Characterization of a Direct Oxygen Sensor Heme Protein from *Escherichia coli*

EFFECTS OF THE HEME REDOX STATES AND MUTATIONS AT THE HEME-BINDING SITE ON CATALYSIS AND STRUCTURE

Received for publication, March 21, 2002, and in revised form, April 16, 2002. Published, JBC Papers in Press, April 22, 2002.

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A protein containing a heme-binding PAS (PAS is from the protein names in which imperfect repeat sequences were first recognized: PER, ARNT, and SIM) domain from *Escherichia coli* has been implied a direct oxygen sensor (Ec DOS) enzyme. In the present study, we isolated cDNA for the Ec DOS full-length protein, expressed it in *E. coli*, and examined its structure-function relationships for the first time. Ec DOS was found to be tetrameric and was obtained as a 6-coordinate low spin ferric heme complex. Its α-helix content was calculated as 53% by CD spectroscopy. The redox potential of the heme was found to be +67 mV versus SHE. Mutation of His-77 of the isolated PAS domain abolished heme binding, whereas mutation of His-83 did not, suggesting that His-77 is one of the heme axial ligands. Ferrous, but not ferric, Ec DOS had phosphodiesterase (PDE) activity of nearly 0.15 min⁻¹ with cAMP, which was optimal at pH 8.5 in the presence of Mg²⁺ and was strongly inhibited by CO, NO, and etazolate, a selective cAMP PDE inhibitor. Absorption spectral changes indicated tight CO and NO bindings to the ferrous heme. Therefore, the present study unequivocally indicates for the first time that Ec DOS exhibits PDE activity with cAMP and that this is regulated by the heme redox state.

Heme proteins and enzymes perform a broad range of functions. Well known examples include O₂ storage with myoglobin, O₂ carriage with hemoglobin, mediators of electron transfer with cytochromes, and catalytic activation of heme ligands with P450s and peroxidases (1–3). Recently, a new class of heme enzymes involved in intramolecular signal transduction is emerging, known as heme-based sensors (4–6). Almost of all the heme-based sensors contain two different functional domains as follows: one is an N-terminal heme domain, which acts a sensor, and the other is a catalytic domain such as a histidine kinase or a soluble guanylate cyclase. These heme sensor enzymes use the heme for mediating transcriptional and regulatory events associated with the presence of gaseous molecules such as CO, NO, and O₂ (4–6). In these enzymes, the ligand association or dissociation from the heme iron leads to protein conformational changes, which transmit signals to the other domain where they initiate catalytic function or DNA binding. For example, the CooA¹ protein from *Rhodospirillum rubrum* is a CO sensor heme protein that regulates the expression of the coo genes associated with CO-dependent growth (Refs. 7 and 8 and references therein). Soluble guanylate cyclase is an NO sensor heme protein that regulates conversion of 5'-GTP to the intracellular second messenger, cGMP (Refs. 9 and 10 and references therein). Hem-AT-Bs and Hem-AT-Hs are oxygen sensors in which the hemes are thought to mediate signal transduction for methylation of the chemotaxis proteins (11, 12).

The Fix proteins, FixL and FixJ, of *Rhizobium meliloti* are well characterized as biological oxygen sensors and regulate the expression of the nitrogen fixation genes of a plant symbiotic bacterium, *Sinorhizobium meliloti* (Refs. 13 and 14 and references therein). Dissociation of O₂ from FixL (Fe(II)) heme initiates its histidine kinase function in another subunit in FixL. The phosphate group transfers to an Asp residue of FixJ. Phosphorylated FixJ then acts as a transcriptional activator of the *nifA* and *fixK* genes, which controls the expression of nitrogen fixation genes and a high affinity terminal oxidase complex, respectively. The N-terminal portion of FixL consists of a heme sensory domain whose sequence and tertiary structure characterize it as a PAS domain. 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) (15), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT) (16), and *Drosophila* single-minded protein (SIM) (17).

Based on the sequence homology of the heme sensor enzyme, FixL, Gilles-Gonzales group identified a heme domain with a PAS sequence from *Escherichia coli* (designated Ec DOS for *E. coli* direct oxygen sensor) (18). They characterized the physicochemical properties of the isolated heme domain of Ec DOS. However, they did not report cloning of the full-length protein or characterize its catalytic function. The same clones cloned a phosphodiesterase (PDE) A1 protein from *Acetobacter xylinum* (designated AxPDEA1) and found that it is a key regulator of bacterial cellulose synthesis (19). AxPDEA1 linearizes cyclic

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¹ The abbreviations used are: CooA, a CO-sensing protein of *R. rubrum*; FixL, an oxygen sensor heme protein of *R. meliloti*; Ec DOS, full-length wild type of a direct oxygen sensor obtained from *E. coli*; PDE, phosphodiesterase; AxPDEA1, a phosphodiesterase A1 protein of *A. xylinum*; ant-cAMP, adenine 3’,5’-cylic monophosphate, 2’-o-anthraniloyl; etazolate, 1-ethyl-4-[[1-(methylthio)ethyl]ene]hydrazino]-1H-pyrazolo[3,4-b]pyridine-5-carboxylic acid ethyl ester; SHE, standard hydrogen electrode; Ni-NTA, nickel-nitrilotriacetic acid.

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Characterization of a Direct Oxygen Sensor Heme Protein

The genomic DNA of E. coli JM109 strain was purchased from Takara Shuzo Co. (Kyoto, Japan). Oligonucleotides were synthesized at Research buffer laboratory (Tokyo, Japan). GENSERT OLIGOS (Sendai, Japan), and ESPECO OLIGO Service Corp. (Tokyo, Japan). Cloning vector, pBluescript SK II (λ) (purchased from Takara Shuzo Co., and Novagen (Darmstadt, Germany), respectively. E. coli competent cells, JM109, XLI-Blue (for cloning), and BL21 (for expression of the protein) were purchased from Takara Shuzo Co., Novagen, and Stratagene (La Jolla, CA), respectively. Taq DNA polymerase, dNTP mixture, and other compounds necessary for PCR were purchased as a Takara Ex TaqTM kit from Takara Shuzo Co. Restriction enzyme, ligase, dNTP mixture, and other compounds necessary for PCR were purchased as a Takara Ex TaqTM kit from Takara Shuzo Co. Restriction enzyme sites. Oligonucleotides between these sites, which introduce a NdeI restriction enzyme site, were designed to contain the heme protein motifs. The reaction mixture containing the heme protein motifs were pooled and concentrated. Before using the protein, imidazole was removed on a Sephadex G-25 gel filtration column. For spectral experiments, DOS-PAS protein was further purified by a gel filtration column of Sephadex G-75 (16 × 60 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.5) and 2 mM MgCl2. To terminate the reaction, DOS was removed by using a Ultrafree-MC centrifugal filter (Millipore Co., Bedford, MA), and 2 units of alkaline phosphatase (Takara Syuzo Co.) was added and incubated for 1 h at 37 °C. The reaction mixture was applied to a column of DEAE-Sepharose and washed with water. Finally, the fluorescence intensity (excitation at 330–350 nm, emission at 430–450 nm) of the eluted fraction was measured to determine the amount of product (20, 21). At least four experiments were conducted to obtain each value; experimental errors are less than 20%.

Four assays for bis(p-nitrophenyl) phosphate, and p-nitrophenyl phosphate were carried out in a solution containing 50 mM Tris-HCl buffer (pH 8.5) and 2 mM MgCl2. To terminate the reaction, DOS was removed by using an Ultrafree-MC centrifugal filter (Millipore Co., Bedford, MA), and 2 units of alkaline phosphatase (Takara Syuzo Co.) was added and incubated for 1 h at 37 °C. The reaction mixture was applied to a column of DEAE-Sepharose and washed with water. Finally, the fluorescence intensity (excitation at 330–350 nm, emission at 430–450 nm) of the eluted fraction was measured to determine the amount of product (20, 21). At least four experiments were conducted to obtain each value; experimental errors are less than 20%.

Enzymatic Assay—E. coli DOS was incubated at 37 °C with ant-cAMP or ant-cGMP in a reaction mixture of 500 µl containing 50 mM Tris-HCl buffer (pH 8.5) and 2 mM MgCl2. To terminate the reaction, DOS was removed by using an Ultrafree-MC centrifugal filter (Millipore Co., Bedford, MA), and 2 units of alkaline phosphatase (Takara Syuzo Co.) was added and incubated for 1 h at 37 °C. The reaction mixture was added to a column of DEAE-Sepharose and washed with water. Finally, the fluorescence intensity (excitation at 330–350 nm, emission at 430–450 nm) of the eluted fraction was measured to determine the amount of product (20, 21). At least four experiments were conducted to obtain each value; experimental errors are less than 20%.

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**RESULTS**

**Optical Absorption Spectra**—Optical absorption spectra of Fe(III), Fe(II), Fe(II)-CO, Fe(II)-NO and Fe(II)-O_2 complexes of Ec DOS are shown in Fig. 1 and summarized in Table I. The Ec and Fe(II) complexes of enzyme, FixL, in which both Fe(III) and Fe(II) complexes are DOS PAS domain (18). The Ec DOS PAS domain has a Soret band at 428 nm and well-formed O_2 solution to the Fe(II) complex. This had an absorption at 417 nm, similar to that of the isolated PAS domain (18). The Fe(II)-O_2 complex was unstable and gradually changed the Fe(III) complex. Autodissociation of the Ec DOS Fe(II)-O_2 complex to the Fe(III) complex as monitored at 579 nm was composed of a single phase with rate constant about 1.5 x 10^{-2} min^{-1}.

In order to identify the axial ligands, the effect of modulating pH on the absorption bands was examined. When the pH of the wild type solution was varied from neutral to acidic, heavy precipitates were formed. On the other hand, isolated Ec DOS PAS domain was stable but was insensitive to changes in pH from 5 to 9.5. Clear decreases in the intensity of the absorption spectra of the PAS domain were observed outside this range (not shown). Denaturation rather than axial ligand substitution is probably responsible for these effects.

**CD, Fluorescence, and Redox Potential**—From the ultraviolet region of the CD spectrum (Fig. 2A), the α-helix content of Ec DOS was estimated as 54% (CONTINLL program; 53% by the SELCON3 program; 56% by the CDSSTR program). β-Sheet content was estimated to be about 10%. Reduction of the heme iron did not change the CD spectrum in the ultraviolet region. The Soret CD band of the resting Fe(III) complex was located at 421 nm on the plus side with Δε = 8 x 10^{4} M^{-1} cm^{-1} (Fig. 2B). Reduction by sodium dithionite shifted the band position to 425 nm and markedly increased the CD intensity up to Δε = 2 x 10^{5} M^{-1} cm^{-1}. The Fe(II)-CO complex had a Soret band at 431 nm on the plus side with Δε = 1 x 10^{5} M^{-1} cm^{-1}.

Ec DOS has 16 Trp residues, whereas Ec DOS PAS has 2 Trp residues. The fluorescence bands attributable to Trp are located at 336 and 333 nm (excitation at 285 nm) for Ec DOS and the isolated Ec DOS PAS domain, respectively (Fig. 2C). The fluorescence intensity of Ec DOS PAS was half that of Ec DOS.

Electrochemical titrations were conducted for the isolated Ec DOS PAS domain. The one-electron midpoint potential of the Ec DOSS heme was determined from the absorbance change at 562 nm. Half saturation points were +70 ± 1 mV for reduction and +63 ± 8 mV for oxidation (Fig. 3). No marked difference between the titration patterns in the two directions was observed. Therefore, the redox potential of heme in the isolated Ec DOS PAS domain was estimated to be +67 mV versus SHE (n = 0.90).

**Mutations at His-77 and His-83**—In order to identify the heme axial ligands of the Ec DOS PAS domain, site-directed mutagenesis of both His residues in the isolated PAS domain (His-77 and His-83) was conducted. As shown in Fig. 4, the His-83 → Gly and His-83 → Ala mutants had tight heme-binding capabilities and showed typical heme absorption maxima at 417 nm, similar to that of the wild type PAS domain. In contrast, the His-77 → Gly and His-77 → Ala mutants had only very small absorption bands at the Soret region, suggesting that very little heme was bound to these proteins consistent with His-77 being one of the heme axial ligands.

The Ec DOS Protein Is a Tetramer and the Isolated PAS Domain Is a Dimer—Purified Ec DOS protein and the isolated Ec DOS PAS domain protein were located at 93- and 19-kDa molecular masses on SDS-PAGE gels (Fig. 5, A and B). These molecular masses, including the His_6 tag (about 2 kDa), are close to values predicted from amino acid sequences of both proteins. Gel filtration was conducted to determine the molecular mass of Ec DOS (Fig. 5, C and D). The estimated molecular...
The molecular mass of the PAS domain was similarly estimated to be 30 kDa (data not shown), suggesting it is a dimer, as previously thought, even though the estimated mass is smaller than the calculated value (18).

**Ec DOS Is a cAMP Phosphodiesterase**—Since the amino acid sequence of the catalytic domain of Ec DOS is homologous to that of AxFDEA1, cAMP or cGMP are the most probable substrates of Ec DOS. Ant-cAMP was assayed for activity with Ec DOS. Only the Fe(II) complex of Ec DOS had enzyme-dependent PDE activity with cAMP (Fig. 6). We did not see detectable activity with the Fe(II)-O2 complex of Ec DOS perhaps due to its very low turnover number or its instability. The Fe(II)-O2 complex steadily decomposed to the Fe(III) complex as mentioned above, which had no PDE activity.

The optimum reaction temperature was found to be between 30 and 40 °C. Thus, all catalytic activities were obtained at

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**TABLE I**

Optical absorption maxima of Ec DOS, AxFDEA1, and FixL; all of them have heme-bound PAS domain (nm)

|           | Ec DOS<sup>a</sup> | AxFDEA1<sup>b</sup> | FixL<sup>c</sup> |
|-----------|---------------------|---------------------|------------------|
| Soret     | Visible             | Soret               | Visible         |
| Fe(III)   | 417                 | 432                 | Not reported     |
| Fe(II)    | 428                 | 532, 563            | 395, 509, 645    |
| Fe(II)-CO | 423                 | 540, 570            | 347              |
| Fe(II)-NO | 419                 | 537, 563            | 427, 548, 560    |
| Fe(II)-O2 | 417                 | 541, 579            | 419, 545, 562    |

<sup>a</sup> Absorption peaks of Ec DOS were essentially the same as those of Ec DOS PAS domain (18).

<sup>b</sup> Ref. 19.

<sup>c</sup> Ref. 18.
37 °C in the present study. The pH optimum for catalysis was found to be around pH 8.5 (not shown). It was reported that divalent metal cations such as Mn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\) can support PDE activity, whereas Ca\(^{2+}\) is virtually ineffective (21). Mg\(^{2+}\) was the most effective dication for supporting catalysis with Ec DOS; Ca\(^{2+}\) and Mn\(^{2+}\) were much less effective, and Zn\(^{2+}\) was ineffective (not shown).

Addition of 1 mM dithiothreitol to the catalytic solution enhanced the activity by 30%. This may be due to reduction of interprotein disulfide bonds, causing a decrease in the amount of aggregated enzyme, as indicated by gel filtration chromatography (not shown). The turnover number of the enzyme under optimum conditions was 0.15 \(\text{mol/min/mol of heme}\).

**Inhibition Studies**—In order to confirm that the Fe(II) heme regulates catalysis, CO and NO were added to the catalytic solution. As shown in Fig. 7, CO and NO markedly inhibited turnover. The IC\(_{50}\) values of CO and NO are about 1 \(\mu M\) or less. Dissociation constants for CO and NO were about 0.3 and 0.5 \(\mu M\), respectively, for Fe(II) Ec DOS, estimated by optical absorption titration (not shown). Note that dissociation constants were calculated from the free CO or NO concentration, whereas the IC\(_{50}\) value reflects total CO or NO concentration.

PDE inhibitors were examined to confirm that catalysis with Ec DOS is PDE activity. Etazolate hydrochloride is a selective inhibitor for cAMP-specific PDE, whereas 3-isobutyl-1-methylxanthine is a nonspecific inhibitor for both cAMP and cGMP PDEs.

**DISCUSSION**

The present work characterized the physicochemical and catalytic properties of full-length Ec DOS and unequivocally demonstrates for the first time that Ec DOS has PDE activity with cAMP, which is regulated by the heme redox state.

**Structural Characterization of Ec DOS**

**Optical Absorption Spectra**—From the absorption spectra, it is clear that despite the close sequence similarity and expected structural similarity between FixL and Ec DOS, the environ-
ment of the heme iron in the two proteins is different (Table 1). The Fe(III) and Fe(II) FixL have high spin penta-coordinate heme iron, whereas the spectra of Fe(III) and Fe(II) Ec DOS full-length wild type are typical of hexa-coordinate low spin heme complexes.

The heme axial ligands of the Ec DOS PAS domain were thought to be His and Met (18). Cytochrome b_{562} (25) and cytchromes c (26), which have this type of coordination, show clear pH-dependent spectral changes with apparent pK\textsubscript{a} values between 9 and 6, caused by axial ligand replacement. In contrast, the Ec DOS PAS domain appears relatively stable to pH variation between 5 and 9.5, with no ligand substitution. Therefore, either the heme environment is massively different or there is a different pair of axial ligands to cytochrome b_{562} and cytchromes c.

**CD, Fluorescence, and Redox Potential**—The α-helix content (54%) of Ec DOS estimated from the UV CD band suggests that it consists of more α-helical structure than FixL (37%), but not much as Mb (78%) (4). The secondry structure of Ec DOS appears to be different from either FixL or Mb (27).

The Soret CD band of Ec DOS is located only on the plus side. This is in contrast with those of cytochrome b_{562} (25) or cytochrome c (28) that have bands on the minus side and both sides, respectively. The Soret CD band of Ec DOS shifted from 421 to 425 nm concomitant with the intensity increase upon reduction, suggesting that heme environment was changed by reduction.

Fluorescence spectra with excitation at 280 nm are generally due to Tyr and Trp. Ec DOS and the isolated Ec DOS PAS domain contain 12 and 2 Trps, respectively. The fluorescence intensities of Ec DOS and Ec DOS PAS appear to correspond with the respective Trp numbers (Fig. 2C). Solvated Trp residues should have a fluorescence maximum in the vicinity of 350 nm, whereas those buried inside the hydrophobic core of the molecule usually have a much lower wavelength (29, 30). The fluorescence bands of Ec DOS and the isolated Ec DOS PAS domain are located at 336 and 333 nm, respectively, whereas both of the unfolded proteins (in the presence of 8 M urea) are located at 349 nm.2 Taken together, it appears that the Trp residues responsible for the fluorescence band must be buried inside the protein or located in a more hydrophobic environment. The reduction of Ec DOS heme did not affect the peak position. This suggests that the environment of these Trp residues was not changed by heme reduction.

The redox potential of Ec DOS was estimated to be +67 mV versus SHE. From this value, it appears that the Ec DOS Fe(III) complex is relatively stable compared with the electron-transferring hemoproteins, cytochrome c (+260 mV versus SHE) (2, 26). However, Ec DOS may be reduced more easily than cytochrome b_{5} (+3 mV) (31), sperm whale myoglobin (+59 mV) (32), and microsomal P450 (~310 mV) (33).

**Axial Heme Ligands of Ec DOS**—In the UV-visible absorption spectra of these mutants, it was clear that His-77, but not His-83, is one of the heme axial ligands in the Ec DOS PAS domain. On the other hand, modeling the structure of the Ec DOS PAS domain based on that of the FixL heme-binding PAS domain led us to predict that only Met-95, a residue on the heme distal side, is capable of coordinating to the heme (18).

**Tetramer**—Many heme sensor proteins such as CooA, soluble guanylate cyclase, and Hem-AT have been known to be homo- or heterodimer. FixL is also a soluble dimeric protein (29). Therefore, it is not surprising that the Ec DOS PAS domain is dimeric (18). However, it is interesting to note that Ec DOS itself is tetrameric, although it has not been reported whether the corresponding PDE with the similar PAS domain is dimeric or tetrameric (24).

**Catalytic Characterization**

**Catalytic Studies**—Ec DOS had PDE activity with cAMP but not with GMP, bis(p-nitrophenyl) phosphate, or p-nitrophenyl phosphate. The latter two compounds are universal substrates for many PDEs (20, 21, 24). Therefore, it appears that Ec DOS has a high substrate specificity for cAMP. PDE inhibitors, etazolate, hydrochloride, and 3-isobutyl-1-methylxanthine, were antagonists for cAMP. The present results strongly support the idea that Ec DOS is actually a PDE and has high specificity for cAMP.

cAMP, synthesized by adenylyl cyclase, is a very important cellular mediator for regulation of catabolism and for E. coli cell division. Therefore, Ec DOS may be involved in intravital signal transduction in E. coli. Imamura et al. (30) reported a gene encoding cAMP PDE which regulates intracellular cAMP levels. We could not see a significant homology in amino acid sequence between Ec DOS and their cAMP PDE. Although we do not know what kind of cellular response is affected by cAMP level regulation by Ec DOS PDE, it is very interesting it could be directly controlled by the redox state of the cell. In addition, we should note that Ec DOS has the highest homology to AxPDEA1. The PDE activity obtained for Ec DOS (0.15 min^{-1}) was about 50-fold lower than that of AxPDEA1 (19) but close to the activity (0.13 min^{-1}) of the cAMP PDE isolated from E. coli cells (30). The activity of Ec DOS was saturated with increasing the concentrations of Ec DOS (Fig. 6). A kind of product-inhibition or unfavorable protein-protein interactions to affect the activity appear to exist, but detailed mechanistic studies remain to be conducted.

The relationship between heme redox state and activity was investigated, and it appears that the Fe(II) form of Ec DOS was active, whereas the Fe(III) form was not. If Ec DOS is an O₂ sensor enzyme, then it is implied that binding of O₂ to the ferrous heme itself or oxidation of heme might cause the structural change necessary to regulate PDE activity under normoxic conditions. This proposed mechanism is very similar to that for FixL activation under hypoxic conditions. On the other hand, the Fe(II)-O₂ complex of Ec DOS was oxidized to Fe(III) heme with a half-life 60 min. This rate is faster than that of AxPDEA1 where a half-life was more than 12 h (19). When E. coli cells were crushed and centrifuged, optical absorption spectrum of an Fe(II) complex at 428 nm was observed in the supernatant solution at the initial stage. The spectrum of the Fe(II) complex slowly converts to that of an Fe(III) complex at 417 nm. This suggests that there is a possibility that the deoxy Fe(II) complex is the native form.

The catalytic domain of most PDEs constitutes the core sequence including consensus metal-binding domains (Zn²⁺ and Mg²⁺) related to those of metal-ion phosphohydrolases (24). Although we have shown that Ec DOS requires Mg²⁺ for activity, it does not have the signature motif HDX₃X₄HₓX₉N, common to many PDEs that is the sequence of the consensus metal-binding site (Zn²⁺ and Mg²⁺). However, a similar motif HDX₃X₄HₓX₉ is present in Ec DOS, which may perform this function.

CO and NO were found to coordinate to the Fe(II) heme immediately, and it is thought that the coordination of CO or NO caused a conformational change in the heme-bound PAS domain. This structural change may transfer a signal to affect the catalytic domain and cause a decrease in activity. A recent

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*S. Hirata, T. Matsui, I. Sagami, and T. Shimizu, unpublished results.*
Characterization of a Direct Oxygen Sensor Heme Protein

resonance Raman spectroscopic study of CO and NO complexes of heme-bound PAS domains suggested that the heme environment of Ec DOS PAS was unusual compared with those of other PAS domains (34).

In summary, the present study unequivocally indicates for the first time that Ec DOS has PDE activity with cAMP, which is regulated by the heme redox state.

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