# Phosphodiesterase Activity of *Ec* DOS, a Heme-regulated Enzyme from *Escherichia coli*, toward 3′,5′-Cyclic Diguanylic Acid Is Obviously Enhanced by $O_2$ and CO Binding

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3′,5′-Cyclic diguanylic acid (c-di-GMP) is emerging as an important bacterial intracellular signaling molecule. Both the Fe(III) and Fe(II) heme complexes of *Ec* DOS, a heme-bound PAS sensor enzyme from *Escherichia coli*, have phosphodiesterase activity toward c-di-GMP (27 and 61 $min^{-1}$, respectively) and that the activity of the Fe(II) heme complex is obviously enhanced by the binding of either $O_2$ (126 $min^{-1}$) or CO (143 $min^{-1}$). Therefore, *Ec* DOS appears to be a novel type of gas sensor enzyme.

The heme-bound domain of *Ec* DOS, a heme-sensor enzyme from *Escherichia coli*, was first cloned by the Gilles-Gonzalez group.[1] Based on its physicochemical characteristics and comparison with the direct oxygen sensor enzyme, FixL, which has a heme-bound PAS sensor domain, they predicted that *Ec* DOS acts as a direct oxygen sensor enzyme and that $O_2$ binding to the heme iron of this enzyme should suppress its phosphodiesterase activity. In separate experiments, this group demonstrated that the full-length enzyme is a cAMP phosphodiesterase.[2,3] This and other groups have also characterized the physicochemical and structural properties of the isolated heme-bound PAS domain.[4–9] They found that the cAMP phosphodiesterase activity is regulated by the heme redox state: it is active when the heme is in the Fe(II) state and inactive with heme in the Fe(III) state.[2,3] Also, binding of CO or NO to the Fe(II) complex makes the enzyme inactive toward cAMP.[2] Profound structural changes in the heme-bound PAS accompanying the heme redox change may explain the heme redox-dependent activity toward cAMP.[6] Knockout of *Ec* DOS in *E. coli* causes the level of cAMP to increase 27-fold compared with the wild type and induces alterations in cell morphology, suggesting that cAMP is a substrate of *Ec* DOS in vivo and that *Ec* DOS is involved in cell differentiation.[10,11]

Recently, 3′,5′-cyclic diguanylic acid (c-di-GMP) has emerged as an important bacterial intracellular signaling molecule associated with motility, virulence, intercellular interaction, and cellulose production.[11–18] The C-terminal domain of *Ec* DOS has a GGDEF domain, which is predicted to have diguanylate cyclase activity (conversion of GTP to c-di-GMP), and an EAL domain, which should act as a c-di-GMP-specific phosphodiesterase (conversion of c-di-GMP to l-di-GMP). Although, the isolated C-terminal domain has c-di-GMP-specific phosphodiesterase activity,[15] the effect of the heme-bound N-terminal domain of full-length *Ec* DOS has not been reported.

We examined the catalytic activity of *Ec* DOS toward c-di-GMP by HPLC, using NADPH as a standard. In the absence of *Ec* DOS, there was no difference in the HPLC patterns under aerobic and anaerobic conditions (Figure 1a). Upon the addition of Fe(III) *Ec* DOS, there were marked changes in the HPLC pattern; specifically, there was a rapid decrease in the c-di-GMP peak and a concomitant increase in the 1-di-GMP peak. This

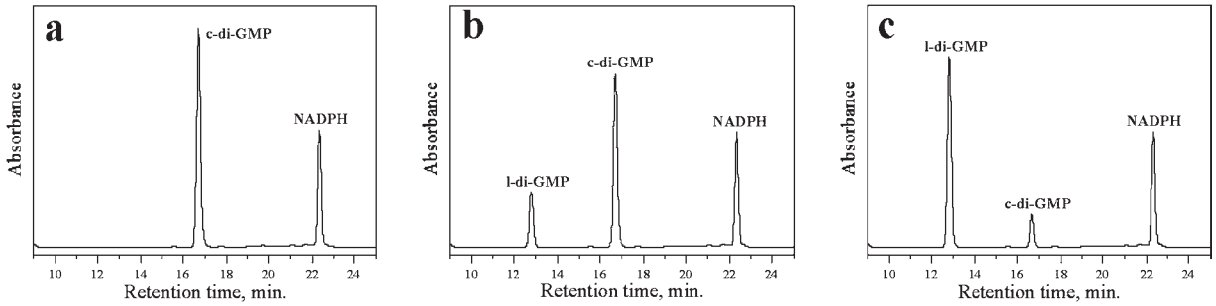


**Figure 1.** HPLC patterns of (a) c-di-GMP (100 μM) and NADPH (standard) in the absence of *Ec* DOS; (b) the reaction mixture containing 1-di-GMP, c-di-GMP, and NADPH 3 min after addition of *Ec* DOS; and (c) the CO-saturated reaction solution containing 1-di-GMP, c-di-GMP, and NADPH 3 min after addition of *Ec* DOS. To avoid any differences in experimental conditions, all experiments were conducted in a glove box at an oxygen concentration of less than 10 ppm. The Fe(III) and Fe(II) enzymes were obtained by mixing *Ec* DOS wth ferricyanide and sodium dithionite, respectively, followed by removal of the excess redox reagents by Sephadex G-25 column chromatography. Phosphodiesterase activity was assayed at 37 °C for 3 min in a reaction mixture containing 50 mM Tris-HCl (pH 8.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM c-di-GMP, and 0.2 μM *Ec* DOS. The reaction was stopped by addition of $CaCl_2$ (final concentration, 10 mM) and adjusted to 0.1 mM $\beta$-NADPH (standard). The mixture was then filtered through a 0.45-μm filter, and the reaction samples (10 μL) were injected into a LUNA 5μ C18 (2) column (15 × 4.6 cm; Phenomenex, Torrance, CA, U.S.A.) and analyzed using a Prominence HPLC system (Shimadzu, Kyoto, Japan) with detection at 254 nm. The following buffers were used in the gradient program: buffer A (100 mM $KH_2PO_4$ with 4 mM tetrabutyl ammonium hydrogen sulfate [pH 6.0]) and buffer B (75% buffer A/25% methanol). The gradient was delivered at a flow rate of 0.7 mL $min^{-1}$ according to 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.

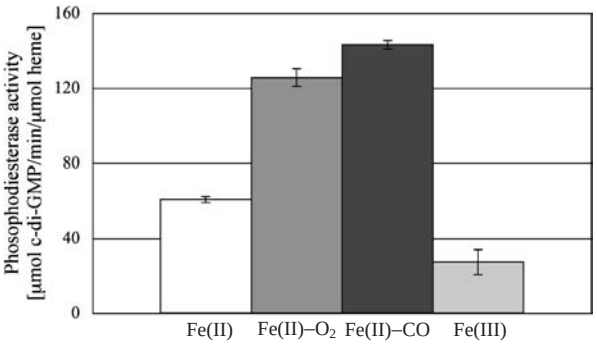


**Figure 2.** The rates of substrate hydrolysis under various conditions. From left to right, activities of Fe(II), Fe(II)–$O_2$, Fe(II)–CO, and Fe(III) forms of *Ec* DOS. The activity of the Fe(II) complex of *Ec* DOS was enhanced by CO and $O_2$ binding. Reaction rates represent means of six determinations.

HPLC pattern is the same as previously reported.[14–16] Figure 1b shows the HPLC pattern obtained after a 3-min incubation at 37 °C with Fe(III) *Ec* DOS. During this 3-min reaction, the turnover number for the Fe(III) enzyme was 27 $min^{-1}$. Addition of Fe(II) *Ec* DOS gave similar time-dependent HPLC pattern, with a turnover number of 61 $min^{-1}$. This two-fold increase in activity for the Fe(II) enzyme contrasts with the enzyme's cAMP phosphodiesterase activity, which is strictly heme-redox dependent and inactive in the Fe(III) state.[2,3]

We next examined the effects of $O_2$ and CO binding on the Fe(II) complex. Surprisingly, we found that the catalytic activity was obviously higher for the $O_2$- and CO-bound *Ec* DOS enzymes (126 and 143 $min^{-1}$, respectively; Figures 1c and 2). Because the rate of autooxidation of the full-length wild-type enzyme is slow (0.015 $min^{-1}$), the optical absorption spectra for the $O_2$-bound Fe(II) enzyme was stable during the course of activity measurement. Only a very small GMP peak was observed even after a 45-min reaction, suggesting that the activity toward 1-di-GMP was very low under the present conditions.

Several $O_2$- and CO-response heme-sensor enzymes and proteins (FixL,[19,20] AxPDEA1,[21] cystathion $\beta$-synthase,[22] heme-bound neuronal PAS protein 2 (NPAS2),[23] and CooA[24,25]) are known. The functions of most of these are suppressed by $O_2$ and CO binding, except for CooA. Therefore, the present study shows that the Fe(II) complex of *Ec* DOS is the first heme-sensor enzyme with a heme-bound PAS domain whose activity is enhanced by the binding of $O_2$ and CO. In particular, AxPDEA1 has both GGDEF and EAL domains the same as *Ec* DOS and the sequence homology of the C-terminal catalytic domains betweeen AxPDEA1 and *Ec* DOS is high, 30% identical and 50% homologous.[1,21] However, both enzymes exhibited the opposite ligand effects in terms of the PDE activity toward c-di-GMP.

Further studies using physical methods are needed to elucidate the unique intramolecular signaling activated in *Ec* DOS by $O_2$ and CO binding.[26]

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