Successful Mesoporous Silica Encapsulation of Optimally Functional EcDOS (E. coli Direct Oxygen Sensor), a Heme-based O₂-Sensing Phosphodiesterase

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The heme-based O₂ sensor from Escherichia coli, EcDOS, exerts phosphodiesterase activity towards cyclic-di-GMP (c-di-GMP), an important second messenger that regulates biofilm formation, virulence, and other important functions necessary for bacterial survival. EcDOS is a two-domain protein composed of an N-terminal heme-bound O₂-sensing domain and a C-terminal functional domain. O₂ binding to the heme Fe(II) complex in the O₂-sensing domain substantially enhances the catalytic activity of the functional domain, a property with potentially promising medical applications. Mesoporous silica is a useful material with finite-state machine-like features suitable for mediating numerous enzymatic functions. Here, we successfully encapsulated EcDOS into mesoporous silica, and demonstrated that encapsulated EcDOS was substantially activated by CO, an alternative signaling molecule used in place of O₂, exhibiting the same activity as the native enzyme in aqueous solution. Encapsulated EcDOS was sufficiently stable to exert its enzymatic function over several experimental cycles under aerobic conditions at room temperature. Thus, the present study demonstrates the successful encapsulation of the heme-based O₂ sensor EcDOS into mesoporous silica and shows that the native gas-stimulated function of EcDOS is well conserved. As such, this represents the first application of mesoporous silica to an oxygen-sensing— or any gas-sensing—enzyme.

Keywords Heme-based oxygen sensor, phosphodiesterase, mesoporous silica

Introduction

Cyclic-di-GMP (c-di-GMP) is an important second messenger in bacteria, reflecting its involvement in biofilm formation, motility, virulence, cell-cycle progression, and many more bacterial functions (Fig. S1, Supporting Information). c-di-GMP homeostasis is critical for the survival of bacteria in the face of external stimuli. At least two heme-based O₂ sensors, YddV and EcDOS, are involved in the homeostasis of c-di-GMP in Escherichia coli. YddV is diguanylate cyclase that synthesizes c-di-GMP from GTP, whereas EcDOS is a phosphodiesterase (PDE) that degrades cdiGMP by converting it to linear di-GMP (l-di-GMP) (Fig. S1). These two enzymes, which have different O₂ affinities, contribute to the regulation of c-di-GMP concentration in bacterial cells in an O₂-concentration-dependent manner, and function synergistically at different O₂ concentrations. Both YddV and EcDOS are typical of heme-based O₂ sensors, which are composed of an O₂-sensing heme-bound domain and a functional domain. The heme-based O₂ sensor PDE, EcDOS, is composed of an N-terminal heme-bound O₂-sensing domain and a C-terminal PDE domain (Fig. S2, Supporting Information). Catalysis by such heme-based O₂ sensors is a multistep process. Binding of an O₂ molecule to the heme Fe(II) complex in the N-terminal domain is the first signal. This alters the protein structure of the heme surrounding the sensing domain, yielding an altered protein structure that constitutes the second signal. This second signal is transduced to the functional domain, where it switches function on/off in response to association/dissociation of the gaseous molecule (Fig. S2). Therefore, EcDOS exerts its function in response to external stimuli such as changes in the concentration of O₂. Binding of CO or NO to the heme Fe(II) complex also markedly enhances catalysis, similar to O₂, reflecting their formation of 6-coordinated low-spin complexes. Also, the binding of O₂, CO, or NO to the heme equally enhanced the activity. Since cyclic-di-GMP is important for bacterial survival, it is possible to envision applications of the O₂-sensor functions of EcDOS to medicinal

Abbreviations
c-di-GMP: Cyclic di-GMP, cyclic dimeric guanosine monophosphate. EcDOS: E. coli Direct Oxygen Sensor, also designated EcDosP (heme-regulated oxygen sensor phosphodiesterase). FSM: Folded-sheet mesoporous material. Heme Fe(II): Protoporphyrin IX Fe(II) complex. Heme Fe(III): Protoporphyrin IX Fe(III) complex or hemin. 1-di-GMP: Linear di-GMP, linear diguanylate monophosphate or pGpG. PDE: Phosphodiesterase. YddV: Heme-based O₂ sensor diguanylate cyclase from E. coli, also designated EcDosC.

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and medical fields.\textsuperscript{14} EcDOS has also been successfully applied to protein sensors and protein microarrays, taking advantage of the relationships between domain-domain interactions and substrate-domain interactions of this enzyme.\textsuperscript{15,16}

Ordered mesoporous materials are useful for numerous biosensor and biocatalysis applications, because biomolecules or enzymes immobilized in the materials retain their functions.\textsuperscript{17-20} We have also reported that encapsulating enzymes in mesoporous silica not only increases the stability of enzymes, it also creates new capabilities.\textsuperscript{21-24} Additionally, we reported the synthesis of nanoporous silicas in anodic alumina pores, and showed that the resultant composite membranes have high potential as artificial membrane supports owing to their large number of interconnected pores.\textsuperscript{24,25} We also successfully developed a highly sensitive prototype sensor with good stability through encapsulation of enzymes into pores of these membranes.\textsuperscript{26-31} Hemoproteins encapsulated in mesoporous silica have UV/visible absorption bands at Soret and visible regions that are the same as those of the hemoproteins in aqueous solutions.\textsuperscript{21-24} The stability and functions of hemoproteins have been examined by introducing horseradish peroxidase\textsuperscript{25,26} into mesoporous silica materials.\textsuperscript{19} Because UV/Visible absorption spectra of hemoproteins are extensively used to understand the coordination and redox structures of these proteins as well as their functional relationships, the encapsulated hemoproteins could create numerous opportunities for applications as a redox sensor, O\textsubscript{2} sensor, or other important hemoprotein function.

In the present study, we first designed mesoporous silica with a pore size based on the volumes of the heme-bound O\textsubscript{2}-sensing domain and the PDE domain such that the PDE domain is located on the surface of the mesoporous silica and the O\textsubscript{2}-sensing domain is inserted into the pore. We then encapsulated full-length EcDOS into the mesoporous silica of the folded-sheet mesoporous material (FSM) type, an application that is the first of its kind application and examined the functional characteristics as well as spectrometric properties of the encapsulated enzyme. We found that the full-length EcDOS protein was almost completely adsorbed into the mesoporous silica. Absorption spectral studies suggested that the heme Fe(III) form of EcDOS was easily reduced by adding sodium dithionite, similar to the native enzyme in aqueous solution. Importantly, the heme Fe(II)-bound EcDOS exhibited basal PDE activity that was substantially enhanced by adding CO, again similar to the native enzyme. Thus, the present study is the first to demonstrate that, because it retains its function, an O\textsubscript{2}-sensing enzyme encapsulated in mesoporous silica can be used in place of the native enzyme in solution.

**Experimental**

**Materials**

c-di-GMP was obtained from BIOLOG (Bremen, Germany). Other chemicals, acquired from Wako Pure Chemical Industries (Osaka, Japan), were of the highest guaranteed grade available and were used without further purification. FSM with a pore diameter of 5.3 nm was prepared from water glass using docosyltrimethylammonium chloride [C\textsubscript{22}H\textsubscript{45}N(CH\textsubscript{3})\textsubscript{3}Cl] and 1,3,5-triisopropylbenzene as previously described.\textsuperscript{22,23} Briefly, 16 g of docosyltrimethylammonium chloride [C\textsubscript{22}H\textsubscript{45}N(CH\textsubscript{3})\textsubscript{3}Cl] was added to 200 mL of water maintained at 70°C, followed by the addition of 60 g of 1,3,5-triisopropylbenzene. The resulting mixture was vigorously stirred for 30 min at 70°C and then added to 200 mL of water (80°C) in the presence of 54.31 g of water glass (SiO\textsubscript{2}/Na\textsubscript{2}O = 2.07). The pH was then adjusted to 8.5 by slowly adding an aqueous solution of 2 M HCl. After the suspension was stirred for 3 h at 70°C, the solid product was filtered out, washed three times with 400 mL of distilled water at 70°C, and dried. The sample was then calcined at 550°C for 6 h in air, and evaluated with respect to pore size, pore volume, and specific surface area.

**Characterization**

Absorption spectra were obtained with a Shimadzu MPS-2400 spectrophotometer (Kyoto, Japan). Nitrogen adsorption isotherms were measured at 77 K on an automatic gas adsorption apparatus (Belsorp Max; BEL Japan, Inc., Osaka, Japan). Specific surface areas were calculated by the Brunauer-Emmett-Teller (BET) method using adsorption data (P/P\textsubscript{0} = 0.05 - 0.30), and pore size distributions were determined by analyzing the adsorption branch using the Barrett-Joyner-Halenda (BJH) method.\textsuperscript{36}

**Cloning of the EcDOS gene, construction of expression plasmids, and overexpression and purification of full-length EcDOS protein**

Cloning of EcDOS and construction of the expression plasmid, pET28a(+)-EcDOS, were performed as described previously.\textsuperscript{9,13} The EcDOS protein was overexpressed in the E. coli strain, BL21(DE3), transformed with the pET28a(+)-EcDOS expression plasmid containing an N-terminal His\textsubscript{6} tag and thrombin cleavage site. Culture conditions for E. coli and protein purification procedures are based on a previous report.\textsuperscript{9,13} After dialysis against 20 mM Tris-HCl (pH 8.0) containing 5% glycerol, oxidized proteins were concentrated using an Amicon Centriprep (Millipore, Billerica, MA, USA). Samples were stored at −20°C in the presence of 40% glycerol.

**Enzymatic assays**

Enzymatic assays were conducted using high-performance liquid chromatography (HPLC) as previously described.\textsuperscript{37,38} PDE activity was assayed at 37°C for 3 min in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 0.1 mM c-di-GMP, and 10 μM EcDOS. The heme Fe(II) form of EcDOS was prepared by adding a trace amount of dithiothreitol (DTT; final concentration, 10 mM). The heme Fe(II)-CO form of EcDOS was prepared by adding CO-saturated buffer, obtained by bubbling with CO gas for at least 30 min at room temperature. The reaction was stopped by addition of CaCl\textsubscript{2} (final concentration, 10 mM) and adjusted to 0.1 mM β-NADPH (standard). The solution was applied to a LUNA 5 μC18 (2) chromatography column (15 × 4.6 cm; Phenomenex Torrance, CA, USA), and products were eluted with a gradient of Buffer A (100 mM KH\textsubscript{2}PO\textsubscript{4}, containing 4 mM tetrabutylammonium hydrogen sulfate, pH 6.0) and Buffer B (75% buffer A/25% methanol) at a flow rate of 0.7 mL/min using the following program: 0.0 min, 40% B/60% A; 15.0 min, 100% B; 20.0 min, 100% B; and 21.0 min, 40% B/60% A. Absorption was monitored at 254 nm.

**Labeling of EcDOS with amine-reactive fluorescent dyes**

To visualize individual EcDOS encapsulated in mesoporous material using fluorescence microscopy, we chemically modified some lysine residues of EcDOS with the amine-reactive fluorescent dye, Alexa Fluor 488 (Abs/Em = 495 nm/519 nm; Molecular Probes). The labeling reaction was carried out by combining 1 mg of enzyme with 1 mL of 20 mM MES buffer (pH 6.0) containing an appropriate amount of fluorescent dye. The sample solution was gently mixed on a rotator for 1 h at 4°C in the dark. The labeled EcDOS was purified by gel
where suspension was then shaken for 12 h at 4 °C to establish an adsorption equilibrium, after which the DOS-FSM conjugate as described above, we prepared an FSM conjugate. The DOS protein has 30 lysine residues. The substrate c-di-GMP (blue ball) is converted into the product, l-di-GMP or pGpG (red ball) by the conjugated/encapsulated EcDOS. [b] By mixing the full-length EcDOS protein with mesoporous silica, the red solution is converted into a transparent aqueous phase (upper), whereas the mesoporous silica in the bottom solid phase becomes red. This phenomenon suggests that the heme protein, EcDOS, is almost completely adsorbed into the mesoporous silica.

Results and Discussion
Encapsulation of full-length EcDOS into FSM
First, we designed the pore size of mesoporous silica such that only the heme-bound O$_2$-sensing domain could be fully incorporated into the resulting pores. Based on the volume of the dimeric heme-bound O$_2$-sensing domain containing 294 amino acids (amino acids 1 – 147 of each monomer), the effective radius of the isolated O$_2$-sensing domain with the heme Fe(III) complex should be 1.41 nm (14.1 Å), whereas that of the heme Fe(II) complex should be 1.58 nm (15.8 Å). On the other hand, the PDE domain containing 414 amino acids (amino acids 148 – 799) was presumed to be much larger than the isolated dimeric heme-bound O$_2$-sensing domain. It should be noted that it is not clear from the crystal structure of part of the PDE domain (the 270-amino-acid EAL domain corresponding to amino acids 148 – 379) is located between the heme-bound O$_2$-sensing domain and the PDE domain. Therefore, we generated and used FSM with a pore size of ~5 – 6 nm so that only the heme-bound domain could be efficiently inserted into the pore. The full-length EcDOS protein is heme-bound; thus, its aqueous solution is colored red. To confirm that the full-length EcDOS protein was conjugated with FSM (Fig. 1a), we gently mixed 18 mg of FSM with an aqueous solution of the full-length EcDOS protein (2.3 mg protein/mL; 1.2 mL) overnight at 4°C using a rotator.
The red-colored aqueous phase subsequently became transparent, whereas the solid phase containing FSM became red (Fig. 1b), suggesting that full-length EcDOS was efficiently adsorbed into FSM. The interaction between EcDOS and FSM has not been clarified, it is probably not ion binding, but hydrophobic bonding. The intensity of the absorption spectrum of the solution containing full-length EcDOS protein was substantially decreased by mixing with FSM, and the resultant band at ∼280 nm ascribed to the protein was also markedly higher than the Soret band, suggesting again that most of the full-length EcDOS protein had adsorbed into the mesoporous silica (Fig. 2a). Based on changes in absorption, the adsorption quantity/ratio was found to be nearly 11% in terms of weight %.

A characterization of the resulting EcDOS-FSM conjugate revealed an average pore size of 5.3 nm, a pore volume of 1.7 cm³ g⁻¹, and a specific surface area of 624 m² g⁻¹ (Table 1).

To determine if the adsorbed full-length EcDOS protein was spectrometrically functional, we added sodium dithionite and observed spectral changes. The Soret band of the heme Fe(III)-bound full-length EcDOS protein at 416 nm was shifted to 428 nm with the concomitant appearance of two clear peaks at about 526 and 560 nm, ascribed to the heme Fe(II)-bound full-length EcDOS protein (Fig. 2b). These spectral changes and patterns observed for the encapsulated full-length EcDOS protein were essentially the same as those observed for the protein in aqueous solution. This suggests that the heme-bound full-length EcDOS protein was properly encapsulated into the mesoporous silica and that encapsulation of the protein did not significantly change the structure of the protein compared to the free protein in solution.

Table 1 Physicochemical properties of the EcDOS-FSM

| Pore size/ nm | Pore volume/ cm³ g⁻¹ | Specific surface area/m² g⁻¹ |
|--------------|-----------------------|----------------------------|
| FSM          | 5.3                   | 1.7                        | 624                       |
| EcDOS-FSM    | 5.5                   | 1.2                        | 475                       |

a. Specific surface areas were calculated by the BET method using adsorption data and the pore size distribution were determined by analyzing adsorption branch by the BJH method.

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Fig. 2 [a] Absorption spectral changes before (A) and after (B) of the solution containing the heme Fe(III)-bound full-length EcDOS protein caused by adsorption into mesoporous silica. Intensities of the Soret band at ∼416 nm and the band at ∼280 nm, ascribed to the protein, were substantially decreased by adding mesoporous silica. [b] The absorption spectrum of heme Fe(III)-bound full-length EcDOS was markedly changed by adding sodium dithionite, demonstrating that heme Fe(III)-bound full-length EcDOS (blue line) in the mesoporous silica was fully reduced by sodium dithionite and converted into heme Fe(II)-bound full-length EcDOS (red line).

Fig. 3 Nitrogen adsorption isotherms for FSM and EcDOS-FSM. The addition of full-length EcDOS decreased STP value in a stepwise manner from line A to line B owing to protein adsorption into the mesoporous silica pore. The inset shows that the distribution of pore sizes, measured as dv/dr values, was correspondingly decreased by the addition of full-length EcDOS.
with that in aqueous solution. Figure 3 shows the nitrogen adsorption isotherm for FSM alone and EcDOS-FSM. The steep increase in nitrogen uptake by FSM observed at a relative pressure of 0.6 is probably attributable to capillary condensation (curve A). By contrast, nitrogen uptake by the EcDOS-FSM conjugate (curve B) was lower than that of FSM. The corresponding pore size distributions of FSM before and after EcDOS loading are shown in Fig. 3 (inset). The addition of full-length EcDOS protein decreased the pore volume and BET surface areas of FSM (Table 1). Note that, in the case of curves C and D in the inset in Fig. 3, approximately 30% of the pore volume of FSM was filled with EcDOS. This finding is reasonable and consistent, given that the effective radius of the heme-bound O₂ sensing domain of the full-length EcDOS protein (1.4 – 1.6 nm) was about one-third the radius of the mesoporous silica pore (5.3 nm). This suggests that the heme-bound domain was fully embedded into the pore, whereas the linker and PDE domains were only partially embedded into the pore. To determine where in the mesoporous silica the full-length EcDOS protein was located, we mixed fluorescence-labeled full-length EcDOS with mesoporous silica and imaged the protein by fluorescence microscopy (Fig. 4). This analysis showed that DIC images of mesoporous silica (Fig. 4a) and fluorescence images of full-length EcDOS (Fig. 4b) overlapped with each other. Dye-labeled, full-length EcDOS was detected as green-colored particles, reflecting the green fluorescence of the Alexa 488 dye (Fig. 4c). The merged image clearly shows that fluorescence-labeled full-length EcDOS was located near the edge of the FSM particle (Fig. 4d), reconfirming that the fluorescence-labeled 231-amino-acid linker and 414-amino-acid PDE domain are situated on the surface of the mesoporous silica (Fig. 1a).

**Catalytic activity of encapsulated full-length EcDOS**

The catalytic activity of mesoporous silica-encapsulated full-length EcDOS was examined with the aid of HPLC (Fig. 5). Encapsulated heme Fe(II)-bound full-length EcDOS exhibited basal activity, as evidenced by the decrease in the concentration of the substrate, c-di-GMP (Fig. 5a, red trace in right-most peak) and concomitant increase in the product, l-di-GMP or pGpG (Fig. 5a, red trace in left-most peak) following addition of the heme Fe(II)-bound enzyme, compared with that observed in its absence (blue traces in Figs. 5a and 5b). Importantly, addition of CO, used to examine the catalytic simulation caused by an external gas molecule, markedly stimulated the PDE activity of the encapsulated heme Fe(II)-bound full-length EcDOS, resulting in more product and less substrate (black traces in Figs. 5a and 5b). It is known that external axial ligands, such as CO and NO, for the heme Fe(II) complex, and cyanide anion and imidazole for the heme Fe(III) complex substantially enhance the PDE catalytic activity of full-length EcDOS.⁹,⁴³ Therefore, the enhancement of PDE activity by CO strongly suggests that the external ligand-induced catalytic enhancement of the encapsulated full-length EcDOS, including that by O₂, is fully functional. DTT was used as a reductant to convert the heme Fe(III) complex with mesoporous silica to the fully functional heme Fe(II) complex.³ However, O₂ dissolved in aqueous solutions quickly binds to the heme Fe(II) complex and forms a heme Fe(II)-O₂ complex; thus, it is difficult to obtain a stable isolated heme Fe(II) complex for comparison under aerobic conditions. To examine the efficiency and stability of the encapsulated full-length EcDOS for subsequent applications, we examined the extent to which enzyme function degraded over time in storage at 4°C in water. As shown in Fig. 6a, the activity of full-length EcDOS in FSM decreased by
only 40% (60% retained) over a period of 30 days, a result that was highly reproducible in repeat experiments (Fig. 6b). By contrast, the native enzyme dissolved in water cannot be used repeatedly. This suggests that the encapsulated full-length EcDOS is stable and its catalytic activity is significantly resistant to protein denaturation after several experimental cycles.

**Conclusions**

In the present study, we successfully encapsulated the heme-bound O₂-sensing domain of the full-length EcDOS protein—a heme-based O₂ sensor PDE—with FSM. A characterization of the resulting EcDOS-FSM conjugate suggested that the linker and PDE domains are located on the FSM surface. The absorption spectral behavior of the encapsulated enzyme was...
the same as that of the enzyme in aqueous solution. Basal PDE and CO-stimulated PDE activities of the native enzyme were also adequately preserved in the encapsulated enzyme. These findings suggest that heme-based O₂ (as well as CO and NO) sensors could be widely applied for medicinal or industrial purposes when they are well encapsulated into mesoporous silica as a finite-state machine.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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