Investigation of the Relationship between Protein−Protein Interaction and Catalytic Activity of a Heme-Regulated Phosphodiesterase from *Escherichia coli* (*Ec* DOS) by Protein Microarray

Yukie Sasakura,[‡,§] Katsuhiro Kanda,[‡,*] Tokiko Yoshimura-Suzuki,[§] Takuya Matsui,[‡] Shinichi Fukuzono,[‡] and Toru Shimizu[§]

Bio-Medical Center, R&D Division, Nanotechnology Product Business Group, Hitachi High-Technologies Corporation, Hitachinaka, Ibaraki, 312-8504, Japan, and Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Aoba-ku, Sendai, 980-8577, Japan

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ABSTRACT: *Ec* DOS, a heme-regulated phosphodiesterase from *Escherichia coli*, is composed of an N-terminal heme-bound PAS domain and a C-terminal phosphodiesterase domain. The heme redox state in the PAS domain regulates *Ec* DOS phosphodiesterase activity. Interestingly, the isolated heme-bound PAS fragment enhances phosphodiesterase activity of full-length *Ec* DOS. The enhancement is also regulated by the heme redox state of the isolated PAS domain. In the present study, we used a newly developed protein microarray system to examine the relationship between catalytic activity and the interaction of full-length *Ec* DOS and the isolated PAS fragment. Adenosine 3′,5′-cyclic monophosphate (cAMP), a substrate of the *Ec* DOS phosphodiesterase, was found to be indispensable for the interaction between *Ec* DOS and the PAS fragment, and two phosphodiesterase inhibitors, 3-isobutyl-methyl-xanthine and etazolate hydrochloride, hindered the interaction. In addition, an enzyme with a mutation in the putative cAMP-binding sites (H590 and H594) was unable to interact with *Ec* DOS and lacked enzymatic activity. These results strongly suggest a close relationship between *Ec* DOS phosphodiesterase activity and interaction with the isolated PAS fragment. Therefore, this study provides insights into the mechanism of how the isolated PAS domain activates *Ec* DOS, which has important implications for the general role of the isolated PAS domain in cells. Moreover, we found that multiple microscale analyses using the protein microarray system had several advantages over conventional affinity column methods, including the quantity of protein needed, the sensitivity, the variability of immobilized protein, and the time required for the experiment.

Heme proteins and enzymes perform a broad range of functions in living cells. Well-known examples include O_2 storage by myoglobin, O_2 transport by hemoglobin, electron transfer by cytochromes, and catalytic activation of heme ligands by P450s and peroxidases (*1*, *2*). Recently, a new class of heme enzymes involved in intramolecular signal transduction, the heme-based sensors, was discovered (*3*, *4*). The FixL protein of *Rhizobium meliloti* is well-characterized as a biological oxygen sensor that regulates the expression of the nitrogen fixation genes of a plant symbiotic bacterium (*5*).

The heme-regulated phosphodiesterase from *Escherichia coli* (*Ec* DOS)[1] was originally identified on the basis of sequence homology to the FixL protein (*6*). Like FixL, *Ec* DOS is a heme-containing signal transduction protein (*7*) composed of an N-terminal heme-containing PAS domain and a C-terminal phosphodiesterase domain (Figure 1A). The heme cofactor in the PAS domain acts as a sensor of the cellular redox state and regulates phosphodiesterase activity toward the substrate, adenosine 3′,5′-cyclic monophosphate (cAMP). Specifically, the protein is active when the heme contains Fe^{2+} and inactive when the heme is bound to Fe^{3+}. Intramolecular transduction of the heme redox state is mediated by global structural changes in the heme-binding PAS domain, which probably triggers alterations in the *Ec* DOS phosphodiesterase domain structure that enhance catalysis (*8*).

Curiously, addition of the isolated Fe^{2+} heme-bound PAS fragment (Figure 1B) to full-length *Ec* DOS (Figure 1A) causes a 5-fold enhancement in phosphodiesterase activity (*9*). Moreover, *Ec* DOS and the isolated PAS fragment can interact with each other only when the heme is in the Fe^{2+}-bound state. Accordingly, protein−protein interactions be-

* To whom correspondence should be addressed. Bio-Medical Center, R & D Division, Nanotechnology Product Business Group, Hitachi High-Technologies Corporation, 882, Ichige, Hitachinaka, Ibaraki, 312-8504, Japan. E-mail, kanda-katsuhiro@naka.hitachi-hitec.com; tel, +81-29-276-6121; fax, +81-29-273-5174.

‡ Hitachi High-Technologies Corporation.

§ Tohoku University.

[1] Abbreviations: *Ec* DOS, heme-regulated phosphodiesterase from *Escherichia coli*; PAS, an acronym formed from the names of Per (Drosophila period clock protein)-Arnt (vertebrate aryl hydrocarbon receptor nuclear translocator)-Sim (Drosophila single-minded protein); cAMP, adenosine 3′,5′-cyclic monophosphate; SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IBMX, 3-isobutyl-methyl-xanthine; SPR, surface plasmon resonance; WT, wild-type; PC, positive control; NC, negative control.

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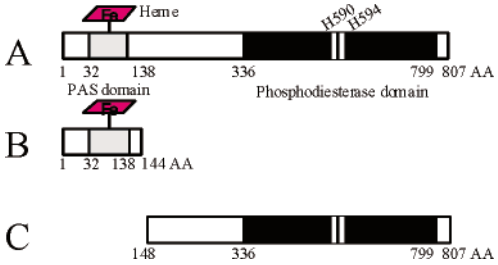

FIGURE 1: Structures of wild-type *Ec* DOS (A), the PAS fragment (B), and ΔN147 mutant of *Ec* DOS (C). The wild-type *Ec* DOS (A) is composed of an N-terminal heme-containing PAS domain (residues 32−138) and a C-terminal phosphodiesterase domain (residues 336−799). The heme cofactor in its PAS domain acts as a sensor of the cellular redox state and regulates phosphodiesterase activity toward the substrate, cAMP. Specifically, the Fe^{2+} protein is catalytically active, whereas the Fe^{3+} is catalytically inactive (*7*). However, an N-terminal truncated heme-free mutant ΔN147 (C) has an activity comparable to the Fe^{2+}-heme-bound wild-type enzyme (*9*). H590 and H594 are putative cAMP binding sites (*9*). Interestingly, addition of the isolated Fe^{2+}-heme-bound PAS fragment (B) to the Fe^{2+}-heme-bound wild-type protein (A) enhanced the activity by 5-fold (*9*).

tween *Ec* DOS and the PAS fragment are thought to be related to control of the enzymatic activity. In a previous study, we showed that 3-isobutyl-methyl-xanthine (IBMX) and etazolate hydrochloride, which have chemical structures similar to that of cAMP, inhibit *Ec* DOS phosphodiesterase activity (*7*). In addition, we have shown that three site-directed mutants of *Ec* DOS, H590A, H594A, and H590A/H594A, are enzymatically inactive (*9*). On the basis of amino acid sequence homology with other cAMP phosphodiesterases, two histidine residues in *Ec* DOS, H590 and H594, are thought to play an important role in binding cAMP (10). However, it has not been clear whether cAMP, inhibitors, and mutations at substrate binding sites influence the protein−protein interactions related to control of catalysis.

Protein microarray systems using an anti-Tag antibody and fluorescence scanning have been shown to detect protein−protein interactions using as little as 1.5 μg of protein (*11*). In this system, an anti-Tag antibody is immobilized onto a solid surface and used to capture Tag−target fusion proteins (Figure 2A). In this way, target proteins appear to sufficiently maintain their native structure because they are immobilized indirectly. Therefore, the system is suitable for detecting protein−protein interactions under relatively native conditions. Moreover, the protein microarray system makes it possible to analyze multiple samples on one solid surface at the same time. Thus, this system can be used to easily examine the effects of substrates, inhibitors, and site-directed mutations on protein−protein interactions associated with catalysis.

In the present study, we used this system to examine the effects of the substrate (cAMP), inhibitors, and various deletion and site-directed mutations on the protein−protein interactions associated with catalysis by *Ec* DOS (Figure 2). It was found that catalysis by *Ec* DOS correlates closely with the interaction between the full-length enzyme and the isolated PAS fragment. We also demonstrate the usefulness of the protein microarray system to detect multiple protein−protein interactions with high sensitivity, indicating that it will be suitable for proteomics analyses such as drug screening and various functional assays.

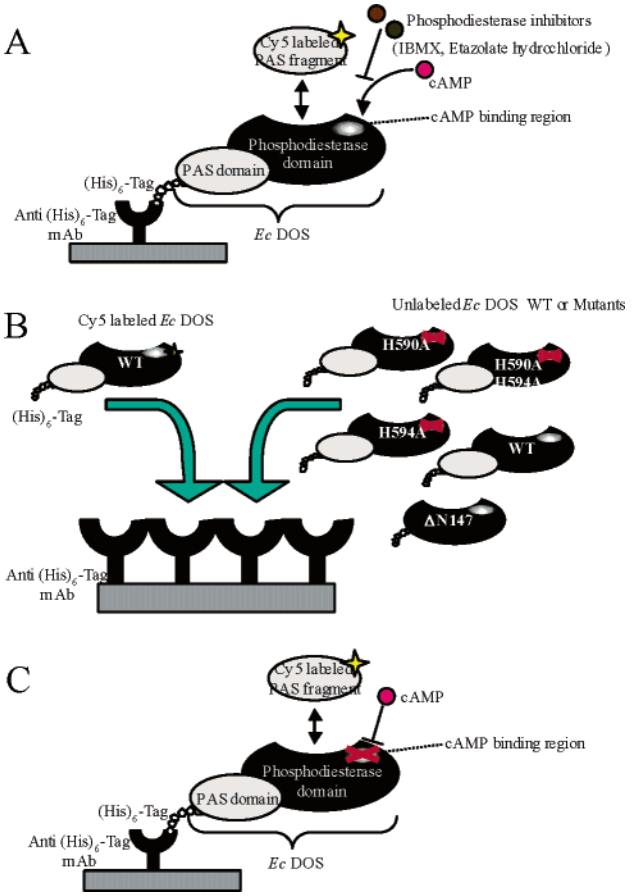

FIGURE 2: (A) Effects of cAMP and phosphodiesterase inhibitors on the interaction between *Ec* DOS and the PAS fragment were examined. Plausible structural model of immobilized *Ec* DOS is shown. *Ec* DOS was immobilized on solid surface via anti-$(His)_6$ mAb, and interaction with the PAS fragment was analyzed with or without cAMP, IBMX, or etazolate hydrochloride. (B) Effects of site-directed mutations on the immobilization of *Ec* DOS were examined. An illustration of competitive binding between Cy5-labeled and unlabeled *Ec* DOS is shown. (C) Effects of site-directed mutations on the interaction between *Ec* DOS and PAS fragment were examined. Plausible structural model for *Ec* DOS (wild-type and three site-directed mutants) immobilized on the solid surface via anti-$(His)_6$-Tag mAb is shown.

EXPERIMENTAL PROCEDURES

Materials. A ProteoChip, prepared by coating aminated slide glass with calixclown-5 derivatives (*12*), was obtained from Proteogen, Inc. (Seoul, Korea). The spotting seal, manufactured by Swasho Techno (Nagano, Japan), was designed to contain 48 small holes (2 mm diameter) on a 25 mm × 55 mm area of the poly(vinyl chloride) seat. The phosphodiesterase inhibitors IBMX and etazolate hydrochloride were purchased from Merck (Darmstadt, Germany). The FluoroLink-Ab Cy5 labeling kit was obtained from Amersham Biosciences (Buckinghamshire, U.K.), and mouse anti-$(His)_6$ mAb was acquired from R&D Systems, Inc. (Minneapolis, MN). Other chemicals were from Wako Pure Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO).

Construction of the Expression Plasmids for Ec DOS and Ec DOS Mutants. Expression plasmids for *Ec* DOS and *Ec* DOS mutants were constructed as described in our previous reports (*7*, *9*). The sequences corresponding to *Ec* DOS and the *Ec* DOS-PAS domain were amplified by PCR using

genomic DNA isolated from the *E. coli* JM109 strain as a template. The obtained clones were inserted into the *E. coli* expression vector, pET 28a(+), which introduces a $(His)_6$-Tag at the N-terminus of expressed proteins. H590A, H594A, and H590A/H594A mutants were generated with a QuikChange site-directed mutagenesis protocol (Stratagene) using pET28a(+)-wild-type *Ec* DOS as a template. The ΔN147 mutant was constructed by PCR using appropriate primers, as described in our previous paper (*9*).

Preparation of Ec DOS and the Ec DOS-PAS Fragment. $(His)_6$-*Ec* DOS (wild-type and H590A, H594A, H590A/H594A, and ΔN147 mutants) and the isolated $(His)_6$-PAS fragment were expressed in *E. coli* BL21 (DE3) and purified as described previously (*7*). Briefly, harvested cells were sonicated, centrifuged at 100 000*g*, and precipitated with ammonium sulfate. Ammonium sulfate was removed using a Sephadex G25 column, and proteins were purified by Ni-NTA affinity chromatography. The purity of proteins was confirmed by SDS−PAGE.

Preparation of the Anti-$(His)_6$ mAb-Immobilized Microarray. The spotting seal was initially adhered to the solid substrate (ProteoChip). Mouse anti-$(His)_6$ mAb was diluted to 100 μg/mL with phosphate-buffered saline (PBS) (pH 7.4) containing 30% glycerol, dispensed at 1.5 μL per spot on the solid surface, and immobilized to the surface by incubating overnight at room temperature. This was followed by immersion in washing buffer (PBS [pH 7.8] containing 0.5% Tween 20) and gentle shaking to remove unbound antibody. The solid surface was blocked with PBS (pH 7.4) containing 3% bovine serum albumin for 1.5 h, washed for 10 min with washing buffer, and rinsed with distilled water. Water remaining on the solid surface was removed using filter paper.

Analysis of the Effects of cAMP and Phosphodiesterase Inhibitors on the Immobilization of Ec DOS. Expressed $(His)_6$-*Ec* DOS was labeled with Cy5 using the FluoroLink-Ab Cy5 labeling kit. Cy5-labeled $(His)_6$-*Ec* DOS (1 mg/mL) was mixed with cAMP (0.1 mM) alone, no addition, cAMP and IBMX (0.1 mM), or cAMP and etazolate hydrochloride (0.1 mM). The solutions were dispensed onto the anti-$(His)_6$ mAb-immobilized solid surface and incubated overnight at room temperature. The solid surface was washed to remove unbound proteins, rinsed with distilled water, and dried under a stream of N_2 gas.

Fluorescence Scanning. Fluorescence scanning of the protein microarray was performed using a ScanArray Express (Packard BioScience, Billerica, MA). Cy5-labeled proteins attached to the microarray were excited with a 633-nm laser. Emitted fluorescence was detected by a photomultiplier and converted into a 16-bit signal, giving a dynamic range of 0−65 535. The obtained signal intensities were quantitatively analyzed using Quantum Array software (Packard BioScience, Billerica, MA).

Analysis of the Effects of cAMP and Phosphodiesterase Inhibitors on the Interaction between Ec DOS and the PAS Fragment Using the Protein Microarray System. The methods for analyzing the interactions between *Ec* DOS and the PAS fragment were described in our previous report (*11*). Briefly, $(His)_6$-*Ec* DOS was dispensed on the anti-$(His)_6$ mAb-immobilized solid surface, after which the Cy5-labeled PAS fragment was added in the presence of cAMP (0.1 mM) alone, no addition, cAMP and IBMX (0.1 mM), or cAMP and etazolate hydrochloride (0.1 mM). After a 3-h incubation at room temperature, the unbound, Cy5-labeled PAS fragment was removed by washing. The surface was then dried under a stream of N_2 gas, and the fluorescence intensity was detected using a microarray scanner. This assay system is depicted in Figure 2A.

Analysis of the Effects of cAMP and Phosphodiesterase Inhibitors on the Interaction between Ec DOS and the PAS Fragment Using the Affinity Column Method. The effects of cAMP and phosphodiesterase inhibitors on the interaction between *Ec* DOS and the PAS fragment were also analyzed using a conventional affinity column method (*9*). Briefly, *Ec* DOS was immobilized on Ni-NTA columns, and the PAS fragment was added to the columns in the presence of cAMP (0.1 mM) alone, no addition, cAMP and IBMX (0.1 mM), or cAMP and etazolate hydrochloride (0.1 mM). After washing with 20 mM imidazole, proteins were eluted with 160 mM imidazole and analyzed by SDS−PAGE.

Analysis of the Effects of Site-Directed Mutations on the Immobilization of Ec DOS. Unlabeled $(His)_6$-*Ec* DOS (0.5 mg/mL of wild-type or H590A, H594A, H590A/H594A, and ΔN147 mutants) and an equal amount of Cy5-labeled, wild-type $(His)_6$-*Ec* DOS were dispensed on the anti-$(His)_6$ mAb-immobilized solid surface and incubated overnight at room temperature. Next, unbound proteins were removed by immersing the surface in washing buffer with gentle shaking. The solid surface was rinsed with distilled water and dried under a stream of N_2 gas. Because competitive binding of unlabeled *Ec* DOS (wild-type or mutants) reduces the immobilization of Cy5-labeled *Ec* DOS, decreases in fluorescence intensity reflect the affinities of wild-type or mutant *Ec* DOS for the anti-$(His)_6$ mAb-immobilized solid surface. This assay system is depicted in Figure 2B.

Identification of Amino Acid Residues Essential for the Interaction between Ec DOS and the PAS Fragment. Ec DOS mutants (H590A, H594A, H590A/H594A, and ΔN147) were dispensed on the anti-$(His)_6$ mAb-immobilized solid surface, and their interactions with PAS fragments were analyzed as described above. This assay system is depicted in Figure 2C.

RESULTS

Analysis of the Effects of cAMP and Phosphodiesterase Inhibitors on the Immobilization of Ec DOS. IBMX and etazolate hydrochloride, which have chemical structures similar to that of cAMP, inhibit *Ec* DOS phosphodiesterase activity (*7*). We first examined the effects of these two compounds on the interaction between *Ec* DOS and the PAS fragment. Because of the parallel analysis of the protein microarray, the same quantity of all target proteins should be immobilized on each spot. Therefore, we examined the quantity of Cy5-labeled $(His)_6$-*Ec* DOS immobilized in the presence or absence of these compounds to confirm that they do not interfere with the immobilization of *Ec* DOS (Figure 3A). The differences of fluorescence intensity in each condition were marginal (<7%; Table 1, column A). Given our previous results (*11*), this variability is almost within the experimental error. Thus, the presence of the phosphodiesterase inhibitors does not affect the efficiency of *Ec* DOS immobilization.

Analysis of the Effects of cAMP and Phosphodiesterase Inhibitors on the Interaction between Ec DOS and the PAS

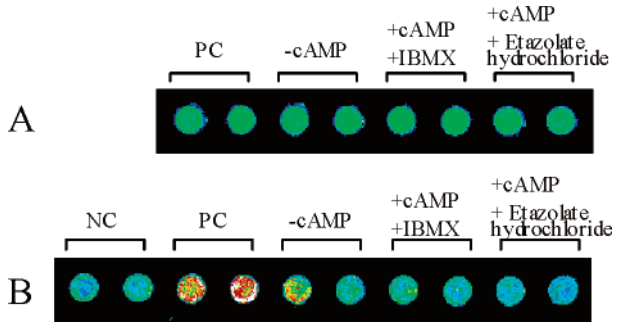

FIGURE 3: Effects of cAMP and phosphodiesterase inhibitors on the immobilization of *Ec* DOS and its interaction with the PAS fragment. (A) Cy5-labeled *Ec* DOS was immobilized on the solid surface via anti-$(His)_6$ mAb. (B) $(His)_6$-*Ec* DOS was immobilized on the solid surface via anti-$(His)_6$ mAb, and the interaction with the PAS fragment was examined. (A and B) Experiments were carried out in the presence of cAMP (PC), in the absence of cAMP (-cAMP), in the presence of cAMP and IBMX (+cAMP/+IBMX), or in the presence of cAMP and etazolate hydrochloride (+cAMP/+ Etazolate hydrochloride). The scanned images were converted into fluorescence intensities. The intensity of the negative control (NC) was subtracted from each sample (for panel B), and the fluorescence intensity of each spot was normalized so that the positive control (PC) intensity was 100. Experimental conditions and results are summarized in Table 1.

Fragment Using the Protein Microarray System. The effects of cAMP and phosphodiesterase inhibitors on the interaction between *Ec* DOS and the PAS fragment were investigated using the protein microarray system, as depicted in Figure 2A. A scanned image of the protein microarray and the experimental conditions for each spot are shown in Figure 3B and Table 1. Following the addition of the Cy5-labeled PAS fragment to spots blocked with bovine serum albumin and lacking *Ec* DOS, there was weak fluorescence (Figure 3B, leftmost two spots). This intensity was due to nonspecific binding and was taken as the negative control. The negative control intensity was subtracted from the intensity for each experimental condition and then normalized so that the positive control intensity was 100. The interaction efficiencies were 26% in the absence of cAMP, 12% in the presence of IBMX and cAMP, and 4% in the presence of etazolate hydrochloride and cAMP (Table 1, column B). These results suggest that cAMP is indispensable for the interaction between *Ec* DOS and the PAS fragment and two phosphodiesterase inhibitors, IBMX and etazolate hydrochloride, hindered the interaction.

Analysis of the Effects of cAMP and Phosphodiesterase Inhibitors on the Interaction between Ec DOS and the PAS Fragment Using the Affinity Column Method. To confirm the results obtained from the protein microarray system, we also examined the effects of cAMP and phosphodiesterase inhibitors on the interaction between *Ec* DOS and the PAS fragment using a conventional affinity column method. In the affinity column method, $(His)_6$-*Ec* DOS was immobilized to the Ni-NTA column, after which the PAS fragment was applied to the column. After washing with 20 mM imidazole, proteins were eluted with 160 mM imidazole and then analyzed by SDS−PAGE (Figure 4). The band corresponding to the PAS fragment (19 kDa) was observed in the positive control (cAMP only) at an intensity 43% of *Ec* DOS (93 kDa). The PAS fragment, however, was not detected in the absence of cAMP or when IBMX or etazolate hydrochloride was present along with cAMP (Table 2, column B). These results were consistent with those obtained from the protein microarray analysis, verifying the reliability of the protein microarray system. On the other hand, variable quantities of *Ec* DOS (maximum 65% variability) were eluted from the Ni-NTA column among the four different runs (Table 2, column A).

Analysis of the Effects of Site-Directed Mutations on the Immobilization of Ec DOS. In the above experiment, it appeared that cAMP was indispensable for the interaction between *Ec* DOS and the PAS fragment. These results reinforced the possibility that there is a relationship between the enzyme's interaction with the PAS fragment and its phosphodiesterase activity. On the basis of amino acid sequence homology with other cAMP phosphodiesterases, two histidine residues in *Ec* DOS, H590 and H594, are thought to play an important role in binding cAMP (*10*). Consistent with this, the three site-directed mutants, H590A, H594A, and H590A/H594A, are enzymatically inactive (*9*). Accordingly, we speculated that these amino acid residues would also be important for the interaction between *Ec* DOS and the PAS fragment.

Insertion of site-directed mutations may affect the affinity of *Ec* DOS for the anti-$(His)_6$ mAb-immobilized microarray. Therefore, we performed a competitive binding assay to assess the quantity of immobilized wild-type and mutant *Ec* DOS. In this experiment, unlabeled wild-type or mutant *Ec* DOS and an equal amount of Cy5-labeled wild-type *Ec* DOS were dispensed onto the anti-$(His)_6$ mAb-immobilized microarray, and their affinities for the anti-$(His)_6$ mAb were determined from the decreases in fluorescence intensity as depicted in Figure 2B. A scanned image of the protein microarray and the experimental conditions of each spot are indicated in Figure 5A and Table 3A. The fluorescence intensities were normalized so that the negative control (only Cy5-labeled *Ec* DOS) was 100. The fluorescence intensities of the wild-type and all mutants of *Ec* DOS were about 30% of the negative control, and the variability of their signals was within 4%, which is smaller than the experimental error (Table 3A). Therefore, it appeared that the introduction of site-directed mutations does not influence the affinity of $(His)_6$-*Ec* DOS for the anti-$(His)_6$ mAb.

Although equal amounts of Cy5-labeled and unlabeled *Ec* DOS were used in the experiment, the fluorescence intensity of the Cy5-labeled *Ec* DOS was 30% of the negative control, which indicates that the immobilized Cy5-labeled *Ec* DOS represented 30% of the total *Ec* DOS. Thus, the Cy5 labeling procedure or simply the presence of the Cy5 molecule may affect the affinity of the $(His)_6$-*Ec* DOS for the anti-$(His)_6$ mAb. However, this does not affect the ability to use the microarray to compare immobilization efficiencies.

Identification of Amino Acid Residues Essential for the Interaction between Ec DOS and the PAS Fragment. We further examined the effects of the site-directed mutations on the interaction between *Ec* DOS and the PAS fragment. The three mutants, H590A, H594A, and H590A/H594A, were immobilized on the solid surface, and their interactions with the PAS fragment were analyzed (Figure 2C). A scanned image of the protein microarray and experimental conditions for each spot are shown in Figure 5B and Table 3B. The negative control intensity was subtracted from the intensity for each spot and then normalized so that the wild-type intensity was 100. The interaction efficiency of H590A

Table 1: Effects of cAMP and Phosphodiesterase Inhibitors on the Interaction between *Ec* DOS and the PAS Fragment Was Assayed Using the Protein Microarray System

	Ec DOS (mg/mL)	cAMP (mM)	IBMX (mM)	etazolate hydrochloride (mM)	A: *Ec* DOS immobilization[a]	B: interaction with PAS fragment[b]
NC	0	0.1	0	0	-	0
PC	1	0.1	0	0	100 ± 1	100 ± 19
-cAMP	1	0	0	0	101 ± 3	26 ± 18
+cAMP +IBMX	1	0.1	0.1	0	107 ± 3	12 ± 9
+cAMP +etazolate hydrochloride	1	0.1	0	0.1	101 ± 4	4 ± 4

[a] Fluorescence intensities obtained from Figure 3A. [b] Fluorescence intensities obtained from Figure 3B.

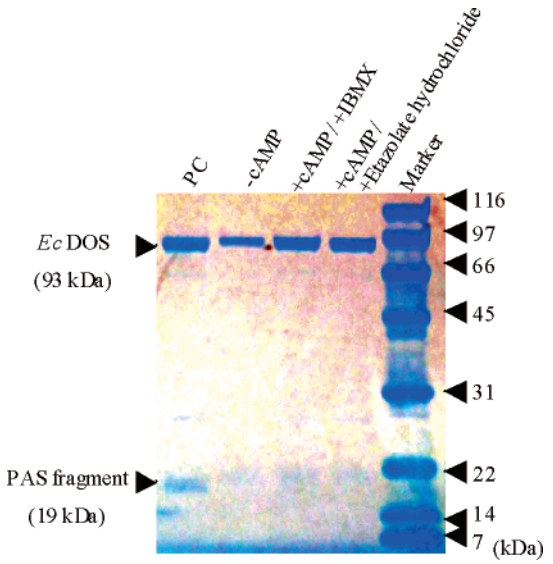

FIGURE 4: Effects ofcAMP and phosphodiesterase inhibitors on the interaction between *Ec* DOS and the PAS fragment using the affinity column method. $(His)_6$-*Ec* DOS was immobilized on a Ni-NTA column and the PAS fragment was applied. After washing with 20 mM imidazole, proteins were eluted with 160 mM imidazole and analyzed by SDS−PAGE. Signals of bands corresponding to *Ec* DOS (93 kDa) and PAS fragment (19 kDa) were normalized so that the *Ec* DOS signal for positive control (PC) was 100. Experimental conditions and results are summarized in Table 2.

and H594A was markedly decreased (50% of the wild-type signal), and the double mutant, H590A/H594A, gave an intensity of only 30% of the wild-type (Table 3B).

Interaction between Ec DOS Deletion Mutant ΔN147 and the PAS Fragment. ΔN147 is a truncated mutant of *Ec* DOS lacking the N-terminal 147 amino acids (Figure 1C). Using the same method depicted in Figure 2B, we determined that the quantities of immobilized wild-type and ΔN147 *Ec* DOS were not markedly different (Table 4A). Their interactions with the PAS fragment are summarized in Table 4B. Consistent with our previous findings (*9*), the ΔN147 mutant had almost the same affinity for the PAS fragment as the wild-type enzyme. These results suggest that the binding of cAMP to *Ec* DOS induces an interaction between the PAS fragment and the region of *Ec* DOS between amino acids 148 and 807.

DISCUSSION

Although not yet commonly used, protein microarray technology is expected to be a useful tool for the functional analysis of multiple proteins. Its use has been slowed by difficulties in retaining native structures and functions in immobilized proteins as well as variability in the quantity of the immobilized protein (*13*). As mentioned in our previous report (*11*), protein immobilization using anti-$(His)_6$ antibody can effectively maintain the native function of proteins on a solid surface. In conventional protein immobilization, the quantities of immobilized proteins usually depend on their size, amino acid sequence, and affinity for the solid surface. For example, Zhu et al. (*14*) reported a yeast proteome chip containing more than 6000 recombinant proteins, but the quantities of immobilized proteins varied from 10 to 950 fg. In the immobilization method used in the present study, the amount of anti-$(His)_6$ antibody is controlled in advance, which determines the amount of immobilized target protein. Therefore, we predicted that the amount of target protein can be kept constant for each condition, regardless of their molecular sizes or amino acid sequences.

Using a competitive binding (Figure 2B), we examined the immobilization efficiency of five kinds of $(His)_6$-fused proteins with different molecular sizes (48, 59, 71, 82, and 93 kDa) toward an anti-$(His)_6$ mAb-immobilized or bare solid surface. The variability in the immobilization efficiency among these five proteins was 7% for the anti-$(His)_6$ mAb-immobilized solid surface but 46% for the bare surface (Table S-1 and Figure S-1 in Supporting Information). This supports the idea that we can regulate the quantity of $(His)_6$-target proteins simply by adjusting the concentration of immobilized anti-$(His)_6$ antibody. Therefore, this method appears to solve the problems in regulating the amount of immobilized protein in microarrays. In addition, the quantities of immobilized proteins were not affected by chemical compounds or site-directed mutations.

Table 5 compares the performance of the affinity column and protein microarray methods for analysis of protein−protein interactions. Here, we discuss our current microarray technology; but some features, for example, the need for only small amounts of sample, are a universal advantage of microarray technology and are not limited to our method. The affinity column method requires 100 μg of each protein for one analysis, whereas the protein microarray uses only 1.5 μg. Generally, the detection sensitivity is proportional to the amount of protein; as the quantity of protein becomes smaller, its sensitivity becomes lower. However, the fluorescence detection used for the protein microarray is theoretically 10^6-fold more sensitive than Coomassie brilliant blue

Table 2: Effects of cAMP and Phosphodiesterase Inhibitors on the Interaction between *Ec* DOS and the PAS Fragment Was Assayed Using the Affinity Column Method

	Ec DOS (mg/mL)	cAMP (mM)	IBMX (mM)	etazolate hydrochloride (mM)	A: *Ec* DOS intensity	B: PAS fragment intensity
PC	1	0.1	0	0	100	43
-cAMP	1	0	0	0	61	<5
+cAMP	1	0.1	0.1	0	123	<5
+IBMX						
+cAMP	1	0.1	0	0.1	126	<5
+etazolate hydrochloride						

Table 3: Identification of Amino Acid Residues Essential for the Interaction between *Ec* DOS and the PAS Fragment

	A: *Ec* DOS immobilization			B: interaction with PAS fragment		
	Cy5-labeled *Ec* DOS (mg/mL)	unlabeled *Ec* DOS (mg/mL)	relative fluorescence intensity[a]	*Ec* DOS (mg/mL)	cAMP (mM)	relative fluorescence intensity[b]
NC	1	0	100 ± 3	0	0.1	0
WT	0.5	0.5	29 ± 1	1	0.1	100 ± 19
H590A	0.5	0.5	27 ± 2	1	0.1	50 ± 6
H594A	0.5	0.5	27 ± 1	1	0.1	55 ± 4
H590A/H594A	0.5	0.5	31 ± 2	1	0.1	30 ± 13

[a] Fluorescence intensities obtained from Figure 5A. [b] Fluorescence intensities obtained from Figure 5B.

Table 4: Effects of a Deletion Mutation on the Interaction between *Ec* DOS and the PAS Fragment

	A: *Ec* DOS immobilization			B: interaction with PAS fragment		
	Cy5-labeled *Ec* DOS (mg/mL)	unlabeled *Ec* DOS (mg/mL)	relative fluorescence intensity[a]	*Ec* DOS (mg/mL)	cAMP (mM)	relative fluorescence intensity[b]
NC	1	0	100 ± 3	0	0.1	0
WT	0.5	0.5	31 ± 1	1	0.1	100 ± 22
ΔN147	0.5	0.5	24 ± 22	1	0.1	114 ± 7

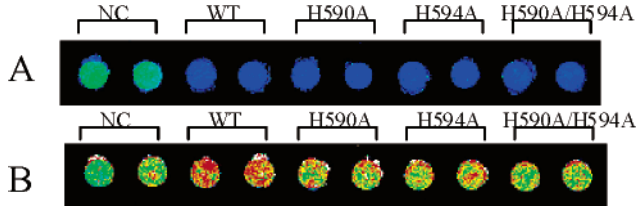

FIGURE 5: Effects of site-directed mutations on the immobilization of *Ec* DOS and its interaction with the PAS fragment. (A) Competitive binding experiment betweenCy5-labeled and unlabeled *Ec* DOS. Unlabeled $(His)_6$-*Ec* DOS (wild-type or mutants) and an equal amount of Cy5-labeled wild-type $(His)_6$-*Ec* DOS were immobilized on the solid surface via anti-$(His)_6$ mAb. Competitive binding of unlabeled *Ec* DOS reduces the immobilization of Cy5-labeled *Ec* DOS. Therefore, decreases in fluorescence intensity reflect the affinities of wild-type or mutant *Ec* DOS for the anti-$(His)_6$ mAb-immobilized solid surface. The scanned image was converted into fluorescence intensities for each spot, which were normalized so that the negative control (NC) intensity was 100. (B) *Ec* DOS (wild-type or mutants) was immobilized on the solid surface via anti-$(His)_6$ mAb, and the interaction with PAS fragment was investigated. The scanned image was converted into fluorescence intensities for each spot. The negative control (NC) intensity was subtracted from the value for each spot and then normalized so that the wild-type (WT) intensity was 100. Experimental conditions and results are summarized in Table 3.

staining, which is used in the affinity column method (*11*), and it remains sensitive even when very low amounts of protein are used. In addition, the affinity column method treats one sample at a time, whereas the protein microarray system can analyze multiple samples in parallel. This ability to analyze multiple samples can markedly reduce the

Table 5: Comparison between Conventional Affinity Column Method and Protein Microarray

	affinity column method	protein microarray
quantity of protein	100 μg	1.5 μg
sensitivity[a]	10 ng	10 fg
time required for analysis of 5 samples[b]	390 min	120 min

[a] Coomassie brilliant blue staining for affinity column method and fluorescence dye for the protein microarray. [b] For the affinity column method, the time required included the following: *Ec* DOS immobilization (20 min × number of samples), incubation with PAS fragment (20 min × number of samples), elution of protein (20 min × number of samples), preparation of samples for SDS−PAGE (60 min), and staining with Coomassie brilliant blue (30 min). For the protein microarray, time required included the following: *Ec* DOS immobilization (30 min), incubation with PAS fragment (60 min), and fluorescence detection (30 min). Preparation time for the Ni-NTA column (for affinity column method) and for the anti-$(His)_6$-mAb-immobilized microarray (for the protein microarray) are not included.

variability in the quantity of *Ec* DOS levels between samples as well as the time consumed for the experiment. Indeed, to analyze five samples, 390 min are needed for the affinity column method, whereas 120 min are needed for the protein microarray system. Therefore, the protein microarray analysis appears to have substantial advantages over the conventional affinity column method with respect to the quantity of protein, sensitivity, variability in the amount of immobilized protein, and time required to complete the assay.

Other detection methods for protein microarrays have been reported, including SPR, mass spectrometry, radioisotope labeling, and chemiluminescence. SPR (*15*) and mass spectrometry (16) do not require labeling of the protein, but their sensitivity is lower than labeling methodologies. In addition, mass spectrometry is low-throughput, expensive, and useful only for studying a limited number of samples. Radioisotope labeling has health and safety issues and requires a long detection time (*17*). Although chemiluminescence has not been widely used for protein microarray analysis, it has excellent sensitivity and speed of detection (*18*). We believe that our microarray technology using fluorescence labeling is also advantageous with respect to speed of detection and multiplicity. However, it would be possible to adopt other detection methodologies if necessary.

The protein microarray system clearly showed that cAMP, a substrate of *Ec* DOS phosphodiesterase activity, and two amino acid residues, H590 and H594, play important roles in the interaction between *Ec* DOS and the PAS fragment. This interaction was strongly inhibited by the addition of the two phosphodiesterase inhibitors, IBMX and etazolate hydrochloride, and by the His mutations. We also confirmed that the *Ec* DOS ΔN147 mutant, which shows a phosphodiesterase activity comparable to the wild-type enzyme, interacts with the PAS fragment as effectively as the wild-type protein. These results strongly suggest that there is a relationship between the interaction with the PAS fragment and the phosphodiesterase activity of *Ec* DOS. It is likely that the binding of cAMP induces the interaction between the PAS fragment and the region of *Ec* DOS between amino acids 148 and 807. We speculate that this interaction would cause a shift in the C-terminal phosphodiesterase domain from a conformational structure that is unfavorable for catalysis to one that favors catalysis.

Generally, cAMP binds to phosphodiesterase in the presence of divalent cations, such as Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, or Zn^{2+} (*19*). In our previous report, we showed that Mg^{2+} is the most effective divalent cation for supporting catalysis by *Ec* DOS (*7*). Interestingly, based on amino acid sequence homology with other cAMP phosphodiesterases (*10*), H590 and H594 in *Ec* DOS are predicted to be located in the Mg^{2+}-binding region and are predicted to work in concert to bind a single Mg^{2+} ion. We found that the H590A and H594A mutants bind the PAS fragment at 50% of the level of the wild-type enzyme, but that the H590A/H594A double mutant had only 30% binding of the PAS fragment (Table 3). This implied that H590 and H594 may play independent roles in Mg^{2+} binding, but that their combination is necessary for the full binding of Mg^{2+}.

The interaction efficiencies of site-directed mutants H590A, H594A, and H590A/H594A with the PAS fragment were 30−50% of the wild-type, whereas cAMP-deficient or inhibitor-treated *Ec* DOS showed about 5−25% of wild-type binding. This raises the possibility that there are other Mg^{2+}-binding regions in *Ec* DOS. In fact, structural analysis suggests that other cAMP phosphodiesterases possess two Mg^{2+}-binding regions (*10*). Therefore, it may be possible that *Ec* DOS also has other amino acid residues that are important for Mg^{2+} binding. In this study, we mutated only one Mg^{2+}-binding site, and therefore, the interaction with the PAS fragment was not completely abolished.

The reason why the isolated PAS fragment can bind and activate *Ec* DOS remains elusive. There are more than 3000 PAS proteins known in nature (*20*), some of which consist of only a PAS domain, such as *E. coli* TraJ (*21*). However, little is known about their physiological functions. It is speculated that isolated PAS domains or the simple PAS protein may act physiologically to activate or inactivate other protein partners in a manner similar to calcium-bound calmodulin (*22*). Also, the existence of proteins that consist of only a PAS domain suggests that there are other proteins resembling the PAS fragment in *E. coli* that can bind to *Ec* DOS and enhance its activity. Further studies are needed to understand the mechanism of the catalytic stimulation by the PAS fragment and other protein partners.

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SUPPORTING INFORMATION AVAILABLE

Immobilization efficiencies of five proteins with different sizes investigated by competitive binding assay (Figure S-1 and Table S-1). This material is available free of charge via the Internet at http://pubs.acs.org.

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