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LETTER

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Controlled and Oriented Immobilization of Protein by Site-Specific Incorporation of Unnatural Amino Acid

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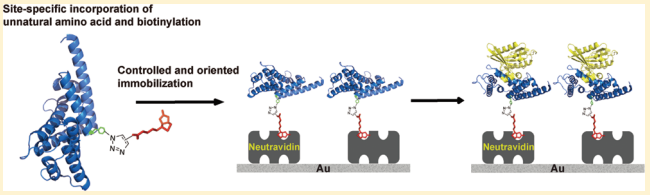
Supporting Information

ABSTRACT: Immobilization of proteins in a functionally active form and proper orientation is crucial for effective surface-based analysis of proteins. Here we present a general method for controlled and oriented immobilization of protein by site-specific incorporation of unnatural amino acid and click chemistry. The utility and potential of this method was demonstrated by applying it to the analysis of interaction between a pathogenic protein DrrA of *Legionella pneumophila* and its binding partner Rab1 of human. Kinetic analysis of Rab1 binding onto the DrrA-immobilized surfaces using surface plasmon resonance revealed that immobilization of site-specifically biotinylated DrrA results in about 10-fold higher sensitivity in binding assay than the conventional immobilization of DrrA with random orientation. The present method is expected to find wide applications in the fields of the surface-based studies of protein−protein (or ligand) interactions, drug screening, biochip, and single molecule analysis.



Protein immobilization is a critical step in the surface-based analysis of protein−protein (or ligand) interactions, including microarray-based proteome analysis,[1−4] single molecule study,[5,6] biochip,[7,8] and drug screening.[9,10] Covalent attachment methods using functional side groups of protein (amine, carboxyl, thiol, etc) have been widely used for protein immobilization to solve the problem of weak attachment in the traditional physical adsorption methods. Furthermore, various indirect attachment methods using chemical cross-linkers or terminal fusion tags through NHS-biotin, biotin ligase, His-tag, or oligonucleotide-mediated immobilization have been developed.[11,12] Attachment using protein A or protein G also has been investigated for improved antibody orientation with minimum inactivation.[13,14] The immobilization method of proteins by azidophenylalanine-mediated photochemical cross-linking was recently reported.[15,16] Most of these methods, however, still provide limited orientation (restriction to N- or C-terminal modification) and applications to antibodies, and they have some problems of a loss of activity and low binding efficiency mainly due to uncontrolled immobilization and random modification.[17−20] Hence, despite many advances, immobilization of proteins in a controlled and oriented manner still remains a challenge.

Here, we report a general strategy for controlled and oriented immobilization of protein by site-specific incorporation of unnatural amino acid and click chemistry. We demonstrate the efficacy and potential of this method by applying it to the analysis of interaction between a pathogenic protein DrrA of *Legionella pneumophila* and its binding partner Rab1 of human. Rab1 is known to be essential for regulating intracellular vesicle transport from endoplasmic-reticulum (ER) to Golgi, serving as a target for *L. pneumophila* infection and proliferation.[21,22] The *L. pneumophila* virulence protein DrrA has dual functionality toward Rab1, and the interaction between DrrA and Rab1 is vital in the pathogenesis of *Legionella* infection. We incorporated site-specifically *ρ*-azido-L-phenylalanine (AzPhe) into DrrA using engineered *Methanococcus jannaschii* tyrosyl-tRNA synthetase (AzPheRS) and tyrosyl-tRNA$_{CUA}$ (MjtRNA$_{CUA}$) as described elsewhere.[23,24] The resulting protein was conjugated with biotin by click chemistry and immobilized on the avidin-modified surface. To demonstrate the effects of oriented immobilization on the efficacy and binding kinetics, surface plasmon resonance (SPR) analysis was conducted for Rab1 binding onto the surfaces immobilized with site-specifically biotinylated and randomly biotinylated DrrA molecules.

EXPERIMENTAL SECTION

Materials. *ρ*-Azido-L-phenylalanine was purchased from Bachem. Biotin-alkyne was obtained from Invitrogen. Sulfo-NHS-Biotin and NeutrAvidin were purchased from Thermo Scientific. Albumin (bovine-biotin labeled) and HABA/avidin reagent were purchased from Sigma. All other reagents were of analytical grade.

Cloning and Mutagenesis. DNA fragments encoding DrrA (317-545, denoted as DrrA) and human Rab1a (1-179, denoted as Rab1) were inserted between *NdeI* and *XhoI* restriction sites of the plasmid pET21a (Novagen). The resulting DrrA and Rab1 had hexahistidine tag at the C-terminus. Asn-364 of DrrA was changed to an amber codon (TAG) for incorporation of unnatural amino acid *ρ*-azido-L-phenylalanine. Engineered *Methanococcus jannaschii* tyrosyl-tRNA synthetase[23] (AzPheRS) was inserted into pRSF-

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Duet-1 (Novagen), resulting in pRSF-AzPheRS. *Methanococcus jannaschii* tyrosyl-$tRNA_{CUA}$ ($MjtRNA_{CUA}$) gene[23] was inserted into pACYC184 vector (New England Biolabs) instead of tetracycline resistance gene, and the resulting construct was designated pTech-$MjtRNA_{CAU}$.

Incorporation of Unnatural Amino Acid and Protein Expression. To incorporate ρ-azido-L-phenylalanine into DrrA, pET21a-DrrA was transformed into *E. coli* BL21 (DE3) cells harboring the plasmids pRSF-AzPheRS and pTech-$MjtRNA_{CAU}$. Cells were cultivated in M9 minimal media containing 1% glycerol, 100 μg/mL carbenicillin, 50 μg/mL kanamycin, and 50 μg/mL chloramphenicol. Cells were grown at 37 °C until OD_{600} reached around 0.6, followed by addition of 0.5 mM IPTG and 1 mM ρ-Azido-L-phenylalanine. After cultivation at 18 °C for 60 h, cells were harvested and lysed. The cell lysate was clarified by centrifugation at 16 000*g* at 4 °C for 30 min, followed by filtration. Filtrated crude extract was mixed with 500 μL of Ni-NTA superflow (Qiagen) and incubated with agitation for 1 h at 4 °C. DrrA was eluted using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), and purified DrrA was desalted with PD-10 desalting column (GE Healthcare, Sephadex G25 Medium) using PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). Rab1 was also expressed using the same procedure except that pET21a-Rab1 was solely transformed into a host cell and was cultivated in LB media. Protein concentration was measured by the Bradford method. Site-specific incorporation of AzPhe into DrrA was analyzed by a tandem liquid chromatography/mass spectrometry (LC/MS) spectrometer (QTOF II, Micromass, Manchester, U.K.) using a silica microcapillary reversed phase column (75 μm × 10 cm) after tryptic digestion in 0.1 M Tris-HCl (pH 8.0) containing 5 mM DTT at 37 °C for 16 h. Total ion chromatograms for native and mutated DrrAs were obtained, and peptide fragments showing different patterns were sequenced. A threshold of 10 counts/s was required to trigger an MS/MS event. The electrospray voltage was set to 2.5 kV, and the specific m/z value of the peptide fragmented by collisionally induced dissociation (CID) was excluded from reanalysis for 100 s.

Biotinylation of Proteins. Biotin-alkyne was conjugated with azide group of ρ-azido-L-phenylalanine-containing DrrA using click chemistry as described elsewhere[25,26] with minor modification. Briefly, 200 μM biotin-alkyne was mixed with protein solution (25 μM) containing 0.05 mM $CuSO_4$ and 0.05 mM TBTA in PBS buffer, followed by addition of 0.05 mM sodium ascorbate. The mixture was incubated at room temperature for 16 h. Sulfo-NHS-biotin was randomly conjugated with amine groups of DrrA according to the supplier's manual. Briefly, 40-fold molar excess of biotin reagent was added to the protein solution and incubated on ice for 4 h. For the biotinylation of DrrA with an average of 1 biotin, various amounts of sulfo-NHS-biotin were added to protein solution and incubated for different times. After time-consuming tasks, 5-fold molar excess of biotin reagent and 2 h incubation was found to generate randomly biotinylated DrrA with an average of 1.06 biotins per protein. All reaction mixtures were desalted with PD-10 desalting column using PBS for the removal of unreacted free biotin reagent. The amount of conjugated biotin with protein was analyzed by the HABA/avidin method. Concentrations of DrrA-AzF-Bio, DrrA-ran-Bio, and DrrA-ran-Bio* were measured by the Bradford method.

Surface Plasmon Resonance (SPR) Analysis. Surface plasmon resonance (SPR) measurements were performed using Biacore 3000 instrument. Neutravidin was covalently immobilized on the carboxyl monolayer, which is constructed by the self-assembly on bare gold surface (SIA kit Au), using EDC/NHS coupling method with the flow of the 10 mM sodium acetate buffer (pH 4.0). The surface was blocked with ethylamine. After the change of buffer to the PBS, 3 μM of site-specifically or randomly biotinylated DrrA were loaded onto the neutravidin-coated surface at a flow rate of 5 μL/min for 10 min and changes in RU were monitored. Various concentrations of Rab1 from 40 nM to 40 μM were serially injected onto the DrrA-immobilized gold surfaces at a flow rate of 10 μL/min for 5 min, and the RU changes were traced for binding events. To check the nonspecific binding of Rab1, biotin-BSA (instead of biotinylated DrrA) was loaded onto the neutravidin-coated surface at a flow rate of 10 μL/min for 5 min, and various concentrations of BSA from 40 nM to 40 μM were also injected onto the DrrA-immobilized surfaces.

Determination of Kinetic Constants. Apparent binding affinity (K_D) of Rab1 on the DrrA-immobilized surfaces was estimated based on the nonregeneration method as described elsewhere.[27] Briefly, various concentrations of Rab1 were serially applied on the respective DrrA-immobilized surface, and apparent K_D values were obtained by a double reciprocal plot of Rab1 concentrations, C, and RU changes, R_{eq}, based on a Langmuir equation at equilibrium

$$\frac{1}{R_{eq}} = \frac{1}{R_{max}} + \frac{K_D}{R_{max}}\frac{1}{C} \quad (1)$$

where the binding affinity, K_D, is equal to k_{off}/k_{on}, R_{max} is the maximum RU change which can be obtained at saturation conditions. The value of K_D is obtained from the slope and intercept. Apparent association and dissociation rate constants, k_{on} and k_{off}, were also estimated as described elsewhere.[27] Briefly, eq 1 can be transformed into the following equation

$$R = R_0 + \left(\frac{k_w}{k_{obs}} - R_0\right)(1 - e^{-k_{obs}t}) \quad (2)$$

where

$$k_w = k_{on}CR_{max} \quad \text{and} \quad k_{obs} = k_{on}C + k_{off}$$

R_0 represents the response just before the binding of Rab1. R denotes the changes in RU during the association time of Rab1 and increases exponentially with time. The RU changes of the association curve were fitted to a three-parameter exponential-rise-to-max function using SigmaPlot (SPSS, Inc.), which takes the form of $y = y_0 + a(1 - e^{-bx})$, where $y_0 = R_0$, $a = k_w/k_{obs} - R_0$, and $b = k_{obs} = k_{on}C + k_{off}$. From the plot of k_{obs} against Rab1 concentrations, C, apparent association and dissociation rate constants were obtained from the slope and intercept.

RESULTS AND DISCUSSION

Since truncated versions of human Rab1 (residues 1−179) and *L. pneumophila* DrrA (residues 317−545) showed a comparable binding affinity to full length proteins,[28] truncated Rab1 and DrrA were used in this study. On the basis of the crystal structure of DrrA in complex with Rab1 (PDB 2WWX)[28] (Figure 1a), we chose Asn-364 of DrrA for incorporation of AzPhe and introduced an amber stop codon (TAG) at this position, because Asn-364 is located on the opposite side of interaction interface with Rab1 and exposed to solvent. With the use of an engineered AzPheRS/$tRNA_{CUA}$ pair derived from *Methanococcus janaschii* TyrRS/tRNA,[23,24] the recombinant DrrA containing AzPhe was obtained, and incorporation of AzPhe into DrrA was confirmed by liquid chromatography−tandem

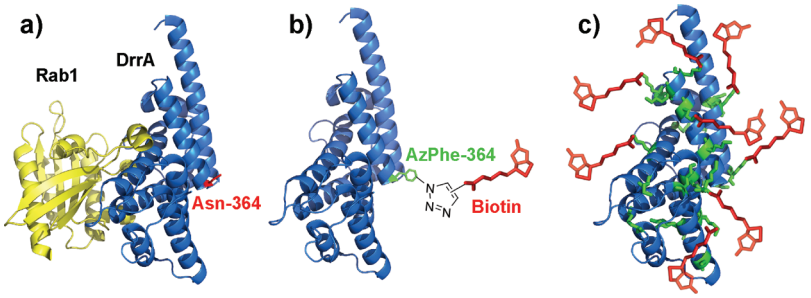

Figure 1. Schematic of site-specific and random biotinylation of proteins. (a) Complex crystal structure of DrrA (blue) and Rab1 (yellow) (PDB 2WWX). The position Asn-364 on DrrA is selected for site-specific incorporation of AzPhe. (b) Conjugation of AzPhe-364 of DrrA with biotin-alkyne by click chemistry. (c) Schematic presentation of randomly coupled biotins on DrrA.

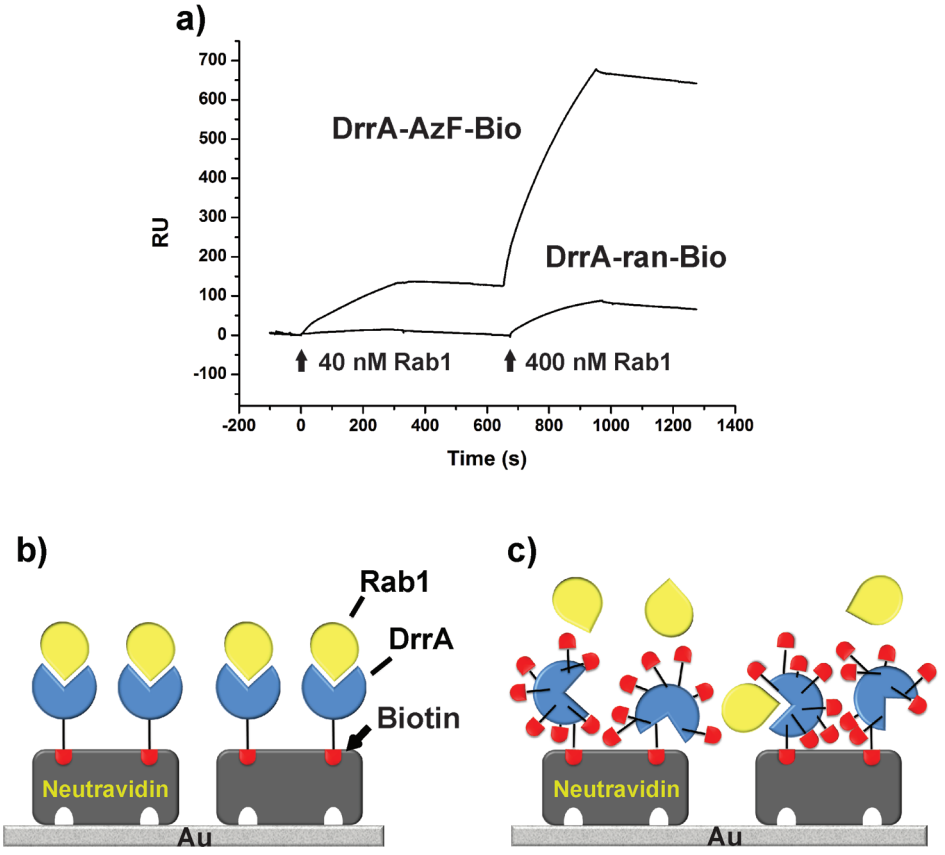

Figure 2. SPR sensorgrams and schematic representation of DrrA immobilization and Rab1 binding. (a) Changes in RU for binding of Rab1 on the surfaces immobilized with DrrA-AzF-Bio and DrrA-ran-Bio, respectively. Two different concentrations (40 and 400 nM) of Rab1 were sequentially loaded onto the DrrA-immobilized surfaces at marked arrows for 300 s and then basal buffer flowed for nearly 300 s. Experiments were carried out in quadruplicate, and typical sensorgrams are shown. (b) Oriented immobilization of DrrA-AzF-Bio and Rab1 binding. (c) Random immobilization of DrrA-ran-Bio and Rab1 binding.

mass spectrometry (LC−MS/MS) analysis (Figure S-1 in the Supporting Information). We also tested incorporation of AzPhe into several positions of Rab1 derived from human, only to find that AzPhe-labeled Rab1 was expressed mostly in the insoluble portion in the bacterial host.

Selective coupling of biotin to DrrA was carried out through a click reaction between AzPhe-364 of DrrA and biotin-alkyne in the presence of 0.05 mM $CuSO_4$, Na-ascorbate, and TBTA in PBS buffer (pH 7.4) as schematically shown in Figure 1b. It is well documented that the copper(I) catalyzed azide−alkyne cycloaddition reaction is simple, efficient, and orthogonal to biological systems.[25,26] *In vivo* incorporation of unnatural amino acids containing azide or alkyne into a protein and coupling with synthetic chemical groups by click chemistry is a useful method for site-specific modification of a target protein. Site-specifically biotinylated DrrA (DrrA-AzF-Bio) was separated from unreacted free biotin by using gel filtration. Biotin to protein ratio of the resulting DrrA-AzF-Bio sample was measured to be 0.7 by the HABA/avidin assay, which is a reasonable value because the yields of the click reactions usually lie in the range of 0.6−0.9.[25]

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Table 1. Kinetic Analysis of Rab1 Binding onto the DrrA-Immobilized Surfaces [a]

	K_D (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
DrrA-AzF-Bio	$(3.77 \pm 0.24) \times 10^{-7}$	$(1.64 \pm 0.46) \times 10^{3}$	$(5.41 \pm 0.02) \times 10^{-3}$
DrrA-ran-Bio	$(2.99 \pm 1.79) \times 10^{-6}$	$(1.26 \pm 0.37) \times 10^{2}$	$(8.70 \pm 0.02) \times 10^{-3}$

[a] Apparent binding affinity (K_D) and association and dissociation rate constants (k_{on} and k_{off}) were estimated for the binding of Rab1 onto the surfaces immobilized with DrrA-AzF-Bio and DrrA-ran-Bio.

Through this selective coupling, the majority of starting DrrA-AzF molecules were site-specifically coupled with biotin-alkyne molecules. All the coupled products are homogeneous since they should have a single biotin at the same position as planned.

For comparison, we coupled biotin to DrrA by conventional method using sulfo-NHS-biotin to generate randomly biotinylated DrrA (DrrA-ran-Bio) (Figure 1c). The HABA/avidin assay showed that approximately eight biotin molecules, on average, were attached to one DrrA molecule. This value seems plausible, considering the fact that there are 26 primary amines including 25 lysine residues and N-terminal in DrrA and that all the amines are in theory potential targets for the biotin coupling reaction, leading to the generation of heterogeneous DrrA molecules with differing numbers of biotins at random positions. These results illustrate that site-specific biotinylation through unnatural amino acid incorporation and click chemistry can produce homogeneous proteins with biotin at a specific position, whereas the conventional coupling method generates heterogeneous proteins with random and multiple biotinylation.

To investigate the binding efficiency between oriented and random immobilizations of DrrA, we performed the kinetic analysis for the binding of a target protein Rab1 using SPR. For immobilization of DrrA, neutravidin was first attached on the carboxyl monolayer of the bare gold surface using the EDC/NHS coupling method. A saturated amount of immobilized neutravidin was around 2 800 RU (resonance units), which can be converted to a surface concentration of 4.60 pmol/cm^2 by employing a molecular weight of 60 kDa for neutravidin and the conversion factor (1 000 RU corresponds to a surface concentration of 1 ng/mm^2).[29] This surface density of immobilized neutravidin is close to that of two-dimensional crystals of streptavidin measured by electron crystallography (4.65 pmol/cm^2).[20] Biotinylated DrrA molecules (DrrA-AzF-Bio and DrrA-ran-Bio) were then captured onto neutravidin-coated surface, respectively. The amounts of immobilized DrrA-ran-Bio and DrrA-AzF-Bio were estimated to be 5.76 ± 0.63 and 5.33 ± 0.94 pmol/cm^2, respectively. Since DrrA-ran-Bio molecules carry multiple biotin tags for immobilization, they are likely to be more easily captured by neutravidin. Nonspecific protein binding was shown to be negligible on the surfaces occupied fully with neutravidin and DrrA (Figure S-2 in the Supporting Information).

The target protein Rab1 was then applied onto the respective surface immobilized with DrrA-AzF-Bio or DrrA-ran-Bio, and the changes in RU were monitored (Figure 2a). The reported binding affinity (K_D) of DrrA for Rab1 by the ITC method was around 80 nM, and the Rab1 solutions were applied in concentration ranges from $^1/_2$ to 5 times the K_D value. When 40 nM Rab1 was loaded onto the surface of DrrA-ran-Bio, binding of Rab1 was almost undetectable. A higher concentration of Rab1 (400 nM) generated only a weak signal of about a 56 ± 5 RU increase, which corresponds to 0.26 ± 0.02 pmol/cm^2 Rab1. On the other hand, when 40 nM Rab1 was applied onto the surface of DrrA-AzF-Bio, binding of Rab1 was clearly observed (around a 100 ± 17 RU increase). When 400 nM Rab1 was sequentially loaded onto the same surface, about 582 ± 45 RU change (2.73 ± 0.21 pmol/cm^2 of Rab1) was observed, which is about 10-fold higher than the value of RU increase on the DrrA-ran-Bio surface. This result clearly demonstrates that oriented immobilization of DrrA through site-specific biotinylation can enhance the binding sensitivity by about 10 times compared with the immobilization of DrrA in a random manner through conventional biotinylation.

In order to gain some insights into high binding efficiency of Rab1 on the surface immobilized with DrrA-AzF-Bio, we estimated apparent binding affinity (K_D) of Rab1 on the DrrA-immobilized surfaces using the method described elsewhere.[27] Various concentrations of Rab1 were serially applied on the respective DrrA-immobilized surface, and apparent K_D values were obtained by plotting reciprocals of Rab1 concentrations and RU changes (Figure S-3 in the Supporting Information and see the Experimental Section). As shown in Table 1, the apparent K_D of Rab1 on the DrrA-AzF-Bio surface was around 377 ± 24 nM, whereas that on the DrrA-ran-Bio surface was 2.99 ± 1.79 μM. This result indicates that oriented immobilization of DrrA gives rise to about 8-fold higher affinity for Rab1 than random immobilization of DrrA, which is well correlated with the observed binding sensitivity shown in Figure 2a. The apparent K_D values are a little higher than the one deduced from the in-solution assay, and similar trends were reported elsewhere.[30]

For more detailed analysis regarding higher affinity, we estimated apparent association and dissociation rate constants, k_{on} and k_{off}, of Rab1 on the DrrA-immobilized surfaces (Figure S-4 in the Supporting Information and see the Experimental Section). As can be seen in Table 1, the k_{off} values on both surfaces were almost similar. On the other hand, the association rate constant (k_{on}) of Rab1 on the surface immobilized with DrrA-AzF-Bio was shown to be about one-order of magnitude higher than that on the DrrA-ran-Bio surface, which consequently leads to high binding sensitivity. From the binding analysis, the amounts of bound Rab1 on the DrrA-AzF-Bio and DrrA-ran-Bio immobilized surfaces were estimated to be 4.75 ± 0.83 pmol/cm^2 and 2.19 ± 0.25 pmol/cm^2, respectively, at the saturated level of Rab1 (Figure S-3 in the Supporting Information). This indicates that about 90% of immobilized DrrA-AzF-Bio molecules are functionally active. On the basis of the results, it is likely that most of the immobilized DrrA-AzF-Bio molecules are homogeneous in terms of orientation and binding affinity for the target ligand as schematically depicted in Figure 2b. In other words, site-specifically biotinylated DrrA molecules carry only a single biotin at a designed position and are immobilized onto neutravidin-coated surface in an ordered and functionally active way, resulting in high binding sensitivity. However, in the case of random immobilization, only one-third of immobilized DrrAs are estimated to be functional and they are vastly heterogeneous in orientation and binding affinity, which causes low binding sensitivity as depicted in Figure 2c.

To check the possible steric interference caused by randomly conjugated biotins, we constructed randomly biotinylated DrrA with an average of 1 biotin (DrrA-ran-Bio*) through rigorous and time-consuming efforts (see the Experimental Section). We conducted the same kinetic analysis using DrrA-ran-Bio* and found that DrrA-ran-Bio* shows 3.6-fold lower binding

sensitivity than that of DrrA-AzF-Bio (Figure S-5a in the Supporting Information). Estimated apparent binding affinity and rate constants were well correlated with the observed binding sensitivity (Figure S-5 and Table S-1 in the Supporting Information). DrrA-ran-Bio* still displayed lower binding efficiency of around ~30% compared with DrrA-AzF-Bio because conventional method (both with and without rigorous modification) resulted in random attachment of biotin to Lys residues on the surface, leading to random immobilization and steric interference of the binding. Interaction between DrrA and Rab1 seems to be further affected by multiple biotins on DrrA. We found that our controlled and oriented immobilization method can prevent steric hindrance caused by the conventional method and improve binding efficiency in the surface-based analysis.

CONCLUSIONS

We have demonstrated a general method for protein immobilization in a controlled and oriented manner via site-specific incorporation of unnatural amino acid and click chemistry. Kinetic analysis of the ligand binding using SPR revealed that oriented immobilization of site-specifically biotinylated DrrA (DrrA-AzF-Bio) results in about a 10-fold higher binding sensitivity than immobilization of randomly biotinylated DrrA (DrrA-ran-Bio). Our results clearly underline the efficacy of the controlled and oriented immobilization of proteins through site-specific incorporation of unnatural amino acid. The present method is anticipated to find wide applications in the fields of the surface-based studies of protein−protein interactions, single molecule analysis, biochip, and drug screening.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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