The heme-regulated phosphodiesterase (PDE) from *Escherichia coli* (Ec DOS) is a tetrameric protein composed of an N-terminal sensor domain (amino acids 1–201) containing two PAS domains (PAS-A, amino acids 21–84, and PAS-B, amino acids 144–201) and a C-terminal catalytic domain (amino acids 336–799). Heme is bound to the PAS-A domain, and the redox state of the heme iron regulates PDE activity. In our experiments, a H77A mutation and deletion of the PAS-B domain resulted in the loss of heme binding affinity to PAS-A. However, both mutant proteins were still tetrameric and more active than the full-length wild-type enzyme (140% activity compared with full-length wild type), suggesting that heme binding is not essential for catalysis. An N-terminal truncated mutant (∆N147, amino acids 148–807) containing no PAS-A domain or heme displayed 160% activity compared with full-length wild-type protein, confirming that the heme-bound PAS-A domain is not required for catalytic activity. An analysis of C-terminal truncated mutants led to mapping of the regions responsible for tetramer formation and revealed PDE activity in tetrameric proteins only. Mutations at a putative metal-ion binding site (His-590, His-594) totally abolished PDE activity, suggesting that binding of Mg^{2+} to the site is essential for catalysis. Interestingly, the addition of the isolated PAS-A domain in the Fe^{2+} form to the full-length wild-type protein markedly enhanced PDE activity (>5-fold). This activation is probably because of structural changes in the catalytic site as a result of interactions between the isolated PAS-A domain and that of the holoenzyme.

Heme-bound phosphodiesterase (PDE)\(^1\) was cloned from *Escherichia coli* (Ec DOS) in our laboratory (1). Ec DOS is a heme sensor enzyme, because its PDE activity toward cAMP is regulated by the heme redox state. The enzyme is active in the Fe^{2+} heme-bound form but inactive in the Fe^{3+} heme-bound form (1). Full-length Ec DOS is composed of two domains, an N-terminal sensor domain and a C-terminal catalytic domain. The sensor domain is comprised of two PAS domains designated PAS-A (amino acids 21–84) and PAS-B (amino acids 144–201) (Fig. 1A). PAS is an acronym of *Drosophila* PER (period clock protein), vertebrate ARNT (aryl hydrocarbon receptor nuclear translocator), and *Drosophila* SIM (single-minded protein). To date, more than 1,100 PAS proteins have been identified (2–5). Despite only 30% homology between PAS proteins, PAS proteins exhibit a characteristic three-dimensional structure with a glove-like fold comprising four juxtaposed β-sheets and flanking α-helices (6–8). Heme is bound to the PAS-A domain in Ec DOS. Resonance Raman spectroscopy of the isolated heme-bound PAS-A domain discloses a characteristic heme coordination structure and environment within this enzyme (9).

Ec DOS is one of the new types of heme-bound proteins that have recently emerged as heme sensors or heme-regulated enzymes (5, 6, 8). Heme sensor enzymes such as soluble guanylate cyclase, FixL (an oxygen sensor heme protein of *Rhizobia meliloti*), CooA (a CO-sensing transcription factor from *Rhodosporillum rubrum*), and HemAT (a heme-containing aerotaxis transducer from *Bacillus subtilis*) contain heme-bound domains, and the heme of these enzymes binds gaseous ligands such as NO, CO, and O\(_2\), respectively. Ligand binding causes a conformational change in the heme environment that is transduced to a signal. The signal is transmitted to the functional domain following which activities such as guanylate cyclase, kinase, DNA binding, and chemotaxis are initiated or terminated in response. Ec DOS is a novel heme sensor enzyme, because the conformational change in the heme environment essential for signal transduction is induced by the redox state and not the association or dissociation of gaseous ligands, which is typical for other well known heme-sensor enzymes (1, 5, 6, 8).

The Gilles-Gonzalez group (10) previously showed that Ec DOS is a heme-regulated protein, assuming that this enzyme takes advantage of heme as an oxygen sensor, and designated it a “direct oxygen sensor from *E. coli*” (Ec DOS) based on the physico-chemical character of the isolated heme-bound PAS-A domain. An earlier study by our group showed that Ec DOS has a relatively fast autoxidation rate (1.5 \times 10^{-2} \text{ min}^{-1}) compared with hemoglobin and myoglobin and that PDE activity is regulated by the heme redox state but not by O\(_2\) binding to the heme iron (1). Another interesting characteristic is that full-length Ec DOS is tetrameric, whereas the isolated heme-bound PAS-A domain is dimeric. Heme-regulated enzymes such as FixL, CooA, and soluble guanylate cyclases are dimers (6–9), whereas HemAT is a tetramer (11). The well known hemopro-

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\(^1\) The abbreviations used are: PDE, phosphodiesterase; Ec DOS, a heme-regulated phosphodiesterase obtained from *E. coli*; PAS, an acronym formed from the names of *Drosophila* PER, ARNT, and *Drosophila* SIM; ant-cAMP, adenosine 3',5'-cyclic monophosphate,2'-o-anthraniloyl, NI-NTA, nickel-nitritriacetic acid.
tein, hemoglobin, is a tetramer, and O$_2$ affinity is allosterically regulated through the heme-proximal site via interactions between individual monomers (Refs. 12 and 13 and references therein). It would be interesting to analyze the role of heme in catalysis, architecture of the tetrameric protein molecule, the specific amino acid(s) or region(s) responsible for tetramer formation, and the mechanism by which the tetrameric state regulates PDE activity.

In this study, we generated six deletion mutants, specifically one N-terminal deleted, one PAS-B-deleted, and four C-terminal deleted proteins. A heme binding site mutant, H77A, and putative metal-ion binding site mutants, H590A, H594A, and H590A/H594A, were additionally examined. Relationships between heme binding affinity, tetramer formation ability, and PDE activity for each deletion mutant were studied. The PAS-B domain is necessary to maintain an appropriate heme-surfacing structure of the PAS-A domain. We mapped the regions responsible for tetramer formation, which appear important for catalysis. Our data show that heme binding is not essential for enzyme catalysis. Interestingly, the addition of the specific amino acid(s) or region(s) responsible for tetramer formation (Tokyo, Japan). The cloning vector, pBluescript SK II(+), was subcloned into pET28a(+). To generate H590A/H594A mutants were generated with a corresponding restriction sites of linearized pET28a(+)Ec DOS. H590A, H594A, and H590A/H594A mutants were generated with a QuikChange site-directed mutagenesis protocol (Stratagene) using pET28a(+)Ec DOS. H590A, H594A, and H590A/H594A mutants were generated with a QuikChange site-directed mutagenesis protocol (Stratagene) using pET28a(+)Ec DOS. H590A, H594A, and H590A/H594A mutants were generated with a QuikChange site-directed mutagenesis protocol (Stratagene) using pET28a(+)Ec DOS. H590A, H594A, and H590A/H594A mutants were generated with a QuikChange site-directed mutagenesis protocol (Stratagene) using pET28a(+)Ec DOS.

**EXPERIMENTAL PROCEDURES**

**Materials—Oligonucleotides were synthesized at the Nihon Gene Research Laboratory (Sendai, Japan) and ESPEC Oligo Service Corporation (Tokyo, Japan). The cloning vector, pBluescript SK II(+), and an expression vector, pET28a(+), were purchased from Toyobo (Osaka, Japan) and Novagen (Darmstadt, Germany), respectively. E. coli competent cells XL1-Blue (for cloning) and BL21 (for protein expression) were purchased from Novagen and Stratagene (La Jolla, CA), respectively. Taq DNA polymerase, dNTP mixtures, and other compounds necessary for PCR were acquired as a Takara Ex Taq™ kit (Takara Bio Inc., Otsu, Japan). Restriction and modifying enzymes for DNA recombination were purchased from Takara Bio Inc., Toyobo, New England Biolabs (Beverly, MA), and Nippon Roche K.K. (Tokyo, Japan). The fluorescence substrate, adenosine 3′,5′-cyclic monophosphate, 2′-o-anthraniloyl (ant-cAMP) was purchased from Calbiochem and Wako Pure Chemicals (Osaka, Japan). Calf intestine alkaline phosphatase was purchased from Takara Bio Inc. DEAE-Sephadex was obtained from Amersham Biosciences. Other chemicals were acquired from Wako Pure Chemicals.

**Construction of Expression Plasmids of Ec DOS mutants—Cloning of expression plasmids of the full-length wild-type Ec DOS, wild-type Ec DOS PAS-A, and H77A Ec Ec DOS PAS-A was expressed and ligated into the expression plasmid containing the remaining full-length wild-type Ec DOS regions using Munl and SacI sites. pET28a(+)ΔPAS-B Ec DOS was constructed by digesting Ec DOS at two PstI sites and ligating into the expression plasmid. ΔN147 and C-terminal deletion mutants were constructed by PCR using the primers listed in Table I. To generate ΔN147, the PCR product was digested with SacI and ligated into a cloning vector, pBluescript SKII(+). Following confirmation by sequencing, the appropriate DNA was subcloned into pET28a(+) using SacI. To construct C-terminal deletion mutants, PCR products digested with BamHI and SacI were ligated into pBluescript SKII(+). Plasmids authenticated by DNA sequencing were digested with BamHI and SacI and ligated into the corresponding restriction sites of linearized pET28a(+) Ec DOS.

**Expression and Purification of Ec DOS and the Isolated Ec DOS PAS Domain—Expression and purification procedures are described in an earlier report (1). Proteins were >95% homogeneous as confirmed by SDS-PAGE. Yields of Ec DOS and the isolated PAS domain from 1 liter of E. coli culture were 210 and 610 nmol, respectively, in terms of heme absorbance at 417 nm (1).

**Enzymatic Assay—Ec DOS was incubated at 37 °C with 0.1 mm ant-cAMP in a 500-μl reaction mixture containing 50 mm Tris-HCl buffer (pH 8.5) and 2 mm MgCl$_2$ under anaerobic conditions as described previously (1). All of the proteins were normalized to a concentration of 0.5 μM. Reduction of the wild-type and mutant proteins was performed by treatment with a trace amount of sodium dithionite. Activities are expressed as nmol product/h/mg protein. At least four experiments were performed to obtain each value. Experimental errors were <20%.

**Gel Filtration—Gel filtration analyses were performed using the AKTA liquid chromatography system equipped with a Superdex 200 HR 10/30 column (Amersham Biosciences). The buffer employed for gel filtration of Fe$^{2+}$ or unreduced protein comprised 50 mm Tris-HCl (pH 7.5), 0.15 m NaCl, and 1 mM diithiothreitol. To determine oligomeriza-

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**TABLE I**

**PCR primers used for the generation of N- and C-terminal truncated and His-590 and His-594 mutant expression constructs**

| Construct | Primer name | Sense primer | Antisense primer | Amino acid number |
|-----------|-------------|--------------|------------------|------------------|
| Wild type | DOS-1       | NdeI         | DOS-3            | 1–807            |
| ΔN147     | N147        | SacI         | DOS-3            | 148–807          |
| A         | DOS-1       | NdeI         | A                | 1–401            |
| B         | DOS-1       | NdeI         | B                | 1–491            |
| C         | DOS-1       | NdeI         | C                | 1–605            |
| D         | DOS-1       | NdeI         | D                | 1–705            |
| H590A     | H590A       | c-H590A      | c-H590A          | 1–807            |
| H594A     | H594A       | c-H594A      | c-H594A          | 1–807            |
| H590A/H594A | H590A/H594A | c-H590A/c-H594A | c-H590A/c-H594A |

Underlined bases signify restriction sites. Bases in italics indicate where mutations were introduced.
tion of the full-length wild-type enzyme in the Fe$^{2+}$ state, protein reduced with excess sodium dithionite was applied to the column equilibrated with the above buffer containing 10 mM sodium dithionite. The molecular masses of the protein peaks were estimated relative to those of standard proteins, specifically ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa).

**Optical Absorption Spectra**—Spectral experiments under aerobic conditions were performed on Shimadzu UV-1650, UV-2500, and Hitachi U-2010 spectrophotometers maintained at 25 °C with a temperature controller. Anaerobic spectral experiments were conducted on a Shimadzu UV-1600A spectrophotometer in a glove box. Following the reduction of heme using sodium dithionite, excess dithionite was removed with a Sephadex G25 column in the glove box. To ensure that the solution temperature was consistent, the reaction mixture was equilibrated in the spectrophotometer for 10 min prior to spectrometric measurements.

**Interactions between Full-length Wild-type Protein and the Isolated PAS-A Domain**—To examine interactions between full-length wild-type or ΔN147 protein and the isolated PAS-A domain, we performed an in vitro binding assay using Ni-NTA-agarose beads. Full-length wild-type and ΔN147 were reduced by treatment with sodium dithionite and incubated in Ni-NTA-agarose equilibrated with a reaction mixture of 500 μl containing 50 mM Tris-HCl buffer (pH 8.5), 2 mM MgCl$_2$, and 0.1 mM CAMP-Na in a microcentrifuge tube on ice for 1 h under anaerobic conditions. The protein-Ni-NTA mixture was packed into a small empty column, and the flow-through was discarded. The (His)$_6$-tag of PAS-A was removed by thrombin digestion and reduced by sodium dithionite treatment. Reduced non-tagged PAS-A was loaded onto the column, which was washed with the same buffer containing 20 mM imidazole to remove unbound proteins. Full-length wild-type/PAS-A binary and ΔN147/PAS-A binary complexes were eluted with the above buffer containing 160 mM imidazole. Non-tagged PAS-A was loaded on a Ni-NTA column in the absence of full-length wild-type or ΔN147 protein. The fraction, eluted with 160 mM imidazole buffer following the above procedure, was additionally analyzed as a negative control to confirm that it does not bind Ni-NTA agarose. All of the steps were performed under anaerobic conditions, similar to enzymatic reactions. The eluate was analyzed by SDS-PAGE.

**RESULTS**

**Deletion and Site-directed Mutagenesis**—We generated deletion and site-directed mutants to determine the role of heme in catalysis and tetramer formation, architecture of the tetrameric protein molecule, the specific amino acid(s) or region(s) responsible for tetramer formation, PDE catalytic site, and the mechanism by which the heme and tetrameric state regulates PDE activity. Six deletion mutants were generated as described under “Experimental Procedures.” The PAS-B domain (amino acids 100–246) was deleted in a construct designated ΔPAS-B (Fig. 1A and Table II). A truncated mutant, denoted ΔN147, contained no N-terminal PAS domain (amino acids 148–807). C-terminal truncated mutant proteins (designated A, B, C, and D) contained amino acids 1–401, 1–491, 1–605, and 1–705, respectively. Electrophoresis patterns of these deletion mutant proteins revealed molecular mass positions consistent with those expected from the amino acid sequences (Fig. 1B). We additionally constructed a H77A mutant of the full-length enzyme in view of the finding that the same mutant of the isolated PAS-A domain loses heme binding capability (1). H590A, H594A, and H590A/H594A mutants of the full-length enzyme were additionally constructed (Fig. 1B), because His-590 and His-594 are speculated to be metal-ion binding sites based on the amino acid sequence of corresponding human PDE4 (15).

**Optical Absorption Spectra**—To determine whether heme binds to mutant proteins, optical absorption spectra in the near UV (near 280 nm), Soret (near 417 nm), and visible (between 460 and 700 nm) regions were obtained for all of the mutants. Absorption at 280 and 417 nm was measured to evaluate the amount of heme bound to mutant proteins (Table II). Spectra of the Fe$^{2+}$ complexes of full-length wild-type protein and non-reduced ΔPAS-B are depicted in Fig. 2. From these spectra, it is clear that heme binds to full-length wild-type enzyme but not to ΔPAS-B. The heme binding abilities of the proteins are summarized in Table II. ΔPAS-B and ΔN147 mutants lost heme binding...
capability and did not display heme absorption at around 400 nm. The C-terminal truncated mutants (A, B, C, and D) and mutants of metal-ion binding sites (H590A, H594A, and H590A/H594A) efficiently bound heme as calculated from the intensity ratio of absorption at 280 and 417 nm of purified proteins (Table II). As expected, the H77A mutant of full-length protein displayed no heme binding ability, similar to the H77A mutant of isolated PAS-A (data not shown) (1).

**Gel Filtration Chromatography**—It is important to determine whether the tetrameric state of full-length wild-type protein is disrupted or maintained following extensive deletion and site-directed mutagenesis. We examined the tetramer formation state by gel filtration chromatography (Fig. 3, A and B). Separation between tetramer, dimer, and monomer states was satisfactory under our experimental conditions, and the oligomeric state was thus determined using molecular masses of standard proteins. As summarized in Table II, H77A, ΔPAS-B, ΔN147 A and C, H590A, H594A, and H590A/H594A mutants were tetrameric in the unreduced forms, whereas B and D mutants were monomeric. We additionally examined whether the tetrameric state is conserved in the Fe^{2+} complex, because the enzyme is active in this state. The full-length wild-type Fe^{2+} complex was a tetramer, similar to the Fe^{3+} complex (cf. legend for Fig. 3).

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### Table II

Deletion and site-directed mutants analysed in this study

| Proteins     | Amino acid | State | 280/417<sup>a</sup> | Heme       | Activity<sup>b</sup> | Reduced<sup>c</sup> | Unreduced<sup>c</sup> |
|--------------|------------|-------|---------------------|------------|-----------------------|----------------------|------------------------|
| Full-length  | 1–807      | Tetramer | 1.0 | + | 100 | <20 |
| PAS-A        | 1–147      | Dimer   | 0.6 | + | <20 | <20 |
| H77A         | 1–807      | Tetramer | >10 | - | 140<sup>d</sup> | 110<sup>d</sup> |
| ΔPAS-B       | 1–99 + 247–807 | Tetramer | >10 | - | 140<sup>d</sup> | 125<sup>d</sup> |
| ΔN147        | 148–807    | Tetramer | >10 | + | <20 | <20 |
| A            | 1–401      | Tetramer | 1.4 | + | <20 | <20 |
| B            | 1–491      | Monomer | 1.1 | + | <20 | <20 |
| C            | 1–605      | Tetramer | 1.2 | + | 50 | <20 |
| D            | 1–705      | Monomer | 1.4 | + | <20 | <20 |
| H590A        | 1–807      | Tetramer | 0.7 | + | <20 | <20 |
| H594A        | 1–807      | Tetramer | 0.9 | + | <20 | <20 |
| H590A/H594A  | 1–807      | Tetramer | 0.8 | + | <20 | <20 |

<sup>a</sup> Intensity ratio of absorption at 280 and 417 nm.

<sup>b</sup> Activities were obtained from at least four experiments and presented as average values. Experimental errors were <20%.

<sup>c</sup> “Reduced” indicates that proteins were treated with sodium dithionite and employed under anaerobic conditions. In these cases, heme-containing mutants were in the Fe^{2+} form. “Unreduced” signifies proteins not treated with reductants and used under aerobic conditions. Therefore, heme-containing mutants are in the Fe^{3+} form.

<sup>d</sup> Although H77A, ΔPAS-B, and ΔN147 mutants do not bind heme, activities were compared with and without treatment of sodium dithionide.
PDE Activity—PDE activity is observed when heme is in the Fe$^{3+}$ state but not when heme is in the Fe$^{2+}$ state (1). However, the heme of Ec DOS is easily oxidized, because autoxidation of heme of full-length Ec DOS is as fast as $1.5 \times 10^{-2}$ min$^{-1}$ and the redox potential of heme (of the isolated PAS domain) of Ec DOS is $+67$ mV (1). Therefore, PDE activities were obtained for the full-length wild-type and mutant proteins under anaerobic conditions following reduction of the enzyme with sodium dithionite. The Fe$^{3+}$ state of heme-bound mutants was maintained during catalysis as confirmed by optical absorption spectra of the final solutions. The H77A mutant, which did not contain heme, displayed PDE activity (140% compared with 100% full-length wild-type enzyme activity) (Table II) under anaerobic conditions. The ΔPAS-B and ΔN147 mutants displayed activities of 140 and 160%, respectively. The C-terminal truncated mutant (c) displayed almost 50% PDE activity compared with the full-length wild-type enzyme. No PDE activity was observed with three other C-terminal truncated mutants, A, B, and D, and metal-ion binding site mutants, H590A, H594A, and H590A/H594A. PDE activities of full-length wild-type and all of the mutant proteins were additionally examined under aerobic conditions in the absence of sodium dithionite. Under these conditions, all of the heme-bound mutants were in the Fe$^{3+}$ form. The H77A, ΔPAS-B, and ΔN147 mutants displayed 110, 125, and 160% activity, respectively, under aerobic conditions, whereas Fe$^{3+}$ full-length wild-type enzyme activity was not observed, in contrast to the Fe$^{2+}$ full-length wild-type enzyme.

Effect of the Isolated PAS-A Domain on PDE Activity—To elucidate the role of the PAS-A domain in catalysis, we examined whether PDE activity is affected upon addition of the isolated PAS-A domain. Surprisingly, PDE activity of the full-length wild-type enzyme was markedly increased (5-fold) following incorporation of the isolated Fe$^{2+}$ PAS-A domain (Fig. 4A). In contrast, PDE activity of the ΔN147 mutant was not altered in the presence of the isolated Fe$^{2+}$ PAS-A domain (Fig. 4B). Upon addition of the isolated Fe$^{3+}$ PAS-A domain, full-length wild-type activity was not altered (Fig. 4A), whereas the activity of ΔN147 was abolished (Fig. 4B). With the isolated heme-free PAS-A domain (H77A mutant), activities of both proteins remained unaffected (Figs. 4, A and B). As a control, we examined the effects of bovine serum albumin on PDE activity. The addition of bovine serum albumin to the full-length wild-type and ΔN147 enzymes had no effects on activity, suggesting that PDE activation by the isolated Fe$^{2+}$ PAS-A domain and catalytic inhibition by the isolated Fe$^{3+}$ PAS-A domain are specific. We additionally examined the effects of Fe$^{3+}$ and Fe$^{2+}$ hemin complexes. No effects of free hemin per se on catalysis by the full-length wild-type protein were observed, suggesting that activation is because of the PAS-A protein molecule containing Fe$^{2+}$ heme but not heme itself.

We next examined the interactions of full-length wild type or ΔN147 with the isolated PAS-A domain using the batch method as described under “Experimental Procedures.” In each lane, two bands corresponding to full-length wild-type or ΔN147 protein and PAS-A were observed (Fig. 5, lanes 2 and 3). Accordingly, we suggest that Fe$^{2+}$ PAS-A interacts with both full-length wild-type and ΔN147 proteins that bind to Ni-NTA-agarose beads. Unreduced PAS-A and reduced full-length wild type or ΔN147 did not interact with each other. Therefore, only reduced full-length wild-type and ΔN147 enzymes interact with the isolated Fe$^{2+}$ PAS-A. Interactions of Fe$^{3+}$ PAS-A with both proteins are evidently specific, because Fe$^{3+}$ PAS-A does not bind to Ni-NTA-agarose beads itself (Fig. 5, lane 4).

Effects of the (His)$_6$-tag on Protein Spectra and Activities—Proteins were expressed with the (His)$_6$-tag at the N-terminal end. To determine the effect of (His)$_6$ on spectra and activities, thrombin treatment was performed on full-length wild-type protein to remove the tag. Spectra, oligomeric state, degradation state, and catalytic activity of the full-length wild-type protein in the absence of (His)$_6$-tag were similar to those in the presence of the tag.

**DISCUSSION**

Heme binding does not correlate with the tetrameric state and PDE activity of Ec DOS. The ΔPAS-B mutant does not contain heme similar to the H77A mutant, suggesting that the PAS-B domain is required to modulate the appropriate structure of PAS-A for maintaining heme binding. Interestingly, under anaerobic conditions, all of the heme-deficient mutants formed tetramers and displayed 140 or 160% activity compared with the full-length wild-type enzyme. Our results indicate that heme binding is not essential for tetramer formation and catalytic activity of Ec DOS. The N-terminal deletion mutant, ΔN147, which does not contain the PAS-A domain, was tetrameric with 160% activity, suggesting that the entire N-terminal domain is not essential for activity. All of the heme-deficient mutants displayed significant activity even under aerobic conditions, whereas no activity was observed with the C-terminal deleted mutants and full-length wild-type enzyme. This reduction had no
effect on activity with H77A and heme-deficient mutants. The results suggest that the heme domain containing Fe\(^{3+}\) inhibits catalysis. Moreover, reduction of the heme iron may induce a conformational change that suppresses this inhibition (Fig. 7, A and B). A similar negative effect of heme on the catalysis of soluble guanylate cyclase was reported recently (14).

The mutants A, ΔPAS-B, and ΔN147 are oligomeric, suggesting that the PAS-B domain and N-terminal region between positions 100 and 247 are not critical to maintain the tetrameric state of the enzyme and that the region between 247 and 401 is important for tetramerization (Fig. 6). This domain may be crucial to maintain the dimeric form of isolated PAS-A. Amino acid residues necessary for the maintenance of the appropriate tetrameric state appear to be located in two regions, specifically between positions 491 and 605 and positions 705 and 807 (Fig. 6), because mutants B and D are monomeric, whereas mutant C is tetrameric. These two regions may contribute more significantly to overall protein architecture than the PAS-A domain, because deletion of these two regions at the C-terminal site resulted in complete dissociation of the tetramer to monomer.

The intensity ratios of absorption at 280 and 417 nm may be used to estimate the extent of heme binding to the protein (Table II). If this ratio is increased upon mutation, heme binding affinity to the protein should be lowered. Among the C-terminal truncated mutants, mutant A with an intensity ratio of 1.4 was still tetrameric. The same ratio was observed for the terminal truncated mutants, mutant A with an intensity ratio of 1.1 (similar to the full-length wild-type protein) (Fig. 4) and 1.2 was tetrameric. H77A and ΔPAS-B contained no heme but were tetramers. Thus, there appears to be no clear relationship between the heme content and oligomeric state. However, the oligomeric states appear to correlate with PDE activity (Table II). Specifically, deletion mutants B and D were monomeric and showed no PDE activity, whereas the tetrameric deletion mutant C exhibited PDE activity. However, the tetrameric deletion mutant A displayed no PDE activity. Accordingly, we propose that the PDE catalytic site is located between residues 402 and 605.

The crystal structures of the catalytic domains of several mammalian cAMP and cGMP PDE have been solved (15–17). Two histidines in the metal-ion binding motif, HXXXH (X\(_{25–30}\)E, have a critical role in catalysis in mammalian PDE (18, 19). A model has been proposed from the crystal structure of human cAMP-specific PDE4B2B in which cAMP is bound to histidine in the motif via two metal-ions (either Mg\(^{2+}\) or Mn\(^{2+}\)) (15). In human cGMP-specific PDE5, these conserved histidines are involved in coordinating Mg\(^{2+}\) and Zn\(^{2+}\) and positioning the substrate for efficient catalysis (16). Ec DOS requires Mg\(^{2+}\) for catalytic activity (1) and contains this consensus metal-ion binding motif. Mutation of both or either of the two histidines (His-590 and His-594) in the motif abolishes catalytic activity (Table II). This is not the result of misfolding or denaturing because data from the gel filtration assay indicate that these mutants form tetramers. Therefore, we propose that the two histidines in the motif play an important role in Mg\(^{2+}\) binding and CAMP binding of Ec DOS.

At present, it is difficult to interpret the reason why the addition of the isolated Fe\(^{3+}\) PAS-A domain enhances PDE activity of the full-length wild-type protein (Fig. 4A) but not that of ΔN147 (Fig. 4B). The domain containing the Fe\(^{3+}\) heme inhibits catalysis by the C-terminal domain in the holoenzyme. A redox change to Fe\(^{2+}\) heme suppresses this inhibition (Fig. 7A). A further increase in the activity of heme-deficient mutants suggests that Fe\(^{3+}\) heme binding to the N-terminal domain induces unfavorable conformational changes for catalysis. The addition of the
isolated PAS-A domain in the Fe$^{2+}$ form alters the unfavorable conformational structure of the catalytic site in the C-terminal domain created by the covalently bound N-terminal domain, resulting in catalytic activation. Therefore, a specific conformational change of the PDE active site is induced upon binding of the isolated PAS-A domain to full-length wild-type protein (Fig. 7A). A similar negative effect of an isolated PAS domain on the catalysis of serine/threonine kinase was reported previously (4). On the other hand, the isolated PAS-A domain interacts with the ΔN147 mutant lacking the entire N-terminal domain (Fig. 5). Binary complexes of isolated Fe$^{2+}$ PAS-A and apoPAS-A with the ΔN147 mutant do not affect activity, whereas isolated Fe$^{3+}$ PAS-A impedes catalysis with the ΔN147 mutant (Figs. 4B and 7B). The solution structure of the PAS kinase N-terminal domain (determined by NMR) implies that regions binding organic compounds (or heme) in the PAS domain and catalytic segments are functionally and structurally linked by a flexible loop between the two segments (3). Therefore, direct interactions between the isolated PAS-A domain and catalytic domain in Ec DOS in the presence of excess PAS-A may enhance catalysis via conformational changes distinct from those in the connected enzyme. Crystal structure studies on the FixL heme domains with PAS indicate that binding of oxygen to the distal site of the heme results in structural alterations in the porphyrin ring that are relayed to the FG loop (6–8). However, in the present case, it is unlikely that this type of structural change near the heme in the full-length wild-type enzyme occurs to facilitate signal transduction and enhance catalysis. Further studies are required to elucidate the catalytic alterations induced by adding the isolated PAS-A domain to the enzyme.

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