Critical Role of the Heme Axial Ligand, Met\textsuperscript{95}, in Locking Catalysis of the Phosphodiesterase from \textit{Escherichia coli} (Ec DOS) toward Cyclic diGMP\textsuperscript{\textregistered}

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Heme-regulated phosphodiesterase from \textit{Escherichia coli} (Ec DOS) is a gas-sensor enzyme that hydrolyzes cyclic dinucleotide-GMP, and it is activated by O\textsubscript{2} or CO binding to the Fe(II) heme. In contrast to other well known heme-regulated gas-sensor enzymes or proteins, Ec DOS is not specific for a single gas ligand. Because Arg\textsuperscript{97} in the heme distal side in Ec DOS interacts with the O\textsubscript{2} molecule and Met\textsuperscript{95} serves as the axial ligand on the distal side of the Fe(II) heme-bound PAS domain of Ec DOS, we explored the effect of mutating these residues on the activity and gas specificity of Ec DOS. We found that R97A, R97I, and R97E mutations do not significantly affect regulation of the phosphodiesterase activities of the Fe(II)-CO and Fe(II)-NO complexes. The phosphodiesterase activities of the Fe(II)-O\textsubscript{2} complexes of the mutants could not be detected due to rapid autoxidation and/or low affinity for O\textsubscript{2}. In contrast, the activities even of the gas-free M95A and M95L mutants were similar to that of the gas-activated wild-type enzyme. Interestingly, the activity of the M95H mutant was partially activated by O\textsubscript{2}, CO, and NO. Spectroscopic analysis indicated that the Fe(II) heme is in the 5-coordinated high-spin state in the M95A and M95L mutants but that in the M95H mutant, like wild-type Ec DOS, it is in the 6-coordinated low-spin state. These results suggest that Met\textsuperscript{95} coordination to the Fe(II) heme is critical for locking the system and that global structural changes around Met\textsuperscript{95} caused by the binding of the external ligands or mutations at Met\textsuperscript{95} releases the catalytic lock and activates catalysis.

Cyclic dinucleotide-GMP (c-diGMP)\textsuperscript{2} is a novel intracellular second messenger that regulates cell motility, differentiation, development, virulence, antibiotic formation, and biofilm formation in bacteria growth and factor-stimulated proliferation in human colon cancer cells (1–10). Enzymes involved in the biosynthesis and breakdown of c-diGMP contain highly homologous GGDEF or EAL subdomains, respectively (1–10). The GGDEF subdomain expresses diguanylate cyclase activity, and catalyzes the synthesis of one molecule of cyclic diGMP from two molecules of GTP via the linear intermediate diguanosine tetraphosphate. The GGDEF subdomain is \textasciitilde 180 amino acids long and has a conserved amino acid sequence, GG(D/E)(D/E)F. The EAL subdomain has a phosphodiesterase activity that hydrolytically cleaves cyclic diGMP into l-diGMP and/or GMP, and is 260 residues in length, including the conserved amino acid sequence EAL. Metabolism of c-diGMP may be physiologically important because the genome of \textit{Escherichia coli} K-12, for example, encodes 19 proteins with GGDEF subdomains and 17 with EAL subdomains.

Heme-regulated phosphodiesterase from \textit{E. coli} (Ec DOS) contains a heme-bound PAS domain in the N-terminal region and a phosphodiesterase domain with GGDEF and EAL subdomains in the C-terminal region (11, 12). Although Ec DOS also contains a GGDEF subdomain, it does not appear to have guanylate cyclase activity. Thus, the precise roles of the GGDEF domain in the catalysis and structure of Ec DOS remain unclear. In contrast, the EAL domain in Ec DOS appears to contain the catalytic domain, so that this domain retains high phosphodiesterase activity that rapidly converts c-diGMP into linear dinucleotide GMP (l-diGMP) (3, 4). Furthermore, \textit{E. coli} with a knock-out of the Ec DOS gene display a morphology and growth rate different from those of the wild-type strain, suggesting that the enzyme is important for cell development and/or proliferation (13).

In well known heme-bound gas-sensor proteins, binding of specific gases to the Fe(II) heme (for example, CO for CooA, NO for soluble guanylate cyclase, and O\textsubscript{2} for FixL) causes a change in the heme environment that is transduced intramolecularly to the other domain to regulate the function (14). Each of these heme-bound gas-sensor enzymes can recognize only a single physiologically relevant gas. We recently reported, however, that the phosphodiesterase activity of full-length, dithionite-reduced Ec DOS is up-regulated upon binding of either O\textsubscript{2} or CO, NO, or CooA.
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FIGURE 1. Comparison between the crystal structure of Fe(II)-O₂ (A, Protein Data Bank code 1V86) and Fe(II) (B, PDB ID 1V92) complex of Ec DOS PAS domains. The atoms of heme (blue), the oxygen molecule bound to the heme (white), the proximal histidine (green), and residues thought to be involved in ligand sensing (M95, green; R97, yellow) are depicted as stick models. The dashed lines show the hydrogen-bond interactions between the side chain of Arg⁹⁷ and the oxygen molecule. The figures were prepared using PyMOL (34).

or CO binding to the Fe(II) heme (15). Although the amino acid sequence and overall structure of the heme-containing PAS domains of Ec DOS and FixL are similar, Ec DOS is activated by both O₂ and CO, whereas FixL is down-regulated only by O₂. Thus, Ec DOS appears to be a novel heme-bound gas-sensor enzyme with the unprecedented ability to recognize more than one type of gas molecule.

To understand why, in contrast to other gas-sensor heme proteins, Ec DOS lacks strict gas selectivity, the roles of the residues around the heme in catalytic activation must be determined. O₂ binding to the Fe(II) heme interacts directly with Arg⁹⁷ on the heme distal side (Fig. 1A) (16). Thus, Arg⁹⁷ appears to be important for recognizing different gas molecules. Met⁹⁵ binds directly to the Fe(II) heme as the endogenous axial ligand (Fig. 1B) (17), and the coordination of Met⁹⁵ to the Fe(II) heme is cleaved by binding of the external axial ligands O₂, CO, and NO. Thus, both Arg⁹⁷ and Met⁹⁵ may play important roles in the regulation of catalysis by gas binding. To explore the role of these two residues, we generated full-length Ec DOS with mutations at Arg⁹⁷ and Met⁹⁵, and examined their catalytic activities and activation by O₂, CO, and NO. As in studies of SmFixL (18), in the current experiments, we measured the catalytic activity in the presence of DTT rather than sodium dithionite to more accurately reflect the physiological conditions in vivo. It was found that mutation of Arg⁹⁷ did not significantly affect the catalytic activation by gases, whereas the mutation of Met⁹⁵ led to high enzyme activity even in the absence of gases as well an inability to be activated by gas binding. Our results imply that Met⁹⁵ plays a critical role in the activation of c-diGMP hydrolysis upon gas binding by Ec DOS.

EXPERIMENTAL PROCEDURES

Materials—All chemical reagents were of the highest grade available and were purchased from Wako Chemicals (Osaka, Japan) and used without further purification. Calf intestine alkaline phosphatase (CIAP) was obtained from Takara Bio (Otsu, Japan), c-diGMP was from BIOLOG (Bremen, Germany), 6-(2-hydroxy-1-methyl-2-nitrosohydrazine)-N-methyl-1-hexanamine (NOC9) was from DOJINDO (Kumamoto, Japan), and BIOMOL GREEN™ reagent was from Biolog (Plymouth Meeting, PA). The QuikChange™ site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Oligonucleotides used for plasmid construction and for site-directed mutagenesis were obtained from Nippon Gene (Sendai, Japan).

Construction of Expression Plasmid and Site-directed Mutagenesis—The cloning of Ec DOS and construction of the expression plasmid pET28a(+) were previously described (19). Mutants were generated as described in the supplementary materials. All constructs were confirmed by DNA sequencing.

Protein Overexpression and Purification—The wild-type and mutantEc DOS proteins were overexpressed in E. coli BL21(DE3) using the pET28a (+) expression plasmid, which contains an N-terminal His₆ tag and a thrombin cleavage site. A single colony was put in 50 ml of Luria-Bertani (LB) medium containing 20 g/ml kanamycin and 0.5% glucose and then shaken overnight at 200 rpm and 37 °C. The cultured medium was added to 1 liter of terrific broth (TB) medium containing 20 µg/ml kanamycin. Then, the medium was shaken for 4 h at 120 rpm and 37 °C. After the medium was cooled down to 20 °C, β-aminolevulinic acid was added to 0.45 mM and incubated with shaking for another 20 h. The E. coli cells were harvested by centrifugation for 10 min at 5000 × g and 4 °C, and finally stored at −80 °C.

The following protein purification procedures were performed on ice or at 4 °C. E. coli cells frozen at −80 °C were suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 20 mM imidazole, 150 mM NaCl, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride (buffer A), and 0.1 mg/ml lysozyme. The solution was sonicated and then centrifuged at 100,000 × g for 30 min. The supernatants were applied to a nickel-nitrilotriacetic acid-agarose column. The Ec DOS fractions were eluted with a linear gradient of 20–300 mM imidazole in buffer A. The solution containing Ec DOS was dialyzed against 20 mM Tris-HCl (pH 8.0) with 5% glycerol (buffer B), and then thrombin protease (Wako Chemicals, Osaka, Japan) (1 unit/mg of protein) was added to cleave the His tag. Subsequently, the sample solution was applied to a nickel-nitrilotriacetic acid-agarose column, and the His-tag-free Ec DOS was eluted in the flow-through fractions using buffer B. The Ec DOS
fractions were then applied to DEAE column, and *Ec* DOS was eluted with a linear gradient of 0–1 M NaCl in buffer B. After the proteins were dialyzed against buffer B, the purified *Ec* DOS was concentrated with Centriprep (Millipore, Billerica, MA) and Centrisalt I (Sartorius AG, Goettigen, Germany). The purified proteins were >95% homogenous as confirmed by SDS-PAGE. The sample was stored at −20 °C in the presence of 40% glycerol.

**Optical Absorption Spectroscopy**—All spectral data were obtained using a UV-1600PC (Shimadzu, Kyoto, Japan) spectrophotometer in a glove box that can maintain the oxygen concentration under 100 ppm (Hirasawa, Tokyo, Japan). The heme of *Ec* DOS proteins was reduced using either 10 mM DTT or small quantities of sodium dithionite in PDE buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 5 mM MgCl₂) under anaerobic conditions. The Fe(II) heme, Fe(II) heme-O₂, and Fe(II) heme-CO complexes were prepared in anoxic (10% H₂ and 90% N₂ gas), O₂-saturated, or CO-saturated buffer, respectively. The gas-saturated buffer solutions were obtained by bubbling the buffer with the appropriate gas for at least 1 h. The Fe(II) heme-NO complexes were prepared by adding of 50 or 100 µM NOC9 to the solution of Fe(II) heme complex.

**Phosphodiesterase Assay**—*Ec* DOS hydrolyzes c-diGMP to l-diGMP as demonstrated by reversed-phase liquid chromatography (supplementary materials Fig. 1S). On the other hand, in our phosphodiesterase assay, we used a colorimetric method, namely, l-diGMP that is converted to a guanylyl (3′,5′)-guanosine by *Ec* DOS. CIAP reacts with only l-diGMP because CIAP lacks phosphodiesterase activity and thus reacts only with terminal phosphate groups. CIAP therefore converts l-diGMP to GpG (guanylyl (3′,5′)-guanosine) and phosphate. One product, phosphate, was quantified by colorimetrically using BIOMOL GREEN reagent (Biomol). The change in absorbance at 630 nm was measured as described above. The initial rates of the reactions were averages of at least three time course experiments.

**RESULTS**

**Optical Absorption Spectra of the Mutants**—We first collected optical absorption spectra for the gas-free and gas-bound Fe(II) heme complexes of the R97A, R97I, and R97E mutants of full-length *Ec* DOS (Fig. 2). The absorption maxima of the mutant proteins are summarized in Table 1. The absorption spectra of these Arg⁹⁷ mutants in the absence or presence of CO and NO were essentially the same as for the wild-type protein, suggesting that the coordination structure (6-coordinated low-spin form) and the heme environment were not substantially changed by the Arg⁹⁷ mutations. The spectra of the Fe(II)-O₂ complexes for the Arg⁹⁷ mutants as measured with a conventional absorption spectrometer, however, could not be detected due to their rapid autoxidation and/or low affinity for O₂.

![Optical absorption spectra of the wild-type (A), R97A (B), R97I (C), and R97E (D) mutant proteins of full-length Ec DOS. Spectra of Fe(II) (solid line), Fe(II)-CO (dotted line), and Fe(II)-NO (dashed line) complexes are presented. Spectra of the Fe(II)-O₂ complexes could not be clearly detected due to their rapid autoxidation and/or low affinity for O₂.](image)

In the remaining experiments, we reduced the wild-type and Arg⁹⁷ mutant enzymes to the Fe(II) heme state using DTT, whereas we reduced the Met⁹⁵ mutant proteins with sodium proteins as precipitates. The supernatant (100 µl) was mixed with 200 µl of BIOMOL GREEN, and the mixture incubated at 25 °C for 30 min. The change in absorbance at 630 nm was measured as described above. The initial rates of the reactions were averages of at least three time course experiments. The experimental errors are shown in the l-diGMP hydrolysis time course data of Fig. 3, and also in Figs. 5S and 6S (supplementary materials). Note that the stick diagrams in Figs. 3B, 4, and supplementary materials Fig. 7S do not show error bars because values were calculated from averaged data points.

**Optical Absorption Spectra of the Mutants**—We first collected optical absorption spectra for the gas-free and gas-bound Fe(II) heme complexes of the R97A, R97I, and R97E mutants of full-length *Ec* DOS (Fig. 2). The absorption maxima of the mutant proteins are summarized in Table 1. The absorption spectra of these Arg⁹⁷ mutants in the absence or presence of CO and NO were essentially the same as for the wild-type protein, suggesting that the coordination structure (6-coordinated low-spin form) and the heme environment were not substantially changed by the Arg⁹⁷ mutations. The spectra of the Fe(II)-O₂ complexes for the Arg⁹⁷ mutants as measured with a conventional absorption spectrometer, however, could not be detected due to their rapid autoxidation (>10 s⁻¹) and/or a low affinity for O₂ (data not shown). It is likely, therefore, that Arg⁹⁷ is important for stabilizing the Fe(II)-O₂ complex.

Optical absorption spectra of the Met⁹⁵ mutants of the full-length enzyme for the gas-free and gas-bound Fe(II) heme complexes were essentially the same as those previously reported for the mutants of the isolated heme-bound PAS domain (20–22). Note that both the M95A and M95L mutants were 5-coordinate high-spin complexes, whereas the M95H mutant was a 6-coordinate low-spin complex in which His must be an axial ligand.

In the remaining experiments, we reduced the wild-type and Arg⁹⁷ mutant enzymes to the Fe(II) heme state using DTT, whereas we reduced the Met⁹⁵ mutant proteins with sodium...
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TABLE 1
Optical absorption spectral maxima (nm) of the Fe(II) complexes for the wild-type, Arg97 mutant, and Met95 mutant proteins in the absence and presence of O2, CO, and NO

| Coordination structures and spin states of the heme iron are shown in parentheses. | EcDOS | Fe(II) | Fe(II)-O2 | Fe(II)-CO | Fe(II)-NO |
|---|---|---|---|---|---|
| WT | 428 | 532 (6cLS)* | 563 | 417 | 542 (6cLS) | 578 |
| R97A | 427 | 532 (6cLS) | 563 | ND* | 424 | 540 (6cLS) | 571 |
| R97I | 427 | 532 (6cLS) | 563 | ND | 424 | 540 (6cLS) | 571 |
| R97E | 427 | 532 (6cLS) | 563 | ND | 424 | 540 (6cLS) | 571 |
| M95A | 434 | 556 (5cHS) | 416 | 541 (6cLS) | 576 |
| M95I | 434 | 559 (5cHS) | 417 | 541 (6cLS) | 579 |
| M95H | 429 | 531 (6cLS) | 562 | 417 | 541 (6cLS) | 579 |
| R97A | 427 | 532 (6cLS) | 563 | 417 | 541 (6cLS) | 579 |
| R97E | 427 | 532 (6cLS) | 563 | 417 | 541 (6cLS) | 579 |

*6cLS, 6-coordinate low-spin heme complex.
*ND, not determined due to rapid autooxidation and/or low affinity for O2.
*5cHS, 5-coordinate high-spin heme complex.

dithionite. It was, therefore, examined whether there were differences in the optical absorption spectra of the Fe(II) heme complexes when the enzymes were reduced with DTT and sodium dithionite. We found that the spectra of the Fe(II) heme complexes were essentially the same, even if the heme iron was reduced by DTT or sodium dithionite, suggesting that the heme environment of the full-length enzyme is not influenced by the reducing agent.

Phosphodiesterase Activity of the Wild-type Enzyme—Activities of other heme-bound oxygen-sensor enzymes, such as SmFixL, in the presence of DTT are much more sensitive to oxygen and appear to reflect the activity of the native enzyme under hypoxic conditions (18). In the present study, therefore, we used 10 mM DTT to reduce the wild-type enzyme, and we measured the catalytic activity in the glove box to provide anoxic conditions. High pressure liquid chromatography (as shown in supplementary materials Fig. 1S) is used in general to measure phosphodiesterase cleavage of c-diGMP. However, this method is time consuming. In this report, we use colorimetric detection of products in the development of a rapid c-diGMP cleavage assay (“Experimental Procedures” and supplementary materials Fig. 2S).

Fig. 3 shows the time course of l-diGMP generation by Fe(II), Fe(II)-O2, Fe(II)-CO, and Fe(II)-NO complexes of wild-type EcDOS in the presence of 10 mM DTT. The initial velocities are compared in Fig. 3B and are summarized in Table 2. The binding of O2, CO, or NO to the heme enhanced the activity 6–7-fold. The finding that even NO binding markedly enhanced the catalytic activity in the glove box to provide anoxic conditions. High pressure liquid chromatography (as shown in supplementary materials Fig. 1S) is used in general to measure phosphodiesterase cleavage of c-diGMP. However, this method is time consuming. In this report, we use colorimetric detection of products in the development of a rapid c-diGMP cleavage assay (“Experimental Procedures” and supplementary materials Fig. 2S).

Effects of Arg97 Mutations on the Phosphodiesterase Activity—The optical absorption spectra of the Arg97 mutants in the absence or presence of CO and NO were similar to those of the wild-type enzyme (Table 1), suggesting that the heme environments of the mutants and wild-type enzyme are essentially the same. Therefore, we examined the activities of the mutant enzymes in the presence of CO or NO. It was found that CO and NO binding markedly enhanced (6.5–13-fold) the catalytic activity of the three Arg97 mutants, although in the absence of these gases, the activities of the R97A and R97E mutants were lower than that of the wild-type enzyme (Table 2 and supplementary materials Fig. 1S). Because the autoxidation of the O2-bound Arg97 mutant proteins was very rapid (>10 s−1) and/or the affinities of the Fe(II) heme complexes for O2 were low, we could not measure the catalytic activity of the O2-bound Arg97 mutant enzymes.

Effects of Met95 Mutations on the Phosphodiesterase Activity—Addition of up to 100 mM DTT was not sufficient to fully reduce the Met95 mutants due to the relatively low redox potential of the heme iron in the mutant proteins. This was also observed for the same mutants of the isolated PAS domain (21). Therefore, we used a trace amount of sodium dithionite to completely reduce the heme to the Fe(II) complex. After removing excess...
sodium dithionite by gel filtration on Sephadex G-25, we measured the catalytic activities of the Met\(^{95}\) mutant proteins in the same buffer containing 10 mM DTT.

Interestingly, the gas-free M95A and M95L mutants showed high activities without gas binding; specifically, the activities of these two mutants were 7–8-fold higher than that of the gas-free wild-type enzyme (Table 1 and supplementary materials Fig. 25). The activities of the gas-bound bound forms of the mutants were almost as high as the gas-free forms (Table 2). In other words, the catalytic activities of the mutant enzymes were not enhanced by gas binding. In contrast, the activity of the gas-free M95H mutant was 2-fold higher than that of the wild-type enzyme, and the gas molecules enhanced its activity 2–3-fold.

### DISCUSSION

The PAS fold is characterized by several \(\alpha\)-helices flanking a five-stranded antiparallel \(\beta\)-sheet scaffold (17, 29–33). PAS domains are found in many signaling proteins, where they are used either as signal sensor domains or for protein dimerization (29–32). Several PAS domain proteins are known to detect signals through the use of prosthetic groups such as heme or flavin (30, 31). Heme-based PAS sensor proteins detect oxygen, CO, and possibly the cellular redox state (14, 31).

Comparison of the crystal structures of the oxygen-free and oxygen-bound PAS domains of \(E_{c}\) DOS (16, 17) shows that Arg\(^{97}\) undergoes a large change in position when oxygen binding occurs. It might thus be expected that Arg\(^{97}\) should play a prominent role in DOS function. Notably, however, our study clearly shows that this is not the case. The R97A and R97I mutants have activity profiles similar to that of the wild-type enzyme. On the other hand, the M95A and M95L mutants are clearly constitutively "on." In the absence of Met\(^{95}\), the PAS domain presumably assumes a conformation similar to that of the gas-bound protein. Consistent with this idea, the M95H mutant, with a histidine bound to the heme, exhibits properties similar to those of the wild-type enzyme. These data strongly suggest that changes in Met\(^{95}\) coordination structure caused by gas binding (mimicked by mutations at Met\(^{95}\)) result in larger conformational changes at the distal side, which subsequently communicate ligand binding information to the catalytic domain.

### Effects of Sodium Dithionite and DTT on the Phosphodiesterase Activity of \(E_{c}\) DOS

Although the optical absorption spectra of the Fe(II) complexes for the wild-type and Arg\(^{97}\) mutant proteins were essentially the same when reduced by DTT or sodium dithionite (Fig. 2 and Table 1), the phosphodiesterase activities of the DTT-reduced enzymes were at least 5-fold higher than those of the dithionite-reduced enzymes (Fig. 3 and supplementary materials Fig. 6S). Similar results have been reported for other heme-bound oxygen-sensor enzymes, including the histidine kinase \(S_{m}\) FixL (18). The low activity of the dithionite-reduced forms of \(S_{m}\) FixL were thought to be due to the formation of an aberrant disulfide bond at Cys\(^{301}\) between Fe(III) homodimers during the preparation of samples under aerobic conditions in the absence of reducing agents. In \(S_{m}\) FixL treated with DTT, the disulfide bond was cleaved, leading to more enhancing the enzyme activity.

The wild-type and some mutants of \(E_{c}\) DOS are sometimes purified as the Fe(II)-O\(_2\) form (data not shown), suggesting that \(E_{c}\) DOS is present as the Fe(II) form in cells. In general, however, the Fe(III) form along with minor components of Fe(II) and Fe(II)-O\(_2\) forms are obtained when \(E_{c}\) DOS is purified under aerobic conditions. \(E_{c}\) DOS contains eight cysteine residues in each monomer. It is possible, therefore, that some cysteine residues in \(E_{c}\) DOS are already oxidized and form disulfide bond(s), leading to lower activity. The following results support the idea that dispensable oxidation caused partial inactivation of \(E_{c}\) DOS. When DTT was removed by G-25 column chromatography, DTT-reduced \(E_{c}\) DOS showed low phosphodiesterase activities for Fe(II), Fe(II)-O\(_2\), and Fe(II)-NO complexes, but showed high activity only for the Fe(II)-CO complex (supplementary materials Fig. 5S). O\(_2\) and NO molecules are oxidants, and thus, may oxidize cysteine residue(s) or cleave disulfide bonds in \(E_{c}\) DOS, partially inactivating the phosphodiesterase activity.

**Role of Arg\(^{97}\) in the Catalysis**—The Arg\(^{97}\) mutants autooxidized rapidly (>10 s\(^{-1}\)) and/or had low affinities for oxygen. Therefore, Arg\(^{97}\) must be important for stabilizing the O\(_2\) molecule bound to the Fe(II) heme by directly binding to the O\(_2\) molecule on the heme distal side (16). In contrast, optical absorption spectra suggest that Arg\(^{97}\) is not important for stabilizing the CO and NO molecules bound to the Fe(II) heme. The enhancement of catalytic activity by CO and NO was similar for the Arg\(^{97}\) mutants and the wild-type enzyme. Therefore, Arg\(^{97}\) must not be critical for the enhancement of catalysis by CO and NO.

The structure of the heme-bound sensor PAS domains of \(S_{m}\) FixL and \(E_{c}\) DOS are similar. Arg\(^{214}\) in \(S_{m}\) FixL, which cor-

### Table 2

Phosphodiesterase activities (nmol l-diGMP/nmol of Ec DOS) of the Fe(II) complexes of the wild-type, Arg\(^{97}\) mutant, and Met\(^{95}\) mutant enzymes in the absence and presence of O\(_2\), CO, and NO  

| Ec DOS | Activities* | -Fold activation* |
|--------|-------------|-------------------|
| WT     | Fe(II)     | Fe(II)-O\(_2\)   | Fe(II)-CO | Fe(II)-NO |  |
| R97A   | 5.6 (0.58) | ND                | 68 (7.0)  | 65 (6.7)  | 12  |
| R97I   | 8.2 (0.85) | ND                | 87 (9.0)  | 87 (9.0)  | 11  |
| R97E   | 5.3 (0.55) | ND                | 37 (3.8)  | 48 (4.9)  | 7.0–9.1 |
| M95A   | 69 (7.1)   | 94 (7.6)          | 78 (8.0)  | 85 (8.8)  | 1.1–1.2 |
| M95L   | 79 (8.1)   | 90 (9.3)          | 96 (9.9)  | 82 (8.5)  | 1.0–1.2 |
| M95H   | 23 (2.4)   | 52 (5.4)          | 82 (8.5)  | 72 (7.4)  | 2.3–3.5 |
| R97E   | 5.3 (0.55) | ND                | 37 (3.8)  | 48 (4.9)  | 7.0–9.1 |
| M95A   | 69 (7.1)   | 94 (7.6)          | 78 (8.0)  | 85 (8.8)  | 1.1–1.2 |
| M95L   | 79 (8.1)   | 90 (9.3)          | 96 (9.9)  | 82 (8.5)  | 1.0–1.2 |
| M95H   | 23 (2.4)   | 52 (5.4)          | 82 (8.5)  | 72 (7.4)  | 2.3–3.5 |

* Namomole of l-diGMP/nmol of Ec DOS.  
* Fold increase in the activity of the gas-bound forms compared with that of the gas-free form.  
* ND, not determined due to rapid autoxidation and/or low affinity for O\(_2\).
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![Graph A](Image)

**FIGURE 4.** Comparison of the catalytic activities of the Fe(II) (white), Fe(II)-O2 (hatched), Fe(II)-CO (gray), and Fe(II)-NO (black) complexes of the Arg97 (A) and Met95 (B) mutant enzymes.

responds to Arg97 in Ec DOS, in the heme distal side directly interacts with the O2 molecule bound to the Fe(II) heme (23). The Fe(II)-O2 complex of SmFixL is inactive, whereas the gas-free Fe(II) complex is active, and Arg214 forms salt bridges with the heme propionate in the gas-free Fe(II) complex (23). Therefore, the O2 molecule has opposite roles in the catalysis of SmFixL and Ec DOS. For SmFixL, the distal Arg214 is thought to be essential for the regulation of kinase activity by ligand binding, O2 binding, and, therefore, reduced autoxidation of the Fe(II)-O2 complex (24, 25). The optical absorption spectra suggest that Arg97 in Ec DOS stabilizes the Fe(II)-O2 complex and that the interaction of Arg97 with the gas ligand may not be important for the activation of catalysis by ligand binding (Table 2 and Fig. 4).

It has been suggested that specific steric and/or electrostatic effects destabilize the Fe(II)-O2 complex (20, 28). In general, an increase in accessibility of the distal pocket to either solvent water molecules or to other polar residues, or protonation of the O2 molecule bound to the Fe(II) heme, accelerate autoxidation of the Fe(II)-O2 complex. The increase in autoxidation rate seen in the Arg97 mutants suggests that Arg97 mutations lead to increases in interaction of polar molecules with the Fe(II)-O2 complex. These findings contrast with data from tests with M95A and M95L mutants, where the mutations significantly decreased the autoxidation rate (20). Isolation of the PAS domain also decreased the autoxidation rate (20).

**Role of Met95 in the Catalysis**—Met95 is the sixth axial ligand in the gas-free Fe(II) complex. Met95 must move away from the heme iron upon binding of exogenous ligand to Fe(II) heme (Fig. 1). Previous studies indicate that mutations at Met95 in the isolated heme-bound PAS domain of Ec DOS substantially increase the affinities of the heme iron for O2, CO, and CN and that the heme redox potential is also markedly changed by Met95 mutations (20, 21, 26). The role of Met95 in catalytic regulation, however, has been unclear.

In the present study, we found that the activities of the gas-free M95A and M95L mutant enzymes were already similar to those of the gas-bound wild-type enzymes (Table 2). Therefore, it appears that Met95 is critical for regulating the catalytic activity of Ec DOS. In the gas-free form, the M95A and M95L mutants are 5-coordinate high-spin complexes, in contrast to the wild-type and Arg97 mutant enzymes, which are 6-coordinate low-spin complexes. In the wild-type and Arg97 mutant enzymes, cleavage of the Met95 coordination to the Fe(II) heme occurs upon gas binding. On the other hand, in the M95A and M95L mutants, as in the gas-bound wild-type and Arg97 mutant proteins, there is no bond between the residue at position 95 and the Fe(II) heme iron. Certain structural change(s) near the main or side chain of Met95 on the heme distal side must occur upon binding of the gas ligand. There may be a similar structure(s) in the Fe(II) complex of the M95A and M95L mutants even in the absence of the gas molecules, which lead to the high activities of the gas-free Fe(II) heme enzymes (Table 2 and Fig. 4).

It is interesting that the M95H mutant is the 6-coordinate low-spin complex, probably as a result of His95 coordination to the Fe(II) heme iron. The catalytic activity of the gas-free form of this mutant was lower than those of other Met95 mutants that are 5-coordinate high-spin complexes. On the other hand, gas binding by this mutant enhanced its activity 2–3-fold, which is higher than that observed for other Met95 mutant proteins. Perhaps a certain conformational change(s) caused by the M95H mutation influences the activity of the gas-free M95H mutant enzyme, leading to activities intermediate between the wild-type/Arg97 and M95A/M95L mutants. Collectively, the results suggest that Met95 coordination to the Fe(II) heme is a key factor in the down-regulation of catalysis so that its removal by gas binding or the M95A or M95L mutations enhances the catalytic activity.

**Comparison with Catalysis of Ec DOS toward cAMP**—Recent studies of the cAMP phosphodiesterase activity of Ec DOS indicate that cAMP is hydrolyzed by the Fe(II) heme form but not the Fe(III) form of Ec DOS (19). Furthermore, binding of CO or NO to the Fe(II) heme complex eliminates the ability to hydrolyze cAMP. X-ray crystal structural studies of the isolated heme-bound PAS domain of Ec DOS indicate that profound structural changes of the heme-bound PAS domain occur upon changes in the redox state (17). Such redox-dependent structural changes appear to reflect the redox-dependent regulation in the cAMP phosphodiesterase activity of Ec DOS. Furthermore, knocking down the Ec DOS gene in *E. coli* causes changes in cell growth rate, cell morphology, and intracellular cAMP concentration, suggesting that Ec DOS plays an important role in modulating the cellular effects of cAMP (13).

In the present study, we showed that, in addition to cAMP, Ec DOS hydrolyzes c-diGMP. The mechanism by which c-diGMP hydrolysis is regulated differs from that regulating cAMP hydrolysis. Namely, the c-diGMP phosphodiesterase activity of Ec DOS is substantially up-regulated by the binding of gas mol-
ecules. Furthermore, it is important to note that, unlike other heme-bound gas-sensor enzymes and proteins (14), Ec DOS does not discriminate between O2, CO, and NO. Further studies are needed to understand the mechanisms of gas sensing and intramolecular signal transduction by this novel enzyme. It will also be interesting to investigate the intracellular cross-talk between the two substrates of Ec DOS, cAMP and c-diGMP (27).

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