Structure−Function Relationships of *Ec*DOS, a Heme-Regulated Phosphodiesterase from *Escherichia coli*

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ABSTRACT

Recent studies have revealed a new class of heme enzymes, the heme-based sensors, which are able to turn on or off cellular signal transduction pathways in response to environmental changes. One of these enzymes is the heme-regulated phosphodiesterase from *Escherichia coli* (*Ec*DOS). This protein is composed of an N-terminal heme-containing PAS domain and a C-terminal functional domain. PAS is an acronym formed from the names of the *Drosophila* period clock protein (PER),[5] vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT),[6] and *Drosophila* single-minded protein (SIM). The heme cofactor in its PAS domain can act as a sensor of the cellular redox state that regulates the adenosine 3′,5′-cyclic monophosphate (cAMP) phosphodiesterase activity. The crystal structures of its heme-containing PAS domain have helped clarify how the heme redox-dependent structural changes initiate intramolecular signal transduction. Here, we review recent findings on the structure−function relationships of *Ec*DOS.

Introduction

All living organisms respond to environmental changes, such as variations in atmospheric levels of O_2, CO, and NO. These diatomic gases are physiologically essential but sometimes toxic to living organisms. Therefore, mechanisms for sensing these gases and responding to them are essential for survival. Recently, a new class of sensing molecules, the heme-containing sensor proteins, has been identified.[1,2] These are distinct from well-known heme proteins, including O_2 carriers (e.g., hemoglobin and myoglobin), oxygen activators (e.g., cytochrome P450 and peroxidases), and mediators of electron transfer (e.g., cytochrome *c*).[3,4] Heme sensor proteins are generally composed of an N-terminal heme-containing sensor domain and a C-terminal effector domain. Binding of gases to the sensor domain induces structural changes in these proteins, which are transmitted to the effector domain, eliciting cellular signal transduction (Figure 1). There are many types of effector domains, including protein kinases, guanylate cyclase, phosphodiesterases, and transcriptional activators.

Examples of heme sensor proteins are shown in Table 1. A heme-bound PAS domain is included in several

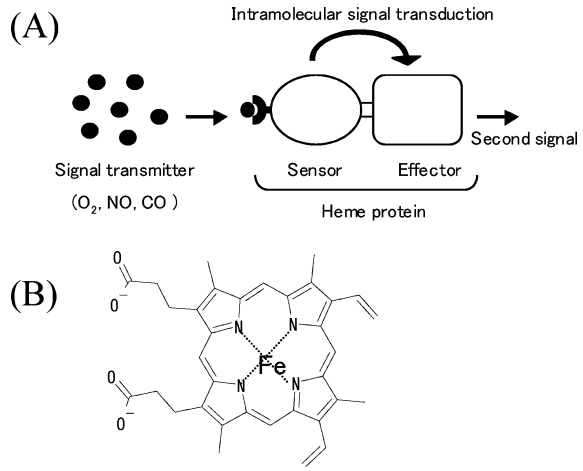

FIGURE 1. (A) The cellular signal transduction system mediated by heme sensor proteins. Binding of gases to the sensor domain induces structural changes in these proteins, which are transmitted to the effector domain, eliciting cellular signal transduction. (B) The molecular structure of Fe-protoporphyrin IX (heme).

Table 1. Examples of Heme Sensor Proteins

protein	ligand or signal	function
*Rm*FixL	O_2	histidine kinase: one heme-bound PAS domain
*Bj*FixL	O_2	histidine kinase: one heme-bound and one heme-free PAS domain
HemAT	O_2	methyl-accepting protein: globin fold
*Ax*PDEA1	O_2	cyclic-di-GMP phosphodiesterase: one heme-bound PAS domain
CooA	CO	transcriptional regulator
NPAS2	CO	transcriptional regulator: two heme-bound PAS domains
sGC	NO	guanylate cyclase
*Ec*DOS	O_2 or heme redox	phosphodiesterase: one heme-bound and one heme-free PAS domain

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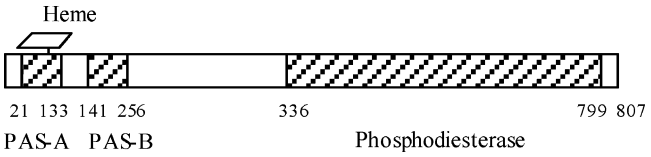

FIGURE 2. Domain structure of *Ec*DOS. The N-terminus of *Ec*DOS contains a tandem repeat of PAS structures, a FixL-like heme-containing PAS-A domain and a heme-free PAS-B domain. The *Ec*DOS C-terminal domain has homology to the enzymatic domain of *Ax*PDEA1, a phosphodiesterase specific for c-di-GMP.

important sensor proteins, such as an O_2 sensor from *Rhizobium* (FixL), an O_2 sensor from *Acetobacter xylinum* PDEA1 (AxPDEA1), neural PAS domain protein 2 (NPAS2), and a heme-regulated phosphodiesterase from *Escherichia coli* (*Ec*DOS).[1] "PAS" is an acronym formed from the names of the proteins in which imperfect repeat sequences were first recognized as follows: the *Drosophila* period clock protein (PER),[5] vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT),[6] and *Drosophila* single-minded protein (SIM).[7] The PAS domain is an important structural motif widely distributed from prokaryotes to mammals and has a characteristic protein fold with several α-helices flanking a five- or six-stranded antiparallel β-sheet scaffold. In Eukarya, the PAS domains are mainly involved in voltage-dependent ion channels and transcriptional factors that regulate the circadian rhythm, the response to hypoxia, and the response to toxins.[8] In bacteria and archaea, PAS domains are mainly found insensors of light, the redox state, and gases. These PAS-sensor proteins often have bound cofactors, such as chromophores, flavin adenine dinucleotide (FAD), [2Fe2S] clusters, and Fe-protoporphyrin IX (heme).[8]

FixL is an O_2-sensing protein from *Rhizobium* that contains a heme-bound PAS domain as a sensor.[9−11] The effector domain of FixL is a protein kinase that phosphorylates the FixJ protein, which regulates the expression of the nitrogen fixation gene. HemAT is the first heme sensor protein identified with a myoglobin-like fold, and it is involved in aerotaxis in bacteria and archaea.[12−14] *Ax*PDEA1 has a heme-bound PAS domain as a sensor, and it catalyzes the hydrolysis of cyclic-di-GMP (c-di-GMP), which is required for the activation of cellulose synthase in cellulose-producing bacteria. CooA and NPAS2 are both CO-sensing heme proteins. CooA is a transcriptional factor from *Rhodospirillum rubrum* that contains a CO-bound heme sensor domain and regulates the expression of the *coo* genes associated with CO-dependent growth.[15−17] NPAS2 is also a transcriptional factor, but unlike CooA, it contains two heme-bound PAS domains in its N-terminus, and it binds to DNA along with BMAL1 and regulates the circadian rhythm.[18] The heme NO sensor, soluble guanylate cyclase (sGC), converts GTP to cyclic GMP, which then acts as intracellular second messenger.[19−21]

*Ec*DOS was originally identified on the basis of sequence homology to the FixL protein.[22] The domain structure of *Ec*DOS is shown in Figure 2. The N-terminus of *Ec*DOS contains a tandem repeat of PAS structures, a FixL-like heme-containing PAS-A domain (residues 21−133), and a heme-free PAS-B domain (residues 141−256). Based on the amino acid sequence homology with the FixL protein, *Ec*DOS was speculated to be a heme O_2 sensor protein and was designated the *Escherichia coli* direct oxygen sensor. The *Ec*DOS C-terminal domain, however, is quite different from that of the FixL protein. It has homology to the enzymatic domain of *Ax*PDEA1, a phosphodiesterase specific for c-di-GMP. Due to recent interest in the heme sensor proteins, we investigated their sensing mechanisms.[23−34] In this Account, we review recent findings on the structure, function, and physiological role of *Ec*DOS.

Physicochemical Properties of *Ec*DOS

*Ec*DOS has been expressed in *E. coli,* and the physicochemical characteristics of the recombinant protein have been investigated. Wild-type full-length *Ec*DOS is tetrameric, but its isolated heme-containing PAS-A domain forms a dimer.[22,23] The PAS-A domains were obtained as a six-coordinate low-spin ferric heme complex.[22,23] CD spectroscopy revealed that it contains approximately 53% α-helix and 10% β-sheet. This α-helical content is more than that of FixL (37%)[35] but less than that of myoglobin (78%).[36] The redox potential of the heme was found to be +67 mV versus standard hydrogen electrode (SHE), indicating that the *Ec*DOS Fe(III) is relatively stable compared to other electron-transferring heme proteins, including cytochrome *c* (+260 mV vs SHE),[37] but reduced more easily than cytochrome b_5 (+3 mV),[38] sperm whale myoglobin (+59 mV),[39] and microsomal P450 (−310 mV).[40] Addition of saturated O_2, CO, and NO solutions to the Fe(II) complex produces the Fe(II)−O_2, Fe(II)−CO, and Fe(II)−NO complexes, respectively.[22,23] Compared to other heme sensor proteins, such as FixL and *Ax*PDEA1, the *Ec*DOS Fe(II)−O_2 complex is not stable to oxidation and gradually changes to the Fe(III) complex. On the other hand, the turnover number of Fe(II) *Ec*DOS is very low (0.15 min^{-1} with cAMP).[23] An *Ec*DOS fragment containing residues 540−807 was reported to hydrolyze c-di-GMP, a specific substrate of *Ax*PDEA1, *Ec*DOS's closest relative, with a turnover number of 100 min^{-1}.[41] However, the autoxidation rate of *Ax*PDEA1 is markedly lower than that of *Ec*DOS. The half-life of *Ax*PDEA1 Fe(II)−O_2 is more than 12 h, whereas that of *Ec*DOS is approximately 1 h.[42] The autoxidation is inappropriate for sensing the association and dissociation of O_2 molecules and, thus, for transmitting these signals to the functional domain. Therefore, *Ec*DOS is expected to be a sensor of the redox state rather than the O_2 concentration. On the other hand, the cytoplasm of *E. coli* is thought to be reductive, and Fe(III) *Ec*DOS may be easily reduced to the Fe(II) form to reproduce the O_2-bound Fe(II) form. Therefore, the *Ec*DOS Fe(II)−O_2 complex may be physiologically important, and further study is required to clarify its physiological roles.

Redox-Dependent Ligand Switching

Mutation of His77 of the isolated PAS-A domain abolishes heme binding,[23,24] suggesting that it is one of the axial ligands for heme. Based on the structure of other related

heme-bound PAS proteins, Met95 was expected to be another axial ligand for heme trans to His77.[43] UV−visible absorption and resonance Raman spectra indicate that the wild-type Fe(II) complex is in the six-coodinated low-spin state, whereas the Met95Ala mutant is in the five-coodinated high-spin state,[24,27] supporting the idea that Met95 is an axial ligand of the *Ec*DOS Fe(II) complex. This was also suggested by the finding that mutations of Met95 dramatically altered the redox potentials and association rates of O_2 and CO for the Fe(II) complex.[27,30] These Met95 mutations were thought to alter the environmental structure of the Fe(II) complex in heme. However, the Fe(III) complex of the Met95 mutants was in the six-coodinated low-spin state like wild-type protein. This result suggested that Met95 mutation did not alter the heme coordination structure in the Fe(III) form, indicating that Met95 is not a ligand for the Fe(III) complex.[24,27]

The crystal structure of the isolated PAS-A domain resolved these conflicting findings. It showed that heme redox-dependent ligand switching occurs in *Ec*DOS; the axial ligands of the Fe(II) complex are His77 and Met95, whereas Met95 is displaced by H_2O (or OH^-) when it is oxidized to the Fe(III) complex.[29] Heme oxidation/reduction is associated with global structural changes in the FG-loop region (residues 86−96), where Met95 is located, followed by switching of the heme ligand. Crystallographic analysis also showed that one subunit of the *Ec*DOS PAS-A dimer is an Fe(II)−O_2 complex and the other is an Fe(II)−Met95 complex,[44] confirming ligand-dependent alteration in the structure of *Ec*DOS. These profound structural changes cause alterations in the pattern of hydrogen bonding around the heme cofactor, as well as global movement of one subunit relative to the other, which may trigger movements of the catalytic domain that initiate catalysis.[29]

Similar switching of the heme axial ligand accompanied by a redox change or binding of an external ligand has been reported for other heme sensor proteins. For example, the heme CO sensor protein CooA also shows redox-dependent ligand switching; its Fe(II) complex has His77 and Pro2 as ligands, whereas the Fe(III) complex has Cys75 and Pro2 as ligands. In this case, Pro2 is replaced by exogenous CO molecules to allow DNA binding by the protein.[45] In the case of sGC, the axial ligand, His105, dissociates from the heme followed by binding of NO molecules, triggering the conversion of GTP to cGMP.[46] The ligand switching system found in *Ec*DOS is similar to but not consistent with these well-known heme sensor proteins but has the characteristic ligand-switching property of this newly discovered enzyme class.

Association of O_2 and CO with the Heme Pocket

The association and dissociation rate constants of *Ec*DOS are 1.9×10^{-3} μM^{-1} s^{-1} and 6.4×10^{-1} s^{-1} for O_2, and 8.1×10^{-4} μM^{-1} s^{-1} and 2.5×10^{-3} s^{-1} for CO, respectively.[30] These association rate constant values were remarkably lower than those of other heme-binding proteins, such as hemoglobin and myoglobin.[47] They are also lower than those of other heme sensor proteins with five-coordinated heme, such as sGC[21,48] or FixL,[49] probably because the dissociation of endogeneous ligand (Met95 of *Ec*DOS) is required prior to the association of exogenous gases. Photodissociation analyses have shown that photolytically dissociated diatomic gases (O_2, CO, NO) rebind to the heme of *Ec*DOS with a very high yield,[50] suggesting that its heme distal pocket has a very tight structure that inhibits the escape of the gases. The isolated heme-bound PAS-A domain had higher association rates and lower K_d values for O_2 and CO than those of the full-length enzyme, indicating that the C-terminal domain may hinder O_2 and CO binding by sterically or electrostatically blocking their access to channels.

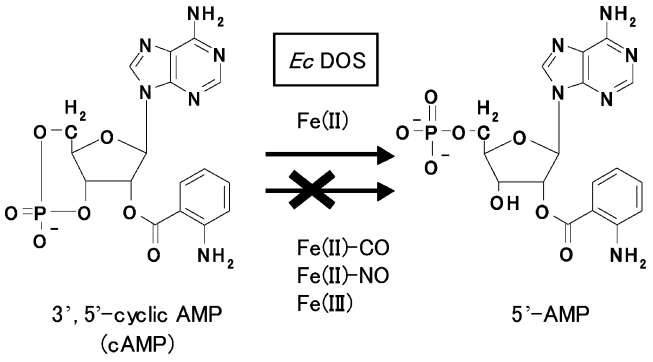

FIGURE 3. Phosphodiesterase activity of *Ec*DOS toward cAMP. The Fe(II) complex, but not the Fe(III) complex, of *Ec*DOS has cAMP phosphodiesterase activity. It is strongly inhibited by CO and NO.

Mutation of Met95 to alanine, leucine, or histidine dramatically enhanced its association rates for O_2 and CO.[30] The Fe(II) complexes of Met95Ala and Met95Leu are in the five-coodinated high-spin state. Therefore, dissociation of the endogenous ligand prior to the binding of exogenous gases is not necessary, which may increase the association rate. On the other hand, the Met95His Fe(II) complex is in the six-coodinated low-spin state because the inserted histidine residue can bind to the heme as an axial ligand. However, the affinities of methionine and histidine residues for the heme would be different, resulting in altered association rates for exogenous ligands.

Phosphodiesterase Activity

The Fe(II) complex, but not the Fe(III) complex, of *Ec*DOS has cAMP phosphodiesterase activity with a turnover rate of nearly 0.15 min^{-1} (Figure 3). In contrast, it does not show phosphodiesterase activity with cGMP as the substrate. This cAMP phosphodiesterase activity is optimal at pH 8.5 in the presence of Mg^{2+} and is strongly inhibited by CO, NO, and phosphodiesterase inhibitors.[23] We constructed several *Ec*DOS mutants to investigate which part of the enzyme is important for catalysis. Mutation of the heme proximal ligand, His77, to alanine and deletion of the heme-containing N-terminal PAS-A domain only slightly increased the phosphodiesterase activity, suggesting that the heme containing PAS-A domain is not crucial for catalysis but rather negatively regulates the catalytic activity of the C-terminal domain in the Fe(III) state.

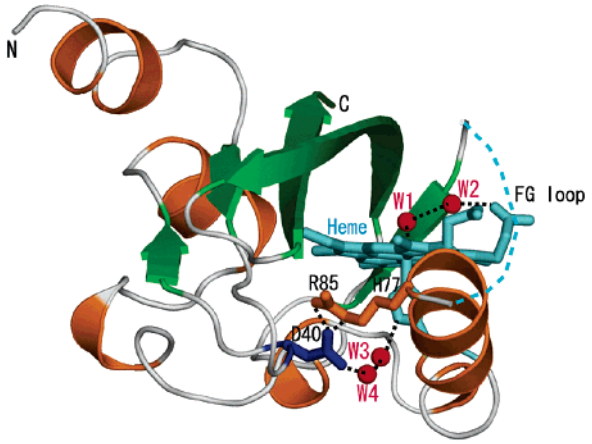

FIGURE 4. Structure of the Fe(III) *Ec*DOS PAS-A domain (PDB code IV9Y). Asp40 interacts with the imidazole ring of His77 via two water molecules (W3 and W4). Asp40 also forms a salt bridge with Arg85 at the surface of the molecule. The hydrogen bonds and flexible FG-loop are depicted by black dotted and cyan broken lines, respectively. The figure was obtained using PYMOL.[65] Reprinted with permission from ref 28. Copyright 2004 Blackwell Publishing Ltd.

Similarly, the three Met95 mutants (Met95Ala, Met95Leu, and Met95His) showed phosphodiesterase activity comparable to the wild-type enzyme, suggesting that the enzymatic activity is not affected by changes in the heme axial ligand or spin state. On the other hand, mutations at Asp40, which forms a salt bridge with Arg85 close to the FG-loop, abolished phosphodiesterase activity,[28] indicating that Asp40 plays an important role in the intramolecular signal transduction that activates catalysis. Several C-terminal deletion mutants were also constructed, and their phosphodiesterase activities toward cAMP were investigated. Interestingly, the mutant proteins that did not form tetramers were enzymatically inactive, suggesting that tetramer formation is essential for the phosphodiesterase activity. Based on the crystal structure of other phosphodiesterases,[51] His590 and His594 are expected to participate in the binding of Mg^{2+}, which is necessary for cAMP binding. Mutations of these histidine residues abolish enzymatic activity, supporting the idea that they are essential for cAMP binding.

Intramolecular Signal Transduction

*Ec*DOS phosphodiesterase activity is abolished when Asp40 is mutated to alanine or asparagine.[28] Structural analysis of *Ec*DOS indicates that Asp40 forms a hydrogen bond via two water molecules with the heme proximal ligand His77 and that it forms a salt bridge with Arg85 in the vicinity of the FG-loop (Figure 4). Asp40 mutants show an increased redox potential and autoxidation rate, which may be caused by disruption of the hydrogen bonding network involving Asp40.

Structural analysis also suggests that the FG-loop is rigid in the Fe(II) complex, whereas it is flexible in the Fe(III) complex. This change in flexibility may regulate the intramolecular signal transduction, thereby adjusting the catalytic activity. A similar mechanism has been reported for mammalian PAS-kinase, in which ligand binding to

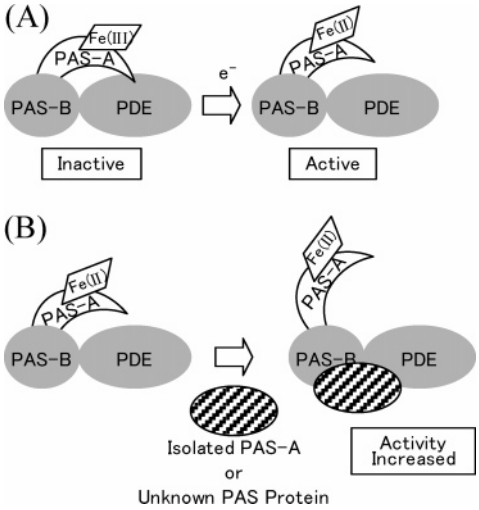

FIGURE 5. (A) Potential interaction between the catalytic domain and the Fe(III) heme-bound PAS domain of the full-length *Ec*DOS. This interaction does not exist in the active Fe(II) state. (B) Potential interaction between the catalytic domain of full-length *Ec*DOS and the isolated Fe(II) heme-bound PAS domain to facilitate catalysis. It is possible that in cells a simple heme-free PAS domain may interact with the catalytic domain of *Ec*DOS to promote catalysis.

the PAS domain causes a structural change in the FG-loop that results in the activation of catalysis.[52] In *Ec*DOS, mutations of Asp40 appear to break the salt bridge between Asp40 and Arg85. This may result in the increased flexibility in the FG-loop and disruption of the transduction of the signal that activates the enzymatic domain.

Interaction between the PAS-A Domain and the Functional Domain

The wild-type *Ec*DOS Fe(II) complex has phosphodiesterase activity, but the Fe(III) complex is catalytically inactive. Thus, it was speculated that direct interaction of the Fe(III) heme-bound PAS-A domain with the C-terminal domain inhibits catalysis, whereas reduction of the heme to Fe(II) suppresses this inhibition by disturbing this interaction (Figure 5A). The activity of heme-deficient mutants is slightly increased, supporting the hypothesis that the heme-binding PAS-A domain negatively regulates catalysis.

Intriguingly, addition of the isolated heme-containing PAS-A domain to the full-length Fe(II) enzyme increases phosphodiesterase activity 5-fold.[26] An in vitro binding assay[26] and protein microarray analysis[32] suggested that Fe(II) *Ec*DOS and the isolated PAS-A domain interact. Given these results, it appears that binding of the isolated PAS-A domain to full-length *Ec*DOS protein causes specific conformational changes in the phosphodiesterase domain that may alter its catalytic activity. The isolated PAS-A domain also bound to the ΔN147 mutant lacking the N-terminal heme-containing PAS-A domain,[26] suggesting a direct interaction between the isolated PAS-A domain and the catalytic domain of *Ec*DOS. In addition, protein microarray analysis showed that the *Ec*DOS phosphodiesterase substrate, cAMP, and the expected substrate-binding residues H590 and H594 are required for the

interaction between *Ec*DOS and the isolated PAS-A domain.[33] These results strongly suggest that the phosphodiesterase activity of *Ec*DOS is related to the interaction with the PAS-A domain.

The issue of why the isolated PAS-A domain can bind and activate *Ec*DOS remains unresolved. There are more than 3000 PAS proteins known in nature,[53] some of which consist of only a PAS domain, such as *E. coli* TraJ,[54] but little is known about their physiological functions. It is speculated (Figure 5) that such simple PAS proteins may, in general, play a physiological role in the regulation of protein partners in a manner similar to calcium-bound calmodulin.[55] Therefore, unknown proteins resembling the PAS-A domain in *E. coli* may bind to *Ec*DOS and enhance its activity (Figure 5B). Further studies are needed to understand the mechanism by which the isolated PAS-A domain alters the catalytic activity of *Ec*DOS and other protein partners.

Physiological Roles of *Ec*DOS

In *E. coli*, cAMP acts as an important second messenger for intracellular signal transduction. It binds to the cAMP receptor protein (CRP) and regulates the transcription of several proteins, for example, components of pathways for catabolic processes, flagellum synthesis, toxin production, DNA replication, and cell division.[56] Adenylate cyclase and cAMP phosphodiesterase control the cellular concentration of cAMP and thereby regulate transcriptional processes. Several mechanisms have been proposed for the response of *E. coli* to O_2 and its survival under O_2-deficient conditions. The heme redox-sensing protein *Ec*DOS is thought to sense the environmental O_2 concentration by modulating the cellular cAMP concentration, thus altering the transcription of specific proteins. Our results have indicated that the cellular cAMP concentration is increased in an *Ec*DOS knockout strain by 26-fold,[34] confirming that *Ec*DOS plays a key role in the control of the cAMP levels in *E. coli*.

*Ec*DOS possesses EAL and GGDEF domains in the C-terminal phosphodiesterase region. Each of them are composed of four conserved regions and have been named EAL and GGDEF according to a conserved sequence within the second and third of these regions, respectively. These domains are present in more than 1000 proteins involved in signal transduction in association with each other,[57,58] but their functions are not well understood. Recently, the EAL and GGDEF domains were shown to function as a phosphodiesterase[59,60] and a cyclase, respectively, for c-di-GMP,[61] a molecule originally discovered as a regulator of cellulose biosynthesis in *Acetobacter xylinum*.[62] Although it has been speculated that there are other downstream targets of c-di-GMP, the role of c-di-GMP in intracellular signal transduction is unclear.[63] *Ax*PDEA1, the closest relative of *Ec*DOS, is a c-di-GMP-specific phosphodiesterase with EAL and GGDEF domains.[57] *E. coli* also has an EAL protein, YahA,[41] and a GGDEF protein, YhcK,[64] which are expected to be a c-di-GMP phosphodiesterase and cyclase, respectively. Therefore, *Ec*DOS may also be involved in c-di-GMP hydrolysis. Schmidt et al. suggested that the isolated EAL domain of *Ec*DOS has c-di-GMP-specific phosphodiesterase activity, suggesting the possibility that it is a physiological substrate of *Ec*DOS.[41] Although it was reported that an increase in the cellular c-di-GMP concentration is accompanied by a change in colony morphology,[64] the function of c-di-GMP in *E. coli* is not completely understood. Therefore, further studies are needed to clarify the relationship between *Ec*DOS and c-di-GMP.

Concluding Remarks

Due to increasing interest in the heme sensor proteins, we carried out a series of studies investigating their sensing mechanisms. We examined the structures, functions, and physicochemical characteristics of the heme-regulated phosphodiesterase from *E. coli*, *Ec*DOS. The most distinctive property of this protein is distal-ligand switching that depends on the heme-redox state; its Fe(II) complex has His77 and Met95 as ligands, while Met95 is displaced by H_2O when it is oxidized to Fe(III). This type of ligand replacement is rare in other well-characterized heme sensor proteins; therefore, it may be a key aspect of the overall mechanism of *Ec*DOS, including redox sensing, intramolecular signal transduction, and catalytic activation. Although *Ec*DOS catalyzes the hydrolysis of cAMP and c-di-GMP, further studies are required to elucidate the physiological functions of *Ec*DOS in *E. coli*. Further characterization of site-directed mutants that have impact on catalysis and determinations of the crystal structures of full-length enzymes of *Ec*DOS may help us to understand the mechanism of the intramolecular signal transduction of this novel heme sensor enzyme.

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