$^{16}$O and $^{13}$C$^{18}$O, respectively, in the 300–650 cm$^{-1}$ region, and spectra d and e in panel B represent the spectra of $^{12}$C$^{16}$O and $^{13}$C$^{18}$O, respectively, in the region 1800–2100 cm$^{-1}$. Spectra c and f display the isotope difference spectra ($c = a - b$, and $f = d - e$). It is obvious from the difference spectra that the 488, 1924, and 1970 cm$^{-1}$ bands of $^{12}$C$^{16}$O are shifted to 477, 1837, and 1879 cm$^{-1}$, respectively, after isotopic labeling. Accordingly, the 488 cm$^{-1}$ band is assigned to the Fe–CO stretching and the 1924 and 1970 cm$^{-1}$ bands to C–O stretching.

On the basis of the empirical $\nu_{CO}$-environment correlation obtained with myoglobin (Mb), the $\nu_{CO}$ frequencies observed at 1910–1930, 1940–1950, and 1960–1970 cm$^{-1}$ correspond to CO with strong hydrogen bonding interaction, moderate hydrogen bonding interaction, and neutral surrounding, respectively (21). Accordingly, we assign the major (1970 cm$^{-1}$) and minor bands (1924 cm$^{-1}$) to non-hydrogen bonded and strongly hydrogen bonded C–O modes of the heme Fe–CO, respectively. The detection of the major band has been reported previously for the Ec DOSH (18–20), but the 1924 cm$^{-1}$ band was not reported before. This is presumably due to overlap of the isotope-shifted ($^{13}$C$^{18}$O) component of the 1970 cm$^{-1}$ band and the 1924 cm$^{-1}$ component of $^{12}$C$^{16}$O in the isotope-difference spectrum (18–20). The use of $^{13}$C$^{18}$O isotope allowed us to clarify the presence of the strongly hydrogen-bonded conformer (1924 cm$^{-1}$).

The low (A) and high frequency regions (B) of RR spectra of the CO complexes for several mutants are shown in Fig. 3 and summarized in Table 1. The mutation of Glu-93 showed no significant effect on the stretching vibration modes. On the other hand, the major C–O mode is downshifted by 3 cm$^{-1}$ in M95A spectrum (c) compared with WT spectrum (a). This implies that the M95A perturbs the electrostatic field near the iron-bound CO ligand. The Fe–CO and CO stretching frequencies of F113L (d) are largely perturbed (Table 1), implying that Phe-113 is also affected by CO and thus functioning in recognizing the axial ligand CO. Furthermore, the mutation of
Arg-97 (e) leads to the disappearance of the strongly hydrogen-bonded conformer. We note that the broad Raman band near 1924 cm$^{-1}$ in the spectrum of R97I (e) is assigned to a heme non-fundamental mode but not to CO stretching. This is because it exhibited no significant change upon isotopic labeling as seen in supplemental Fig. S2.

Thus, the RR results indicate that the strongly hydrogen bonding conformer at $\sim$1924 cm$^{-1}$ is observed for WT, E93I, M95A, and F113L proteins and is absent only for R97I mutant. This means that Arg-97 forms a hydrogen bond with the bound CO in the hydrogen bonding conformer.

We also examined NO-isotope dependence in RR spectra of NO-bound form of WT and several mutants (Table 1 and see also supplemental Fig. S3). The 557 cm$^{-1}$ band is assigned to the Fe–NO stretching, and the 1580 and 1635 cm$^{-1}$ bands to N–O stretching. The 1635 cm$^{-1}$ component of the NO-bound form means that NO is placed in a hydrophobic environment without a hydrogen bond, but the other N–O stretching frequency (1580 cm$^{-1}$) is characteristic of NO having a negative charge and being stabilized by strong hydrogen bonding (22–24). Because the strongly hydrogen bonding conformer is observed for WT, E93I, M95A, and F113L and is absent only for R97I, it is reasonable to suggest that Arg-97 forms a hydrogen bond with the heme-bound NO in the hydrogen bonding conformer.

**Time-resolved Resonance Raman Investigations of WT Ec DOS**—TR$^3$ spectroscopy has been successfully used to characterize photodissociated heme-CO complexes in various heme proteins (19, 25–28). It provides information about dynamic structures in the heme and its pocket. In the present study, TR$^3$ is used to follow dissociation of CO from Ec DOSH heme. The TR$^3$ spectra for WT Ec DOSH obtained from the pump/probe experiments are displayed for the high (panel A) and low (panel B) frequency regions in Fig. 4, and they are compared with those of the equilibrium CO (a) and reduced (l) forms. In the spectrum of the equilibrium-reduced form (l), the oxidation state marker ($\nu_1$) appears at 1361 cm$^{-1}$, characteristic frequency of the heme in the reduced state (18–20). The $\nu_2$ band is observed at 1581 cm$^{-1}$ and the core-sensitive band ($\nu_3$) at 1494 cm$^{-1}$, characteristic of 6c-Is heme (Table 2). This indicates that heme adopts the 6c-Is state in the reduced form, where Met-95 is the heme axial ligand in the distal side (Fig. 1A).

Upon binding of CO to the reduced heme, Met-95 is displaced by CO and $\nu_2$, $\nu_3$, and $\nu_1$ bands are shifted to 1370, 1499, and 1581 cm$^{-1}$, respectively (Fig. 4A, trace a) (19). The spectrum for $\Delta t_d = 0.5$ ms (Fig. 4A, trace b) is similar to that observed without the pump beam (Fig. 4A, trace a). This indicates that the recombination of CO to photodissociated species is completed in one turn of the spinning cell. In addition, the fact that the $\nu_{Fe-CO}$ band at 488 cm$^{-1}$ almost disappeared at $\Delta t_d = 20$ ns and thenceforth (Fig. 4B, traces c–k) demonstrates that photodissociation is achieved by the pump beam.

In the transient species at 20 ns (Fig. 4A, trace c), $\nu_1$, $\nu_2$, and $\nu_3$ bands appeared at 1353, 1470, and 1559 cm$^{-1}$, respectively (Fig. 4A, traces c–k) demonstrates that photodissociation is achieved by the pump beam. The $\nu_1$ band is assigned to a heme characteristic frequency of the heme in the reduced state (18–20). The $\nu_2$ band is observed at 1581 cm$^{-1}$ and the core-sensitive band ($\nu_3$) at 1494 cm$^{-1}$, characteristic of 6c-Is heme (Table 2). This indicates that heme adopts the 6c-Is state in the reduced form, where Met-95 is the heme axial ligand in the distal side (Fig. 1A).

Furthermore, the spectrum of the equilibrium-reduced form of WT (Fig. 4B, l) displays Raman bands at 343, 380, and 413 cm$^{-1}$, which can be assigned to $\nu_0$, propionate bending frequency (1580 cm$^{-1}$) is characteristic of NO having a negative charge and being stabilized by strong hydrogen bonding (22–24). Because the strongly hydrogen bonding conformer is observed for WT, E93I, M95A, and F113L and is absent only for R97I, it is reasonable to suggest that Arg-97 forms a hydrogen bond with the heme-bound NO in the hydrogen bonding conformer.

**Time-resolved Resonance Raman Study of Ec DOSH**—TR$^3$ spectroscopy has been successfully used to characterize photodissociated heme-CO complexes in various heme proteins (19, 25–28). It provides information about dynamic structures in the heme and its pocket. In the present study, TR$^3$ is used to follow dissociation of CO from Ec DOSH heme. The TR$^3$ spectra for WT Ec DOSH obtained from the pump/probe experiments are displayed for the high (panel A) and low (panel B) frequency regions in Fig. 4, and they are compared with those of the equilibrium CO (a) and reduced (l) forms. In the spectrum of the equilibrium-reduced form (l), the oxidation state marker ($\nu_1$) appears at 1361 cm$^{-1}$, characteristic frequency of the heme in the reduced state (18–20). The $\nu_2$ band is observed at 1581 cm$^{-1}$ and the core-sensitive band ($\nu_3$) at 1494 cm$^{-1}$, characteristic of 6c-Is heme (Table 2). This indicates that heme adopts the 6c-Is state in the reduced form, where Met-95 is the heme axial ligand in the distal side (Fig. 1A).

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**TABLE 1**

| Proteins | $\nu_{Fe-CO}$ | $\nu_{Fe-CO}$ | $\nu_{Fe-NO}$ | $\nu_{Fe-CO}$ |
|----------|---------------|---------------|---------------|---------------|
| WT       | 488           | 1924/1970     | 557           | 1580/1635     |
| E93I     | 488           | 1924/1969     | 557           | 1580/1635     |
| M95A     | 488           | 1924/1967     | 557           | 1580/1635     |
| F113L    | 496           | 1924/1965     | 551           | 1580/1632     |
| R97I     | 486           | 1972          | 551           | 1635          |
Time-resolved Resonance Raman Study of Ec DOS

FIGURE 4. TR spectra of photodissociated WT Ec DOS in the 1290–1745 cm−1 (A) and 300–550 cm−1 (B) regions. The delay times (Δτ) of the probe pulse from the pump pulse are specified at the right side of panel A; in both panels Δτ = −0.5 (b), 0.02 (c), 0.5 (d), 1.0 (e), 5.0 (f), 10 (g), 50 (h), 100 (i), 200 (j), and 1000 (k) μs, whereas spectra a and l denote the stationary spectra for equilibrium CO-bound and -reduced forms, respectively. The sample concentration was 200 μM in 50 mM Tris-HCl buffer, pH 7.5. The observed ν bands (black) is fitted (red lines) with the minimum number of Gaussian functions (blue lines).

TABLE 1
Raman frequencies of porphyrin skeletal modes, peripheral group modes, heme iron coordination, and delay times after photodissociation of CO for WT Ec DOS

| Proteins | Delay time | ν1 | ν2 | ν3 | ν4 | ν5 | ν6 | Coordination |
|----------|------------|----|----|----|----|----|----|-------------|
| Reduced  | 1361       | 1494| 1581| 380| 413| 5c-hs (Met-95) |
| CO       | 1370       | 1499| 1581| 377/384| 409/427| 6c-ls (CO) |
| Transient| 0.02       | 1353| 1470| 1559| 5c-hs |
|          | 1.0        | 1559| 1470| 1559| 5c-hs |
|          | 100        | 1559| 1470| 1559| 5c-hs |

Time-resolved Resonance Raman Investigations of Arg-97 Mutants—Met-95 is heme-coordinated in the reduced form, and the side chain of Arg-97 is oriented toward the surface of the protein (Fig. 1A). Whereas, in the O2-bound form, Met-95 is pointing toward the protein surface and Arg-97 reorients inward to the heme distal pocket to provide stabilization to heme-coordinated O2 (Fig. 1B). Thus, it is possible that the side-chain rotation of Arg-97 in the opposite direction upon ligand dissociation plays a role in regulating the Met-95 binding to the heme upon photodissociation of CO. To examine this idea, in particular the role of the side chain of residue 97, we have prepared R97I, R97A, and R97E variants, and performed their TR measurements. In the case of the R97I mutant (Fig. 5 and Table 3), spectra a−g (panel A) show 5c-hs heme, for which the ν bands appeared at 1351, 1471, and 1559 cm−1, respectively, similar to those observed for WT. In contrast to these, new ν bands appeared at 1369, 1497, and 1580 cm−1, respectively, at 50 μs (spectrum h) after CO photolysis.

TABLE 3
Raman frequencies of porphyrin skeletal modes, heme iron coordination, and delay times after photodissociation of CO for different variants

| Proteins | Delay time | ν1 | ν2 | ν3 | ν4 | ν5 | ν6 | Coordination |
|----------|------------|----|----|----|----|----|----|-------------|
| M95A     | 0.02−1000  | 1353| 1470| 1561| 5c-hs |
|          | 100−1000   | 1370| 1498| 1582| 6c-ls (CO) |
| R97I     | 0.02−1000  | 1351| 1471| 1559| 5c-hs |
|          | 50−1000    | 1369| 1497| 1580| 6c-ls (CO) |
| R97E     | 0.02−1000  | 1355| 1472| 1557| 5c-hs |
|          | 100−1000   | 1362| 1494| 1581| 6c-ls (Met-95) |
| R97A     | 0.02−10    | 1355| 1471| 1559| 5c-hs |
|          | 10−1000    | 1361| 1497| 1581| 6c-ls (Met-95) |
| F113L    | 0.02−200   | 1355| 1471| 1559| 5c-hs |
|          | 5−1000     | 1360| 1497| 1580| 6c-ls (Met-95) |
| F113T    | 0.02−1000  | 1352| 1471| 1557| 5c-hs |
|          | 5−1000     | 1360| 1497| 1580| 6c-ls (Met-95) |
These frequencies are similar to those observed for the equilibrium CO-bound form (a), implying generation of 6c-ls heme. Furthermore, the $\nu_{4\text{Fe-CO}}$ band appeared at 50 $\mu$s, and its intensity gradually increased till 1 ms (panel B, spectra h–k). Thus, the conversion from the 5c-hs to 6c-ls state after CO photolysis is attributed to rebinding of CO to the heme, although a small fraction of 5c-hs heme remained till 1 ms as indicated by the bands at 1351, 1471, and 1559 cm$^{-1}$. Therefore, CO, but not Met-95, rebinds to the heme after the photolysis of CO in the R97I mutant. These results demonstrate that the side chain of Arg-97 is important in regulating the motion of Met-95 in protein dynamics.

On the other hand, the mutation of Arg-97 by Glu (R97E) produces significantly different dynamics (Fig. 6 and Table 3) than those observed for WT (Fig. 4). Specifically, instead of 5c-hs heme, both 5c-hs (major; 1355, 1472, and 1557 cm$^{-1}$) and 6c-ls (minor; 1362, 1494, and 1581 cm$^{-1}$) species were simultaneously observed at a 20 ns delay (panel A, spectrum c). The transient spectra contain both 5c-hs and 6c-ls species till 1 ms. This means that the binding of Met-95 to the heme is not complete till 1 ms (panel A, c–k), whereas Met-95 binding occurs within 100 $\mu$s in WT (Fig. 4).

Furthermore, we performed similar experiments for R97A (Table 3 and see also supplemental Fig. S6) and the results suggested that the binding of Met-95 and the rebinding of CO to the heme after CO photolysis are faster in R97A variant than those occurring in WT. Thus, the mutation of Arg-97 by a residue with different side chains significantly perturbs the movement of Met-95 in Ec DOSH.

Time-resolved Resonance Raman Study of Ec DOS

To further investigate the role of Phe-113, especially in the dynamics of Ec DOSH protein, we have performed TR$^3$ experiments for F113L mutant, and the results are displayed in Fig. 7 and Table 3. At 20 ns
after photolysis of CO, the 5c-hs heme is formed as indicated by the \( \nu_\text{v} \), \( \nu_\text{p} \), and \( \nu_\text{s} \) bands at 1353, 1471, and 1559 cm\(^{-1}\), respectively. The 5c-hs heme species was dominant till 10 \( \mu \)s but thenceforth new \( \nu_\text{v} \), \( \nu_\text{p} \), and \( \nu_\text{s} \) bands appeared at 1361, 1497, and 1581 cm\(^{-1}\), respectively, meaning that 6c-ls heme is generated. These results are similar to those observed for WT (Fig. 4).

Accordingly, these bands arise from the Met-95-bound heme. The transient \( \nu_\text{s} \) band is broad, and its band fitting analysis suggested the presence of three \( \nu_\text{s} \) bands at 1353, 1361, and 1372 cm\(^{-1}\) at 100 \( \mu \)s delay (i). The 1353 and 1361 cm\(^{-1}\) bands correspond to the 5c-hs and 6c-ls (Met-95 binding) heme, respectively. Because the \( \nu_\text{Fe-CO} \) band appeared at 100 \( \mu \)s and its intensity gradually increased till 1 ms (panel B, spectra i–k), we suggest that the \( \nu_\text{s} \) band at 1372 cm\(^{-1}\) arises from the 6c-ls heme in which CO is the sixth ligand of heme. The transient spectrum at 200-\( \mu \)s delay (panel A, spectrum j) also showed the simultaneous presence of 5c-hs and the Met-95- and CO-bound forms. The intensity of the \( \nu_\text{s} \) band at 1372 cm\(^{-1}\) corresponds to the CO-bound form and is more increased at 1 ms (spectra k) delay, whereas that of the 5c-hs state (1353 cm\(^{-1}\)) almost disappeared. Thus, Met-95 and CO bind to the 5c-hs heme generated upon photolysis of CO. Similar results were observed for the F113T mutant (supplemental Fig. S7). Thus, the mutation of Phe-113 significantly perturbs the Ec DOS protein dynamics.

**Phosphodiesterase Activity**—We have shown recently the role of distal residues in catalysis (15). For instance, Met-95 coordination to the reduced heme is critical for locking the system and that global structural change around Met-95 caused by the binding of the external ligands releases the catalytic lock and activates catalysis. In the present study, we examined the importance of Phe-113 for the catalytic reaction. We prepared the full-length mutants for Phe-113 (F113L and F113T) and determined their PDE activities under anaerobic conditions. The results are summarized in Table 4 (see also supplemental Fig. S8). It is evident from Table 4 that the binding of \( \text{O}_2 \), CO, or NO to the reduced heme of WT enhances the activity by 6- to 7-fold (15), but the enhancement depends little on a ligand species. The ligand-free reduced form of F113L and F113T exhibited the activities higher and lower than that of WT, respectively. On the other hand, the ligand-bound forms of Phe-113 mutants exhibited similar activities but higher than those of WT irrespective of a ligand species. Thus, our data clearly demonstrate that the mutation of Phe-113 perturbs the PDE activities. This implies that the side chain of Phe-113 plays an important role in determining the conformation of the heme pocket related to that of the catalytic site toward 3',5'-cyclic diguanylic acid.

**DISCUSSION**

**Interactions between Heme-bound Ligand and Distal Residues**—In the present study, we have investigated the interactions of heme-bound CO and NO with the surrounding residues by monitoring the RR spectra for several mutants (E93I, M95A, R97I, and F113L). These mutants were designed to perturb the electrostatic field near the iron-bound gaseous ligand and also allow us to investigate the communication pathway between distal residues of the protein and heme. We found the formation of both hydrogen-bonded and non-hydrogen-bonded conformations in the CO- and NO-bound forms for WT, E93I, M95A, and F113L proteins. On the basis of the similarity of the CO- and NO-bound spectra of E93I to those of
WT, we conclude that no communication pathway exists between Glu-93 and heme-bound CO or NO.

On the other hand, the mutation of Met-95 alters only $\nu_{C-O}$ but not $\nu_{N-O}$ frequency. The $\nu_{Fe-O_{2}}$ frequency (560 cm$^{-1}$) is not affected by mutation of Met-95 (supplemental Fig. S9). These results may be related to the binding geometry of different gaseous ligands, where CO binds to the heme iron linearly, whereas a bent geometry is known for both O$_2$- and NO-bound forms (31). These results also suggest that the distal residues resulting from the M95A mutation weakly interact with the bound ligand species and perturb the ligand field only a little. Surprisingly, the PDE activity of the ligand-free form of M95A mutant was similar to those of ligand-bound forms irrespective of the ligand species (15) and similar to that of the ligand-bound forms of other mutants (Table 4). Accordingly, it is deduced that the protein conformation of heme pocket (FG loop, including the locations of Arg-97 and Phe-113), which is generated when Met-95 is displaced from the distal coordination site of heme iron, is critical to make the PDE catalytic domain of Ec DOS active. In other words, Met-95 coordination to heme locks the protein conformation in an enzymatically inactive form (15), and such conformation is somewhat relaxed in F113L.

The present RR results indicate that Arg-97 forms a hydrogen bond with heme-bound CO or NO in the hydrogen-bonded conformation. In addition, the absorption spectra of O$_2$-bound forms of Ec DOS could not be detected due to rapid auto-oxidation and/or a low affinity for O$_2$. Similar results were observed for the Arg-97 mutants of the full-length Ec DOS (15). Thus, it is likely that the hydrogen bonds from Arg-97 play a critical role for protecting the heme from oxidation by O$_2$ and thus stabilizing the heme-bound O$_2$, but this interaction may not be essential for CO- or NO-bound forms. Recently, Tanaka et al. reported the effects of Arg-97 mutation on the PDE activity (15). Interestingly, the PDE activities of Arg-97 mutants depend on the side chain of the 97th residue. For instance, the PDE activities of the CO- and NO-bound forms of R97A are similar to those of WT, whereas those of R97I and R97E are increased (by 24–28%) and decreased (by 29–47%), respectively, compared with those of WT (15). Because the geometry and electric polarization of heme-bound ligand depend on the ligand species, the observed facts indicate the importance of interactions between Arg-97 and heme-bound ligand.

The F113L mutant significantly perturbs the $\nu_{Fe-CO}$, $\nu_{Fe-N_{2}}$, $\nu_{Fe-O_{2}}$, $\nu_{CO}$, and $\nu_{NO}$ vibrations. A Phe residue has been known to stabilize polar ligands in several natural and variant Mbs, including elephant Mb (32–34). Similarly, Phe-113 would be critical for stabilizing the heme-bound ligands in the heme distal pocket of Ec DOSH protein. In the reduced form, the main-chain peptide oxygen of Gly-94 of the rigidified FG loop forms hydrogen bonds with the main-chain oxygen of Phe-113 and nitrogen of Leu-115 from H$_2$ sheet via a water molecule (10, 11). This hydrogen bond network is disrupted upon O$_2$ binding (10). In addition, the crystal structures of Ec DOSH revealed that there is noticeable movement of the H$_\mu$ sheet upon O$_2$ binding. Specifically, the root mean square deviation of C$_n$ atoms in the H$_\mu$ sheet (residues 108–120) with respect to the reduced form was 0.41 Å for the O$_2$-bound form. The root mean square deviation was calculated with Swiss-PdbViewer (35). These observations are compatible with our previous UV resonance Raman results in which Trp-110 from the H$_\mu$ sheet undergoes environmental changes upon O$_2$ binding (36). Furthermore, the PDE activities of ligand-bound forms of Phe-113 mutants were enhanced by 25–55% compared with those of WT (Table 4), demonstrating that the interactions of Phe-113 with bound ligand perturb the protein conformation to regulate the function of Ec DOS. We also propose that the interactions of Phe-113 with bound ligand would be involved in communicating the heme structural changes to the H$_\mu$ sheet upon ligand binding.

**Activation via Heme Peripheral Groups**—The propionate binding modes, $\delta(C_{\mu}C_{\alpha}C_{\alpha})$, of the equilibrium-reduced and CO-bound Ec DOSH were observed at 380 and 377/384 cm$^{-1}$, respectively. The $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ mode of the O$_2$-bound Ec DOSH was also observed at 384 cm$^{-1}$ (not shown). The frequency of $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ is indicative of hydrogen bonding between the heme propionates and the surrounding residues (37). In Mb, the heme 7-propionate constitutes a hydrogen bond with His-97 and Ser-92, and the $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ mode appears at 376 cm$^{-1}$. The disruption of this hydrogen bond by mutations leads to downshifts of the band by ~10 cm$^{-1}$ (38). In the O$_2$-bound form of Ec DOSH, the 7-propionate makes strong hydrogen bonds with Arg-97, whereas, in the reduced form, it makes weak hydrogen bonds with the backbone NH of Met-95 and two water molecules (10). This structural change is compatible with the higher frequency of the $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ mode (384 cm$^{-1}$) in the O$_2$- or CO-bound forms.

A schematic model for the structural change occurring upon ligand photodissociation is illustrated in Fig. 8. After CO photolysis of the CO-bound WT Ec DOSH, the 5c-hs species is formed in which no new prominent bands are observed in the 250–450 cm$^{-1}$ region in the picosecond TR$^3$ spectra for Ec DOSH (19). This indicates that the heme peripheral modes of the transient species are identical to those of the CO-bound form in this timescale. After a 20 ns delay, however, a weak $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ band appeared at 363 cm$^{-1}$. Because the lower $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ frequency accounts for a weak (or no) hydrogen bond of heme 7-propionate, we suggest that the hydrogen bond between 7-propionate and Arg-97 is cleaved within 20 ns of photolysis (Fig. 8).

The 363 cm$^{-1}$ band in the WT spectrum is upshifted to 375 and 380 cm$^{-1}$ within 1.0 and 100 µs, respectively. Because the $\delta(C_{\mu}C_{\alpha}C_{\alpha})$ frequency of 380 cm$^{-1}$ at 100-µs delay was observed for the equilibrium-reduced form (Fig. 4B, spectrum l) in which Met-95 is hydrogen-bonded with 7-propionate, the 380 cm$^{-1}$ band would arise from the hydrogen-bonded species, and its formation must be completed by 100 µs after CO-photolysis. Then, what is the origin of the 375 cm$^{-1}$ band observed at 1 µs? The crystal structure showed that the 6-propionate constitutes hydrogen bonds with Asn-84 and Gly-94 in the reduced form, but it forms a hydrogen bond only with Asn-84 in the O$_2$-bound form, for which Gly-94 from FG loop moves far away from the 6-propionate (10). Thus, we speculate that the formation of a hydrogen bond between Gly-94 and heme 6-propionate is the origin of the 375 cm$^{-1}$ band observed at 1 µs (Fig. 8). Therefore, we propose the following model upon photolysis of CO from heme: the hydrogen bond networks from both heme propi-
onates experience changes, leading to large conformational changes of protein moiety. Specifically, Gly-94 and Met-95 move inward to the heme distal pocket, while Arg-97 orients toward the protein surface as a result of the changes in the propionate hydrogen bonding interactions upon CO photolysis, communicating the heme structural changes to the FG loop. In addition, the inward movement of Gly-94 would reform its hydrogen bonding network with Phe-113 and Leu-115 via a water molecule (10, 11), communicating the heme structural changes to the H\textsubscript{b} sheet as we proposed earlier.

Recently, we have shown that the heme structural changes upon ligand binding in Mb are communicated to the globin moiety through heme propionates (39). In addition, the heme propionates play a crucial role in signaling mechanism of FixL (4) and HemAT-Bs (40) proteins upon ligand binding/dissociation. Similarly, the present results for Ec DOS\textsubscript{H} strongly suggest that the structural changes of heme upon ligand binding are communicated from the peripheral propionates to the FG loop.

Furthermore, the vinyl bending modes, $\delta(C_\beta C\alpha C\beta)$, of the equilibrium-reduced and CO-bound Ec DOS\textsubscript{H} were observed at 413 and 409/427 cm$^{-1}$, respectively. In the transient states, the $\delta(C_\beta C\alpha C\beta)$ bands appeared at 408 and 413 cm$^{-1}$ at 0.02 and 100 $\mu$s, respectively. This indicates the presence of different intermediates in which the interactions of vinyl side chains of heme with surrounding residues are altered upon CO-photodissociation. Thus, the cleavage of the hydrogen bond between 7-propionate and Arg-97 at 20 ns and the formation of a new hydrogen bond between 7-propionate and Met-95 at 100 $\mu$s are accompanied by structural changes around heme vinyl groups. These results are in agreement with our previous UV resonance Raman study in which the heme structural changes are communicated through the 2-vinyl group to Trp-53, an important residue for function of Ec DOS (36).

**Distal Residues Regulate Met-95 Binding to the Heme—Liebl and co-workers (30) reported the CO-rebinding kinetics upon its photodissociation from Ec DOS\textsubscript{H}. They showed that Met-95 binding to the heme occurs in 100 $\mu$s in competition with bimolecular CO recombination, and subsequent replacement of Met-95 by CO occurs at ~8 ms (30). In contrast, in other heme proteins with a hexa-coordinate heme, such as CooA (41) and neuroglobin (42), the binding of an internal ligand does not occur prior to CO-recombination. To understand why Met-95 binding occurs before the recombination of CO in Ec DOS\textsubscript{H} protein, it is essential to investigate the role of the distal residues such as Arg-97 and Phe-113 (Fig. 1) in the regulation of protein dynamics.

Arg-97 is located on the protein surface in the ligand-free reduced form, but it reorients inward to the heme distal pocket in the O\textsubscript{2}-bound form to provide stabilization to heme-coordinated O\textsubscript{2} (Fig. 1). Thus, it is possible that the rotation of Arg-97 in opposite directions upon ligand dissociation plays a role in

![FIGURE 8. Proposed scheme for the structural changes in the heme active site of Ec DOS\textsubscript{H} following CO dissociation. Hydrogen bonds are represented with black broken lines.](image-url)
regulating the Met-95 binding to the heme. To examine the role of Arg-97, in particular its electrostatic interactions, we mutated Arg-97 by different side chains (Ala, Ile, and Glu) and performed TR3 experiments for these mutants.

The results indicate that the mutation of Arg-97 significantly perturbs the Met-95 binding to the heme upon photodissociation of CO. Specifically, in the R97I mutant, in which Ile-97 does not provide electrostatic interactions with its surroundings but has a bulky side chain, Met-95 does not bind to the heme but CO rebinds. This must be related to the PDE activities of R97I, which are higher than those observed for WT by 24–28% (15). On the other hand, Met-95 binding is very slow in Mb, the distal His-64 forms a hydrogen bond with its PDE activity, which is lower than that of WT by 29–47% (15). In Mb, the distal His-64 forms a hydrogen bond with its PDE activity, which is lower than that of WT by 24–28% (15). The results indicate that the mutation of Arg-97 significantly perturbs the Met-95 binding to the heme upon photodissociation of CO. Specifically, in the R97I mutant, in which Ile-97 does not provide electrostatic interactions with its surroundings but has a bulky side chain, Met-95 does not bind to the heme but CO rebinds. This must be related to the PDE activities of R97I, which are higher than those observed for WT by 24–28% (15). On the other hand, Met-95 binding is very slow in Mb, the distal His-64 forms a hydrogen bond with its PDE activity, which is lower than that of WT by 29–47% (15). In Mb, the distal His-64 forms a hydrogen bond with its PDE activity, which is lower than that of WT by 24–28% (15).

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