Accelerated Articles

Protein Microarray System for Detecting Protein–Protein Interactions Using an Anti-His-Tag Antibody and Fluorescence Scanning: Effects of the Heme Redox State on Protein–Protein Interactions of Heme-Regulated Phosphodiesterase from *Escherichia coli*

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A highly sensitive microarray system for detecting protein–protein interactions has been developed. This method was successfully applied to analyze the interactions of heme-regulated phosphodiesterase from *Escherichia coli* (*Ec DOS*). To immobilize (His)$_6$-Tag fused *Ec DOS*, anti-(His)$_6$-Tag monoclonal antibody (anti-(His)$_6$-Tag mAb) was initially immobilized on the solid surface, and (His)$_6$-Tag fused *Ec DOS* was fixed by antigen–antibody interactions. For this experiment, ProteoChip, generally suitable for antibody immobilization, was used as solid substrate. In this report, we confirm the antibody immobilization ability of ProteoChip and specific binding to the F(c) region of the antibody. Based on this finding, interdomain interactions between *Ec DOS* and the isolated heme-bound PAS domain were investigated on the solid surface. *Ec DOS* immobilized via anti-(His)$_6$-Tag mAb maintained interactions with the PAS fragment, in contrast to directly immobilized *Ec DOS* in the absence of anti-(His)$_6$-Tag mAb. Heme-redox-sensitive interactions between *Ec DOS* and the PAS fragment were additionally detected using anti-(His)$_6$-Tag mAb as a mediator. Our results collectively suggest that the immobilization method using anti-Tag antibody is suitable for maintaining native protein characteristics to facilitate elucidation of their structures and functions on solid surfaces.

Microarrays of immobilized biological molecules on solid surfaces have undergone rapid development in recent biotechnological research efforts. Progress in DNA arrays has allowed the analysis of expression patterns of multiple genes in a single experiment. Protein microarray is another useful tool for the concomitant analysis of a large number of proteins. However, this technology is not yet well established. One of the reasons for this lag is that proteins are chemically and structurally more complicated than DNA systems.

A number of immobilization techniques of proteins on solid surfaces have been proposed. For example, physical adsorption, covalent binding, and chemical binding methods, including avidin–biotin interactions, are well documented. The physical adsorption method immobilizes proteins via hydrophobic interactions, whereas immobilization by the covalent binding method involves forming covalent linkages between proteins and functional groups on the solid surface. These methods are simple and widely

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used. However, there are drawbacks in that orientations of attached proteins cannot be controlled, and thus the observed interactions often do not reflect the functions of native proteins. Although the chemical binding method can control orientation, proteins must be chemically modified before immobilization. These modifications often influence protein function. Recently, thin films of porous hydrophilic matrix, such as polyacrylamide or agarose gels, have been used for the immobilization of proteins. These are usually designated ‘3D arrays’ and avoid denaturation of immobilized proteins due to their homogeneous aqueous environment. However, dispensed protein solutions are easily spread in the porous matrix in 3D arrays. Consequently, it is difficult to control the size and shape of protein spots. In addition, contamination to adjacent spots frequently occurs during the washing steps. Thus, novel protein immobilization methods are required to solve these problems and establish protein microarray as an efficient technology.

In the present study, we developed a novel protein immobilization method using recombinant Tag fusion proteins. Tag peptides used for purification are fused to the N- or C-terminal regions of target recombinant proteins. Antibodies against Tags are immobilized onto the solid surface, and target fusion proteins are captured by antigen—antibody interactions between Tag and immobilized anti-Tag antibody. It was expected that target proteins would maintain their native structure and function, since proteins are immobilized loosely and indirectly on the solid surface via anti-Tag antibodies. Moreover, overexpression of proteins with a Tag is commonly employed. Therefore, this immobilization method using an anti-Tag antibody may be effective and highly sensitive.

The model protein, heme-regulated phosphodiesterase from *Escherichia coli* (Ec DOS), is a heme-containing signal transduction protein. Ec DOS is composed of an N-terminal heme-containing PAS domain and a C-terminal phosphodiesterase domain. The redox state of the heme cofactor (Fe²⁺ or Fe³⁺) in the PAS domain regulates phosphodiesterase activity on cAMP. Specifically, the Fe²⁺ protein performs catalysis, while the Fe³⁺ protein is catalytically ineffective. This intramolecular signal transduction is induced by redox-regulated structural changes in the PAS domain, which possibly trigger alteration of the Ec DOS phosphodiesterase domain structure. Addition of the isolated heme-bound PAS fragment (Figure 1, lower picture) to full-length Ec DOS (Figure 1, upper picture) enhances phosphodiesterase activity by 5-fold.

The results obtained are consistent with our previous biochemical findings.

**EXPERIMENTAL SECTION**

**Materials.** ProteoChip, prepared by coating an aminated slide glass with calixclown-5 derivatives, was obtained from Proteogen Inc. (Seoul, Korea). Spotting seal manufactured by Swasho Techno (Nagano, Japan) was designed to contain 48 small holes (2-mm diameter) on 25 mm × 55 mm of the poly(vinyl chloride) seat. FITC-conjugated anti-mouse IgG F(c) fragment, unlabeled mouse IgG F(ab) fragment, and unlabeled mouse IgG F(ab) fragment were purchased from Rockland (Gilbertsville, PA). The FluoroLink-Ab Cy5 labeling kit was obtained from Amersham Biosciences (Buckinghamshire, U.K.), and mouse anti-(His)₆-Tag 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).

**Preparation of Ec DOS and Ec DOS–PAS.** Ec DOS and the isolated PAS fragment containing (His)₆-Tag at the N-termini were expressed in *E. coli* BL21 (DE3) and purified, as described previously. Briefly, harvested cells were broken by sonication and centrifuged at 10000g, and protein was collected by ammonium sulfate precipitation. Ammonium sulfate was removed using a Sephadex G25 column, and proteins were purified by Ni-NTA affinity chromatography. Purity of the proteins was confirmed by SDS–PAGE.

**Competitive Immobilization of Antibody Fragments.** Spotting seal was initially adhered to a solid substrate (ProteoChip). A mixture of 1 μg/ml FITC-labeled mouse IgG F(ab)₂ fragment and 10–1000 μg/ml unlabeled mouse IgG F(ab)₂ fragment was prepared in PBS (pH 7.4) containing 30% glycerol. The mixture was dispensed at 1.5 μL/spot on the solid surface and incubated overnight for immobilization. This was followed by immersion in washing buffer (PBS, pH 7.8, containing 0.5% Tween 20) and gentle shaking for 10 min to remove unbound antibody fragments. The solid surface was rinsed with distilled water and dried with an N₂ gas stream.

**Fluorescence Scanning.** Fluorescence scanning of the protein microarray was performed using ScanArray Express (Packard BioScience, Billerica, MA), and intensity was analyzed with Quantum Array software (Packard BioScience).

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Preparation of Anti-(His)_{6}Tag mAb Immobilized Microarray. Spotting seal was initially adhered to the solid substrate (ProteoChip). Mouse anti-(His)_{6}Tag mAb was diluted to 100 μg/mL with PBS containing 30% glycerol, dispensed at 1.5 μL/spot on the solid surface, incubated overnight at room temperature for immobilization on the solid surface, and gently washed for 10 min to remove unbound antibody. This was followed by blocking with PBS (pH 7.4) containing 3% BSA for 1.5 h, washing for 10 min with washing buffer, and rinsing with distilled water. Water remaining on the solid surface was removed using filter paper.

Immobilization of Ec DOS on the Microarray. Ec DOS was diluted with dilution buffer (50 mM Tris-HCl, pH 8.5, containing 10% BSA, 30% glycerol) and dispensed at 1.5 μL/spot on an anti-(His)_{6}Tag mAb-immobilized solid surface. Following overnight incubation at room temperature, Ec DOS was fixed on the solid surface via a (His)_{6}Tag at the N-terminus and washed with buffer A (50 mM Tris-HCl, pH 8.5, containing 0.1 mM cAMP and 2 mM MgCl_{2}) for 10 min.

In parallel, Ec DOS was immobilized directly on the solid surface without anti-(His)_{6}Tag mAb. In this case, the protein was diluted with PBS containing 30% glycerol, dispensed at 1.5 μL/spot on the solid surface, followed by overnight incubation at room temperature, and washing with PBS (pH 7.8) for 10 min. Next, the surface was blocked with PBS (pH 7.4) containing 3% BSA for 1.5 h and washed for 10 min with buffer A.

Interactions between Ec DOS and Ec DOS—PAS (Reduced Fe^{2+} Form). The Ec DOS-immobilized microarray was immersed in buffer A containing 1 mM sodium dithionite to reduce the heme cofactor. The expressed PAS fragment was digested in advance with thrombin to remove the (His)_{6}Tag at the N-terminus and labeled with Cy5 using the FluoroLink-Ab Cy5 labeling kit. The Cy5-labeled PAS fragment was reduced using 10 mM sodium dithionite and diluted with 50 mM Tris-HCl (pH 8.5) containing 10% BSA, 30% glycerol, 0.1 mM cAMP, and 2 mM MgCl_{2}. An amount of 1.5 μL/spot was dispensed on the Ec DOS-immobilized microarray, incubated for 3 h at room temperature, washed with buffer A for 20 min, and dried with N_{2} gas stream. The experiment was performed in a glove bag filled with N_{2} gas to maintain anaerobic conditions. Fluorescence intensity was detected using a microarray scanner.

Determination of the Quantity of Immobilized Ec DOS. (His)_{6}Tag fused Ec DOS was labeled with Cy5 using the FluoroLink-Ab Cy5 labeling kit and immobilized on the solid surface with or without the anti-(His)_{6}Tag mAb, as described above. The quantity of immobilized Ec DOS was analyzed from fluorescence intensity.

Redox-State Dependency of Interactions between Ec DOS and the PAS Fragment. Interactions of oxidized (Fe^{3+}) Ec DOS were analyzed using the method for reduced Ec DOS under aerobic conditions with no reduction by sodium dithionite. Moreover, to confirm the oxidation state, an equimolar amount of potassium ferricyanide was added to immobilized Ec DOS and Cy5-labeled PAS fragment.

RESULTS AND DISCUSSION

Solid Substrate (ProteoChip) Binds the Antibody F(c) Region. A previous study has shown that the surface of ProteoChip immobilizes antibody in the orientation specifically bound with the F(c) region and leaves the paratope region free to interact with antigen. This immobilization is dependent on host-guest interactions between ProLinker, a novel calixcrown derivative coated on ProteoChip, and amino cations on the protein surface. To evaluate this ability of ProteoChip, the mixture of FITC-labeled mouse IgG F(c) and unlabeled mouse IgG F(c) or IgG Fab fragment was dispensed, and fluorescence intensities of each spot were plotted versus competitor concentration. Competition between FITC-labeled F(c) and unlabeled F(c) fragment, and FITC-labeled F(c) and unlabeled F(c) fragments, are shown by the solid line and broken line, respectively.

Figure 2. Ability of ProteoChip to bind the antibody F(c) region. (a) Left: illustration of the IgG molecule, Right: competitive binding experiment between FITC-labeled F(c) fragment and unlabeled F(c) or Fab fragment. (b) Scanning images of protein microarray: competition between FITC-labeled F(c) and unlabeled F(c) fragments (upper picture), and FITC-labeled F(c) and unlabeled F(c) fragments (lower picture). (c) Scanning images were analyzed using QuantumArray software, and fluorescence intensities of each spot were plotted versus competitor concentration. Competition between FITC-labeled F(c) and unlabeled F(c) fragment, and FITC-labeled F(c) and unlabeled F(c) fragments, are shown by the solid line and broken line, respectively.

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ProteoChip recognized the F(c) region more effectively than the Fab region. ProteoChip is manufactured by coating an aminated slide glass with ProLinker, a novel calixcrown-5 derivative. The antibody immobilization ability of ProLinker was investigated using AFM and QCM by Lee et al. They suggested that antibodies immobilized on the ProLinker-coated surface form a homogeneous monolayer with the ideal orientation.

For immunoassays on solid surfaces, the orientation of immobilized antibody is important to maintain antigen binding activity. Biotinylation of antibody is usually required for immobilization on the avidin-coated surface in an ordered orientation. However, antibody modification is time-consuming and may impair activity. In the present study, we unequivocally demonstrate that the surface of ProteoChip specifically binds the F(c) region of the antibody. This finding strongly supports Lee’s explanation that the ProLinker immobilizes antibody in the ideal orientation without protein modification. Accordingly, we employ ProteoChip to immobilize target proteins via anti-Tag antibody as a mediator.

Effects of Anti-(His)_{6}-Tag mAb on Interactions between Ec DOS and the PAS Fragment. We investigated the effects of anti-(His)_{6}-Tag mAb on protein immobilization. Anti-(His)_{6}-Tag mAb was initially immobilized on the solid surface, and (His)_{6}-Tag fused Ec DOS was fixed by antigen–antibody interactions between Tag and anti-Tag mAb. In parallel, Ec DOS was immobilized directly onto the solid surface in the absence of anti-(His)_{6}-Tag mAb. The Cy5-labeled PAS fragment was dispensed to each spot at a concentration of 200–800 µg/mL, and fluorescence intensities were compared (Figure 3). Following the addition of Cy5-labeled PAS fragment to empty spots where Ec DOS was not immobilized and only blocking treatment with BSA was performed, fluorescence intensity was increased slightly in a concentration-dependent manner (Figure 3b, d; lower picture). This intensity was dependent on nonspecific binding, and therefore taken as a negative control. Scanning images of the protein microarray are depicted in Figure 2b (with anti-(His)_{6}-Tag mAb) and d (in the absence of anti-(His)_{6}-Tag mAb). Fluorescence intensity from which the negative control is subtracted is shown in Figure 3e. Following the addition of 800 µg/mL Cy5-labeled PAS fragment, the fluorescence intensity of Ec DOS immobilized via anti-(His)_{6}-Tag antibody was 24 000. On the other hand, Ec DOS directly immobilized without anti-(His)_{6}-Tag mAb displayed a lower intensity of 4600. Note that fluorescence intensities described in this paper are relative values.

Determination of the Quantity of Immobilized Ec DOS. To confirm the amount of immobilized Ec DOS, the quantities of Cy5-labeled Ec DOS immobilized with or without anti-(His)_{6}-Tag mAb were compared. In Figure 3, we employed 1 mg/mL Ec DOS for immobilization and analyzed interactions with the PAS fragment. The fluorescence intensity of directly immobilized Ec DOS (1 mg/mL) was 2.5 times higher than that of indirectly immobilized Ec DOS using anti-(His)_{6}-Tag mAb (Figure 4c and d).
These results indicate that the amount of directly immobilized Ec DOS is higher than that of indirectly immobilized Ec DOS. Accordingly, it is proposed that directly immobilized Ec DOS cannot maintain interactions with the PAS fragment on the solid surface despite its larger quantity, in contrast to Ec DOS immobilized via anti-(His)_6-Tag mAb.

The number of immobilized protein molecules is dependent on molecular size. Specifically, as molecular size increases, the number of immobilized molecules decreases. The molecular size of Ec DOS is 92 kDa (60% that of IgG (150 kDa)). The amount of Ec DOS immobilized via anti-(His)_6-Tag mAb depends on the quantity of immobilized IgG. Therefore, it is appropriate that the quantity of Ec DOS immobilized via anti-(His)_6-Tag mAb is less than that of directly immobilized protein.

Previous studies have suggested that antibody bound to the ProLinker-coated surface forms a monolayer. It is predicted that Ec DOS is also immobilized as a monolayer via (His)_6-Tag at the N-terminus, maintaining the same orientation. Plausible models of immobilized Ec DOS with or without anti-(His)_6-Tag mAb are depicted in Figure 5. As shown in Figure 5b, tight binding to the solid surface may induce protein denaturation. This problem may be avoided by using anti-(His)_6-Tag mAb as a mediator (Figure 5a), thus maintaining the structure and function of Ec DOS on the solid surface.

Concentration Dependence of Interactions between Ec DOS and the PAS Fragment. The concentration dependence of interactions between Ec DOS and Cy5-labeled PAS fragment was investigated to confirm specificity. We show that the quantity of immobilized Ec DOS increases in a concentration-dependent manner, from 10 μg/mL to 1 mg/mL (Figure 4). A constant concentration (500 μg/mL) of Cy5-labeled PAS fragment was added to the spots on which 10 μg/mL, 100 μg/mL, or 1 mg/mL Ec DOS was immobilized, and fluorescence intensity was monitored. Fluorescence intensity increased with increasing concentrations of immobilized Ec DOS (Figure 6). Based on the data, we propose that interactions between Ec DOS and the Cy5-labeled PAS fragment are dependent on the quantity of immobilized Ec DOS.

Redox-State Dependence of Interactions between Ec DOS and the PAS Fragment. Ec DOS containing reduced heme (the Fe^{2+} complex) binds the PAS fragment, in contrast to enzyme-containing oxidized heme (the Fe^{3+} complex). In the present study, we attempted to detect heme redox-dependent interactions between Ec DOS and the PAS fragment on the solid surface. Scanning images (Fe^{2+} and Fe^{3+} Ec DOS) are illustrated in Figure 7a and b, respectively. The fluorescence intensity from which negative control was subtracted is shown in Figure 7c. Upon the
addition of 1000 µg/mL Cy5-labeled PAS fragment, the fluorescence intensity of Fe²⁺ Ec DOS was 32 000, while that of Fe³⁺ Ec DOS was only 7700. Here, note again that these fluorescence intensities are relative values. These results are consistent with our previous data, confirming that Ec DOS maintains its redox-dependent character. In an earlier study, interactions between Fe³⁺ Ec DOS and PAS fragment were evaluated as almost zero. However, we detected very weak interactions of Fe³⁺ Ec DOS in this experiment.

In a previous investigation, we employed the “affinity column method” to analyze these interactions. Fe²⁺ or Fe³⁺ Ec DOS was incubated with Ni-NTA agarose and packed into a small syringe, and nontagged PAS fragment was loaded on the column. The column was washed with 20 mM imidazole-containing buffer, and the Ec DOS–PAS fragment complex was eluted with 160 mM imidazole. The eluted solution was analyzed by SDS–PAGE and observed by CBB staining (with a detection limit of 10 ng). In our experiment, the Cy5-labeling efficiency of PAS (Cy5-dye: protein ratio) was 1:1. Therefore, theoretically we can detect 3 × 10⁵ molecules (10 fg) of Cy5-labeled PAS/spot with a diameter of 2 mm, with 10⁶-fold higher sensitivity than CBB staining. Here we observed very weak interactions of Fe²⁺ Ec DOS in this experiment.

In a previous investigation, we employed the “affinity column method” to analyze these interactions. Fe²⁺ or Fe³⁺ Ec DOS was incubated with Ni-NTA agarose and packed into a small syringe, and nontagged PAS fragment was loaded on the column. The column was washed with 20 mM imidazole-containing buffer, and the Ec DOS–PAS fragment complex was eluted with 160 mM imidazole. The eluted solution was analyzed by SDS–PAGE and observed by CBB staining (with a detection limit of ~10 ng). In the present study, we use fluorescence dye for detection. According to the manufacturer, the scanner can detect 0.1 fluorescence molecule/µm². In our experiment, the Cy5-labeling efficiency of PAS (Cy5-dye: protein ratio) was ~1:1. Therefore, theoretically we can detect 3 × 10⁵ molecules (10 fg) of Cy5-labeled PAS/spot with a diameter of 2 mm, with 10⁶-fold higher sensitivity than CBB staining. Here we observed very weak interactions of Ec DOS under aerobic conditions, which were not detected with the previous method.

There are two possibilities on why interactions between Ec DOS and the PAS fragment occur under aerobic conditions. Oxidized Ec DOS may interact with the PAS fragment very weakly, or a very small amount of reduced Ec DOS is mixed in solution despite aerobic conditions. The latter possibility is more plausible, since reduced Ec DOS is sometimes observed under aerobic conditions in crude lysates immediately following sonication. Moreover, the oxidized form is thought to be in equilibrium with the reduced form. To examine the latter possibility, we treated immobilized Ec DOS and Cy5-labeled PAS fragment with an oxidizing agent, potassium ferricyanide, and investigated the interaction between them (Figure 7d and e). Upon the treatment, there was almost no interaction seen, suggesting that a trace amount of reduced form was mixed in the so-called “oxidized form” in the reaction solution. Therefore, the very weak interaction under aerobic conditions that was not monitored by any biochemical methods can be efficiently detected by the present system with high sensitivity.

In the present study, we demonstrate that Ec DOS maintains its redox-state dependency on the solid surface. This finding indicates that Ec DOS immobilized via anti-(His)₆-Tag mAb maintains its native structure and function. Moreover, the sensitivity of the fluorescence dye system is 10⁶-fold higher than that of the conventional method. Our novel analysis system may be employed to investigate other unknown interactions of Ec DOS. The redox state of wild-type Ec DOS also regulates enzymatic activity. Therefore, there may be a specific relationship between protein–protein interactions and Ec DOS activity. Moreover, the multimerization state of Ec DOS affects enzymatic activity. Wild-type and some mutant Ec DOS enzymes that form tetramers are catalytically active, whereas monomeric mutants lose activity. The relationship between tetramer formation and catalytic activity is intriguing. We aim to investigate these unknown interactions using the novel protein analysis method presented here and elucidate...
the relationship between protein interactions and enzymatic activity. Our system may also be applied to determine other characteristics of Ec DOS, including enzymatic activity.

CONCLUSIONS

In the present study, we employed an efficient protein immobilization strategy using anti-Tag antibody and a superior solid substrate, ProteoChip, to analyze interactions between Ec DOS and the PAS fragment. We initially investigated the antibody immobilization ability of ProteoChip and showed that its surface specifically binds the F(c) region of antibody. Accordingly, it was expected that ProteoChip would immobilize antibody in the ideal orientation and leave the paratope region free to interact with antigen. Based on this finding, we immobilized a model protein, Ec DOS, via anti-(His)6-Tag mAb on ProteoChip and detected interactions between Ec DOS and PAS fragment, which were not observed when Ec DOS was directly immobilized without anti-(His)6-Tag mAb. Ec DOS immobilized via anti-(His)6-Tag mAb also maintained its redox-state dependency. Based on these results, we propose that the protein immobilization method using anti-Tag antibody is effective to maintain the native structures and functions of Ec DOS. Since protein expression with a Tag is a commonly used procedure, this immobilization method using anti-Tag antibody is a potentially useful strategy. Additionally, we observed very weak interactions of Ec DOS under aerobic conditions for the first time, due to highly sensitive fluorescence detection. The cellular redox state is very important for all organisms to maintain life, and signal transduction is often regulated by interactions of biomolecules. Therefore, analysis of redox-dependent protein—protein interactions of Ec DOS is essential to understand the cellular mechanisms of E. coli.

At present, there is no single method that is best suited for all proteins or applications of protein microarrays. Therefore, it is anticipated that many methods will be developed for protein microarray applications. In this study, we have demonstrated that a combination of anti-Tag antibody and ProteoChip is effective to maintain Ec DOS structure and function. However, further experiments are required to prove the potential of this method.

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; IgG, immunoglobulin; FITC, fluorescein isothiocyanate; Cy5, cyanine5; PBS, phosphate-buffered saline; BSA, bovine serum albumin; AFM, atomic force microscope; QCM, quartz crystal microbalance; CBB, Coomassie Brilliant Blue.

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