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Design and Construction of Glutamine Binding Proteins with a Self-Adhering Capability to Unmodified Hydrophobic Surfaces as Reagentless Fluorescence Sensing Devices

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Abstract: The chemically and genetically remodeling of proteins with ligand binding specificities can be utilized to synthesize various protein-based microsensors for detecting single biomolecules. Here, we describe the construction and characterization of fluorophore-labeled glutamine binding proteins (QBP) and derivatives coupled to the independently designed hydrophobic polypeptide (E12) that can adhere onto solid surfaces via hydrophobic interactions. The single cysteine mutant (N160C QBP) modified with the three environmentally sensitive fluorescent dyes (IAANS, acrylodan, and IANBD ester) showed increased changes in fluorescence intensity induced by glutamine binding. The use of these conjugates as reagentless fluorescence sensors enables us to determine the glutamine concentrations (0.1−50 μM) in homogeneous solution. The fusion of N160C QBP with E12, $(Gly_4\text{-}Ser)n$ spacers (GS*n*), and IANBD resulted in the novel fluorescence sensing elements having an adhering capability to hydrophobic surfaces of unmodified microplates. In ELISA and fluorescence experiments for the microplates treated with a series of the conjugates, IANBD-labeled N160C QBP-GS1-E12 displayed the best reproducibility in adhesion onto the hydrophobic surfaces and the precise correlation between fluorescence changes and glutamine concentrations. The performance of the biosensor-attached microplate for glutamine titrations demonstrated that the hydrophobic interaction of E12 with solid surfaces is useful for effective immobilization of proteins that need specific conformational movements in recognizing particular biomolecules. Therefore, the technique using E12 as a surface-linking domain for protein adhesion onto unmodified substrates could be applied effectively to prepare microplates/arrays for a wide variety of high-throughput assays on chemical and biological samples.

Introduction

Engineering of proteins with ligand binding specificities is one of many valuable approaches to the construction of analytical tools for measuring single molecular species.[1−4] In the past decade, several periplasmic binding proteins have been chemically and genetically reconstructed as biosensors whose ligand-induced structural changes are integrated into optical signal transduction.[5−9] Currently, the immobilization of proteins maintaining relevant biological activities on solid surfaces is a real challenge in the fabrication of protein arrays for a wide variety of biological analyses[10−15] because most attachments of the proteins on the surfaces cause denaturation and interfere with their critical conformational movements. Especially, during treatment of substrates with proteins as sensing devices, it is crucial to control their orientations and concentrations at surfaces, as this in turn can strongly dominate the detectability of the analyte. Furthermore, although many methods in the immobilization of proteins on solid surfaces have been reported so far,[10,16−18] there are several technical and cost problems to be solved (e.g.,

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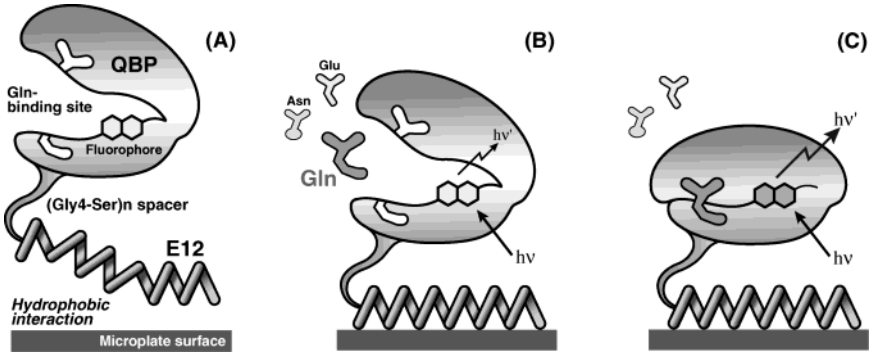
Scheme 1. Schematic Illustration of the Mechanism in Glutamine Detection by the Reagentless Fluorescence Sensing Device on Hydrophobic Surfaces[a]



[a] (A) E12 unit of the fluorophore-labeled conjugate QBP-GS*n*-E12 adheres spontaneously onto unmodified microplate surfaces through hydrophobic interactions. (B) After adhesion of the conjugate, QBP unit retaining specific recognition for glutamine is displayed on the surface. (C) The conformational movement of QBP upon glutamine binding induces an environmental change around the fluorophore from hydrophilic to hydrophobic states, which results in the amplification of fluorescence emission signals.

reductions of advanced surface modifications through multistep processes and high prices of frequently used plates). Hence, it is desirable to establish a more simplified methodology that enables proper immobilization of proteins with molecular sensing abilities on surfaces of reasonable substrates. Thereupon, we have taken particular note of hydrophobic interactions as a driving force for immobilization of proteins on unmodified solid surfaces and then designed the artificial polypeptide with a specific affinity to hydrophobic substrates. Here, the independently designed polypeptide with high hydrophobicity was genetically constructed and incorporated into ligand binding proteins to afford biosensing elements with a self-adhering capability on microplate surfaces. After site-specific chemical labeling of the fusion proteins with fluorophores, their efficiency in adhesion onto hydrophobic surfaces and optical sensing ability for a target molecule on the microplate were assessed.

Glutamine is already known as an excellent source of energy and a crucial nutrient within the living body. Recently, since it has been discovered that the physiology of glutamine can promote the adjuvanticity and wound healing,[19−22] the utilization of glutamine in transfusion and nutrient preparation has been examined in medical and clinical cures. Therefore, the versatile method, which can sense the presence of glutamine in biological samples and measure the concentration changes in controlled solution, should contribute to its effective investigation and further widespread use. Against a backdrop of that, we focused on the application of a high-throughput assay[14−18] performed by microplates coated with biosensors and then constructed glutamine binding proteins with a self-adhering capability at unmodified hydrophobic surfaces as reagentless fluorescence sensing devices. This makes it possible to establish not only the microplate-based assay system for rapid determination of glutamine concentrations but also a novel immobilization technique for proteins with molecular sensing abilities.

The protein constructed here as a glutamine sensor consists of three individual functional groups: (1) glutamine binding protein (QBP) for specific recognition of glutamine, (2) fluorescent probe as a reporter of the ligand-induced conformational change, and (3) artificial hydrophobic polypeptide configured for the interaction with hydrophobic surfaces of, for example, a microplate. The periplasmic glutamine binding protein in *E. coli* is composed of a single polypeptide chain that folds into two domains linked by hinge strands.[23−25] The structure of QBP upon glutamine binding is switched from "open form", where the two major domains are relatively far apart, to "closed form" which envelops the bound ligand.[24,25] Examining the structures of the ligand-free and ligand-bound states suggests that the environment around the asparagine residue at position 160 close to the glutamine binding cleft is substantially changed following ligand binding. Therefore, site-directed mutagenesis was performed to replace the Asn by a Cys residue that was then chemically modified with thiol-reactive fluorophores to transduce the binding event into optical signals. Oligomeric repeats of the polypeptide sequence Ala-Pro-Gly-Val-Gly-Val (E) are found in intravital structural protein "elastin".[26,27] Such a hexapeptide consisting of hydrophobic amino acids has been known to form a rigid β-spiral structure with hydrophobic ridges[28−30] that stabilizes protein structures. Based on the unique structure and hydrophobic property derived from a repeating motif of the polypeptide unit, tandemly 12-repeated polypeptide (E12) was genetically synthesized as a surface-linking domain.[31,32] The incorporation of E12 into QBP can be expected to provide QBP with an adhering capability onto unmodified surfaces through hydrophobic interactions. Moreover, the QBP and E12 were linked by (Gly-Gly-Gly-Gly-Ser)*n* ($n = 1$, GS1; $n = 2$, GS2) spacers to exclude steric hindrance between QBP and E12. After chemical modifications with environmentally sensitive fluorescent reagents, the resulting conjugates are designated fluorophore-labeled QBP-GS*n*-E12s. According to the

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design concept described above, the E12 unit of the conjugates adheres preferentially onto unmodified surfaces via hydrophobic interactions and thereby the conformational change of QBP unit upon ligand binding occurs independently. As illustrated in Scheme 1, the processes enable us to transduce the presence of glutamine into fluorescence signals.

In this study, the single cysteine mutant N160C QBP and fusion proteins N160C QBP-GS*n*-E12s were genetically prepared and were chemically labeled with the environmentally sensitive fluorophores (IAANS, acrylodan, and IANBD ester). These conjugates were investigated in terms of performance as reagentless fluorescent biosensors in homogeneous solution. Furthermore, we particularly focused attention on unique properties of IANBD-labeled N160C QBP-GS1-E12 and evaluated its adhering capability onto unmodified hydrophobic surfaces and utility value as a reagentless optical sensing device on the microplate.

Materials and Methods

Construction of QBP-E12 Genes. The mutant N160C QBP in pET-28b (Novagen), whose asparagine residue at position 160 is replaced by a cysteine, was made by oligonucleotide directed mutagenesis using the Stratagene QuikChange Kit. The fusion protein N160C QBP-E12 was prepared as follows: an *Nco*I restriction site was previously introduced to pBluescript II SK(−) (pBS) by insertion of a synthetic oligonucleotide, and then the fragment encoding the N160C QBP gene was introduced between the prepared *Nco*I-*Xho*I sites to construct pBS-QBP. The fragment encoding the tandemly 12-repeated Ala-Pro-Gly-Val-Gly-Val sequence (E12), which was available from the digestion of pGEX-E12 plasmid[31] with *Bam*HI and *Bgl*II, was inserted at the C-terminal site of the N160C QBP gene in the pBS-QBP. Finally, the fragment encoding N160C QBP-E12 in pBS was transferred at the *Nco*I-*Xho*I sites of pET-28b to produce a fusion protein with His_6-tag. The resulting plasmid is designated pET-QBP-E12. Furthermore, based on the method above, two plasmids pET-QBP-GS1-E12 and pET-QBP-GS2-E12, where the fragment encodings (Gly-Gly-Gly-Gly-Ser)*n* (*n* = 1; GS1, *n* = 2; GS2) were inserted between QBP and E12 sequences, were constructed.

Protein Expression and Purification. *E. coli* BL21 (DE3) cells (Novagen) were transformed with the plasmids pET-QBP, pET-QBP-E12, pET-QBP-GS1-E12, and pET-QBP-GS2-E12. They were grown at 37 °C in LB medium supplemented with 50 μg/mL kanamycin until $OD_{660} = 0.7$. Protein expression was induced with 1 mM isopropylthio-β-D-galactoside (IPTG). Cells were cultured for another 6 h at 20 °C and then harvested by centrifugation. They were washed with saline buffer (25 mM imidazole, 500 mM NaCl, and 20 mM phosphate, pH 7.4) and disrupted by sonication. After centrifugation, the supernatant was applied to an Ni^{2+}-NTA column to purify the fusion proteins by using the affinity of histidine-tag. The resulting proteins were designated N160C QBP (28.8 kDa), N160C QBP-E12 (35.3 kDa), N160C QBP-GS1-E12 (36.0 kDa), and N160C QBP-GS2-E12 (36.3 kDa), respectively. The purity and identity of the recombinant proteins were confirmed by SDS−PAGE and Western blotting (see the Supporting Information).

Fluorophore Coupling. 2-(4′-(Iodoacetoamido)anilino)naphthalene-6-sulfonic acid (IAANS), 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), and (((2-(iodo-acetoxy)ethyl)methyl)amino)-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester) were purchased from Molecular Probes and used without further purification. The fluorophores were covalently attached to the engineered QBP mutants via the single free cysteine at position 160. Typical labeling conditions were described as followed: the fluorophores (10-fold-excess) in DMF or MeCN were reacted with the freshly purified QBP mutants (~1 mg) in phosphate buffer A (150 mM NaCl, 50mM sodium phosphate, pH 7.0) overnight at 4 °C. Removal of unreacted dye was performed by dialysis. The extent of labeling was calculated from both the protein concentration and the extinction coefficients for individual fluorophores.[6,7] To achieve dye-to-protein ratio = ~1, labeling conditions were optimized with respect to fluorophore/protein concentration and time.

Fluorescence Assays. All fluorescence measurements were performed on a Jasco FP-777 spectrofluorometer unless otherwise stated. Fluorescence emission spectra were obtained by excitation of the labeled conjugates at 325 nm for IAANS, 390 nm for acrylodan, and 485 nm for IANBD. The protein concentration was 500 nM in phosphate buffer A, and aliquots of glutamine were added from stock solutions (0.45−0.3 mM) in the same buffer. The titrations of glutamine were carried out by monitoring the fluorescence intensities at emission maximum following appropriate excitation for each fluorophore ($\lambda_{ex} = 295$ nm and $\lambda_{em} = 335$ nm for tryptophan; $\lambda_{ex} = 325$ nm and $\lambda_{em} = 432$ nm for IAANS; $\lambda_{ex} = 390$ nm and $\lambda_{em} = 500$ nm for acrylodan; $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm for IANBD), and the averaged values were fitted to the equation

$$\Delta F_{obs} = \Delta F_{max} S / (K_d + S) \quad (1)$$

where ΔF_{obs} is the change in fluorescence intensity, ΔF_{max} is the maximum attainable change in fluorescence intensity, S is the molar concentration of glutamine, and K_d is the dissociation constant.

Typical immobilization of the fluorophore-labeled proteins to unmodified solid surfaces of a microplate was employed by the following procedure: 150 μL aliquots of each IANBD-labeled QBP conjugate in various concentrations were added to individual wells of 96-well polystyrene microplates (Sumitomo Bakelite Co., Tokyo, Japan). After incubation overnight at 4 °C, these wells were washed several times with phosphate buffer including 0.05% Tween 20 (PBS-T) to remove any weakly adsorbed conjugates. After addition of 150 μL of phosphate buffer A to each well, the fluorescence measurements on the conjugate-immobilized microplate were performed with the CytoFluor 4000 (PerSeptive Biosystems). The titrations of glutamine in solution were carried out by monitoring the changes in fluorescence emission signal derived from IANBD-labeled conjugates adhering to the microplate surfaces ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm).

Characterization of the Inverse Transition. The temperature-dependent aggregation of N160C QBP and N160C QBP-GS1-E12 was characterized by measuring the absorbance at 350 nm as a function of temperature. The turbidity measurements were performed using the Beckman DU7500 spectrometer connected with a micro T_t (inverse transition temperature) analyzing system. The concentration of the proteins in phosphate buffer A was 0.25 μg/μL, and the temperature was raised at a constant rate of 1.0 °C/min.

Enzyme-Linked Immunosorbent Assay (ELISA). Aliquots of each IANBD-labeled conjugate (150 μL, 200 nM) were added to each well of a 96-well polystyrene microplate and incubated overnight at 4 °C. After the microplate was washed several times with PBS-T, 150 μL of 0.25% Block Ace (Dai-nippon Pharmaceuticals) in phosphate buffer A was applied to the wells and incubated for 1 h at 25 °C. All the wells were washed several times with PBS-T, and then 150 μL of 0.1 U/mL anti-polyhistidine-peroxidase (Roche) was added and incubated for 1.5 h at 25 °C. After the wells were washed several times with PBS-T and once with phosphate buffer A, 150 μL of a solution containing substrates of peroxidase and H_2O_2 (Konica) were applied to the wells and incubated for 30 min at 25 °C. The enzyme reaction was stopped with 100 μL of 2 N sulfic acid, and the absorbance at 450 nm (ref 655 nm) was measured on the Microplate-reader (BIO-RAD).

Results and Discussion

Properties of the Mutant N160C QBP. A glutamine binding protein (QBP), which belongs to a member of periplasmic protein groups in *E. coli*, is composed of two similar globular

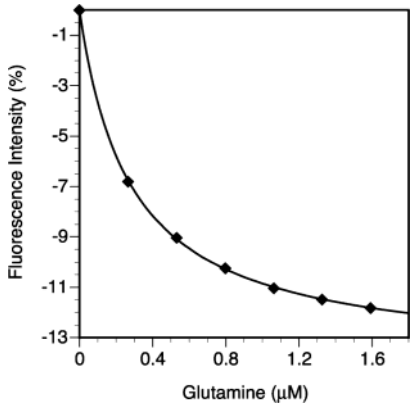

Figure 1. Change in intrinsic tryptophan fluorescence emission of the mutant N160C QBP upon glutamine binding (λ_{ex} = 295 nm, λ_{em} = 335 nm). The binding curve is the average of three independent titrations (error bars are smaller than the data points shown).

domains connected with a peptide hinge.[23−25] Glutamine induces a hinge bending motion between two domains, resulting in the conformational change from a "ligand-free" open form to a "ligand-bound" closed form. Recently, the structures in both of them were determined by X-ray crystallographic analyses.[24,25] The structural data of each conformation allowed us to identify that the asparagine residue at position 160 is close to the glutamine binding site and shows a large environmental change upon ligand binding. The asparagine was therefore mutated to a cysteine for labeling with environmentally sensitive fluorescent probes. The covalently attached fluorophores are positioned to respond to such a ligand-induced conformational change.

First, to confirm the glutamine binding affinity of QBP after the single cysteine mutation, intrinsic tryptophan fluorescence was measured in the presence of glutamine. Figure 1 exhibits that the fluorescence intensity decreases with increasing the glutamine concentration in solution. Using the fluorescence emission data and eq 1, the dissociation constant K_d was calculated to be 0.29 μM. The K_d value is in the range of dissociation constants of wild-type QBP previously reported (0.2−0.5 μM).[9,23,24] This result indicates that the affinity of the mutant (N160C QBP) is maintained even if the asparagine is replaced by a cysteine residue. However, the fluorescence change observed here is much too small to be useful as a sensing signal. Basically, such a tryptophan fluorescence is unsatisfactory for a sensitive detection of glutamine, because of both the small magnitude of the intensity change and high autofluorescent background in biological samples at the short wavelength. Therefore, it will be essential to amplify the fluorescence intensity and shift the excitation/emission characteristic to a longer wavelength by environmentally sensitive fluorescent probes attached to N160C QBP. Here, we selected and used three different fluorescent reagents IAANS, acrylodan, and IANBD ester. All of them are very sensitive to changes in the solvent accessibility, probe mobility, and polarity of environment.[33,34]

Characterization of the Fluorophore-Labeled N160C QBPs. The N160C QBP was modified with IAANS, acrylodan, and IANBD ester to afford IAANS-, acrylodan-, and IANBD-labeled N160C QBPs, respectively. The fluorescence emission spectra on each labeled protein were measured in the presence of an excess amount of glutamine. As expected, it was observed that fluorescence intensities in the ligand-bound forms of all labeled QBPs dramatically increased over those in the ligand-free forms (Figure S1). These spectral changes imply an increase in hydrophobicity of the environment around the attached fluorophores.[7,8] Particularly, the blue shifts in the wavelength of emission maximum on IAANS- and acrylodan-labeled conjugates can be interpreted as being due to the dye being in nonpolar rather than aqueous environments.[8,33,34] In fact, according to the structural examinations, the conformational change accompanying the ligand binding results in the fluorophores being buried in the cleft of QBP and shielded from water. This should bring about the microenvironmental alteration from hydrophilic to hydrophobic states.

In glutamine titrations by the use of the IAANS-, acrylodan-, and IANBD-labeled N160C QBPs, significant changes in emission intensity were observed in a wide variety of glutamine concentrations (Figure 2). From the plot of the intensity data against glutamine concentration and subsequent fitting to eq 1, the K_d values of 0.30, 2.0, and 6.6 μM were determined for IAANS-, acrylodan-, and IANBD-labeled N160C QBPs, respectively. The K_d value for the IAANS-labeled conjugate is identical to that of nonlabeled N160C QBP, which suggests that steric interference of the attached ANS to the ligand-induced conformational movement is very small. However, by irradiation of excitation light over a period of time, the conjugate was gradually degraded. In contrast, acrylodan- and IANBD-labeled N160C QBPs were relatively stable during fluorescence measurements, but they showed the affinity for glutamine 1 order of magnitude lower than that of IAANS-labeled conjugate. This may be due to steric hindrance that the fluorophores offer to the conformational change upon ligand binding. Fortunately, when structurally similar amino acids, asparagine and glutamic acid, were added to the solution of each labeled protein, the increased propensity in fluorescence emission intensity was not observed on all mutants. This observation verifies that the specific recognition for glutamine in QBP is conserved even after the chemical modifications with the fluorophores. Considering all results above, by assorted use of these labeled conjugates, the glutamine concentration in the range of ca. 0.1−50 μM can be measured correctly. Hence, IAANS-, acrylodan-, and IANBD-labeled N160C QBPs are useful for reagentless fluorescence biosensing in homogeneous solution.

Design and Construction of the Fluorophore-Labeled N160C QBP-GS*n*-E12s. Typical optical biosensors have been built from two major functional components: a biological receptor which recognizes a particular ligand and a transducer which converts a recognition event to measurable optical signals.[3,4] For introduction of a self-adhering capability on unmodified solid surfaces as a new function into the biosensors, we carried out the construction of an independently designed hydrophobic polypeptide interacting with hydrophobic surfaces. The combination of the artificial hydrophobic protein with QBP and adequate chemical modification with fluorophores can transform QBP into a novel optical biosensor adhering at substrate surfaces by itself.

Repeats of an elastin-based hexapeptide consisting of the hydrophobic amino acids (Ala-Pro-Gly-Val-Gly-Val) (E) have been known to form a rigid β-spiral structure containing hydrophobic ridges in solution.[28−30] Oligomerization of the gene

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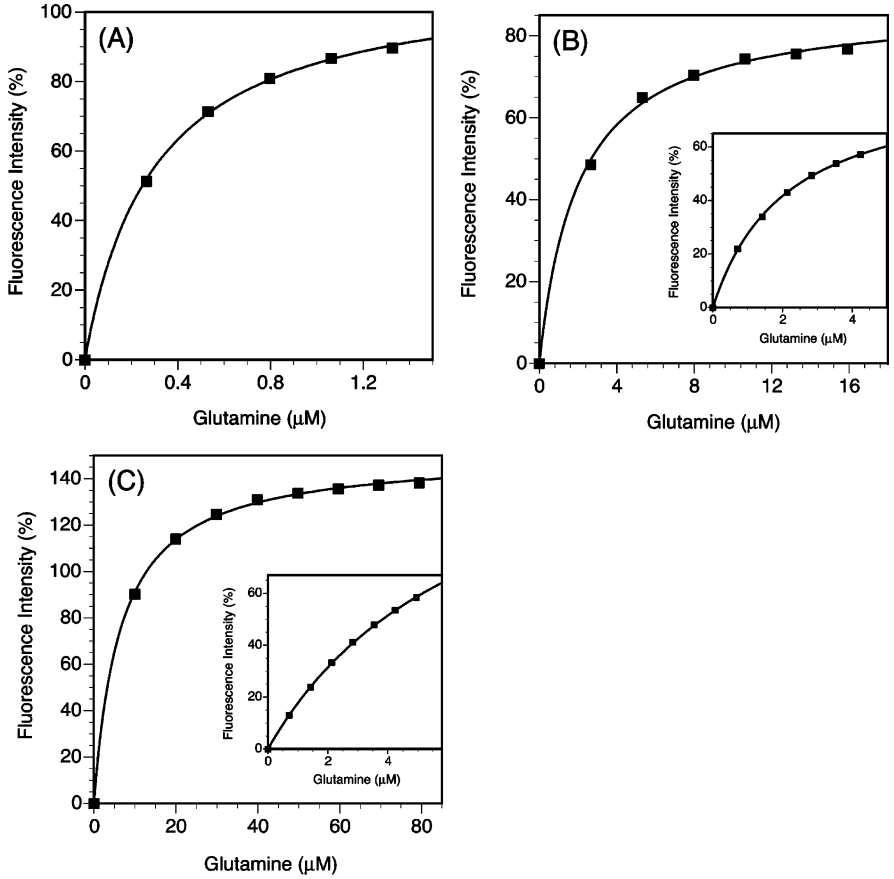

Figure 2. Titrations of glutamine by fluorophore-labeled N160C QBPs ((A) IAANS-, (B) acrylodan-, (C) IANBD-). (Insets) Binding curves at low glutamine concentrations. The data points are the respective averages of three separate measurements (error bars are smaller than the data points shown).

encoding such a unique polypeptide sequence can produce structural proteins with precisely defined chain lengths and specified masses. Based on the methodology, the tandemly 12-repeated unit of the E (E12)[35] was genetically constructed as a surface-linking domain with appropriate hydrophobicity. On the fusion protein (N160C QBP-E12) generated by the coupling of E12 with QBP, the E12 unit is expected to adhere positively onto solid surfaces via hydrophobic interactions and thereby the QBP unit retaining the affinity for glutamine is displayed at the surfaces. Furthermore, to wiggle out of steric interferences between QBP and E12, we also prepared two new fusion proteins N160C QBP-GS1-E12 and N160C QBP-GS2-E12 by introduction of (Gly-Gly-Gly-Gly-Ser)*n* ($n = 1$, GS1; $n = 2$, GS2) spacers into N160C QBP-E12. In fluorophore labeling of all mutants N160C QBP-GS*n*-E12s, IANBD ester was selected as a fluorescent dye because we observed the highest stability of IANBD-labeled N160C QBP in all the labeled conjugates (the stability of fluorophore-labeled N160C QBPs for irradiation of individual excitation light at room temperature: IANBD- > acrylodan- > IAANS-).

To identify influences on the glutamine affinity of the QBP unit by fusion with E12, glutamine binding assays were performed for N160C QBP-E12, N160C QBP-GS1-E12, and N160C QBP-GS2-E12 that were labeled with IANBD ester. The fluorescence changes along with addition of glutamine were here monitored under the condition without attachment of the conjugates on solid surfaces of a microplate. Regardless of the kinds of the used conjugates, good correlations between fluorescence intensities and glutamine concentrations were observed, which were similar to those found in the use of the fluorophore-labeled N160C QBPs. Particularly, as represented in Figure 3, IANBD-labeled N160C QBP-GS1-E12 showed the greatest increase in fluorescence intensity and favorable reproducibility. It is interesting that the K_d value 2.1 μM, calculated from the observed emission data and eq 1, manifests the higher affinity for glutamine than that of IANBD-labeled N160C QBP

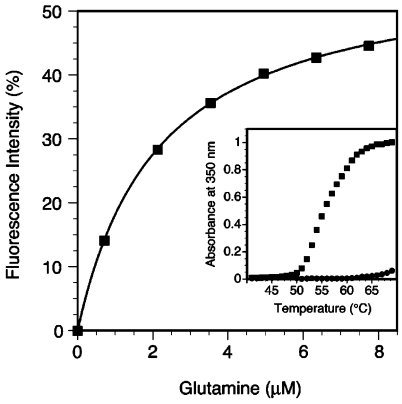

Figure 3. Binding of glutamine to IANBD-labeled N160C QBP-GS1-E12 under the condition without adhesion of the conjugate to microplate surfaces ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm). The binding curve is the average of three independent measurements (error bars are smaller than the data points shown). (Inset) Turbidity (A_{350}) as a function of temperature for N160C QBP-GS1-E12 (■) and N160C QBP (●). All turbidity profiles were measured with a 0.25 μg/μL protein concentration in phosphate buffer A at 1.0 °C/min heating rate.

(35) CD spectrum of E12 showed a characteristic peak of a β-spiral structure at ~198 cm^{-1}.

without E12. This may be derived from the structural difference of amino acid sequences following QBP, but the exact understandings will require further study.

Next, we focused attention on the property of N160C QBP-GS1-E12 and found out whether the hydrophobic feature of the E12 domain in the conjugate is present or not. The thermally dependent aggregation characteristic for elastin-like polypeptides is known as coacervation.[31] Such polypeptides are soluble in aqueous solution below the inverse transition temperature (T_t), but when the temperature is raised above the T_t, a sharp transition occurs leading to an increase in turbidity due to intermolecular aggregation. T_t values of N160C QBP-GS1-E12 and N160C QBP in phosphate buffer A were measured by spectroscopic monitoring absorbance at 350 nm as a function of temperature. A turbidity profile for N160C QBP-GS1-E12 exhibited an exponential increase in turbidity during the course of increasing temperature (Figure 3 inset). The observed pattern is typical to those found in elastin-like polypeptides.[36,37] The T_t value of N160C QBP-GS1-E12 was 56.0 °C for the protein concentration of 0.25 μg/μL. In contrast, N160C QBP showed no turbidity depending on such a temperature change. These results apparently demonstrate that the E12 unit can uniquely form the elastin-like structure and display a strong hydrophobic character even after fusion with the QBP unit. Therefore, the coacervation data convinced us that E12 can interact independently with hydrophobic surfaces of a microplate.

Self-Adhering Capability of the IANBD-Labeled N160C QBP-GS1-E12 to Unmodified Hydrophobic Surfaces. For the purpose of confirming the adhering capability of IANBD-labeled N160C QBP-GS1-E12 to hydrophobic surfaces, fluorescence on the unmodified microplate treated with the conjugate was measured (see Materials and Methods). Depending on increases in concentration of the conjugate added to the wells, increases in emission intensity were observed (Figure S2, $\lambda_{ex} = 485$ nm and $\lambda_{em} = 530$ nm). This result indicates that IANBD-labeled N160C QBP-GS1-E12 bears the adhesion ability to the unmodified hydrophobic microplate and the surface density can be controlled by changing the concentration of the conjugate standing at the wells. Moreover, since the saturation of fluorescence intensity was observed in a ca. 200 nM solution, the concentration (200 nM) is used for immobilization of the conjugate throughout the subsequent experiments. This is because the use of the largest intensity change in the presence of glutamine is of great advantage to achieve the higher sensitivity and perform the measurements for wide-range glutamine concentrations.

To assess the adhesion efficiency of the conjugate improved by the introduction of E12, the existential quantities of N160C QBP-GS1-E12 and N160C QBP on microplate surfaces was compared by ELISA using an anti-polyhistidine antibody attached with peroxidase. As shown in Figure 4A, the absorbance simply indicates degrees of amounts of each conjugate adhered to the surfaces. The fusion of E12 obviously enhanced the adhesion activity of N160C QBP-GS1-E12. Consecutively, to examine the binding activity of the QBP unit after adhesion on the same plates used in ELISA, glutamine binding assays on the plates were carried out by measuring fluorescence

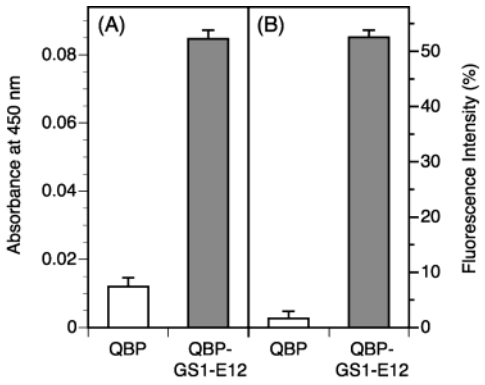

Figure 4. (A) Comparison of adhesion efficiencies of IANBD-labeled N160C QBP and IANBD-labeled N160C QBP-GS1-E12 onto unmodified surfaces of the hydrophobic microplate. Absorbance at 450 nm (ref 650 nm) was measured on ELISA using anti-polyhistidine antibody attached with peroxidase. (B) Glutamine binding assay for the same plate used in the absorption analysis. The fluorescence experiments were performed in the presence of 10 μM glutamine ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm). The absorbance and fluorescence data are the mean ± SD from three separate assays, respectively.

emission changes. Upon addition of glutamine to the plate treated with IANBD-labeled N160C QBP-GS1-E12, a large increase in fluorescence intensity was observed (Figure 4B). On the other hand, despite some adhesion of IANBD-labeled N160C QBP on the surface, confirmed by ELISA, the fluorescence intensity on the plate treated with the conjugate hardly changed in the presence of glutamine (Figure 4B). It is understandable in this case that nonspecific adsorption of QBP on the surface occurs to cause denaturation disturbing the glutamine binding event. Taking both ELISA and fluorescence data into account, it is clarified that the E12 unit adheres preferentially onto the hydrophobic surface to prevent deactivation of the QBP unit due to nonspecific interaction with the surface. Therefore, the simple fusion of E12 enables the intrinsic activity of QBP to be retained even after adhesion on the microplate surface.

Glutamine Binding Assay for the IANBD-Labeled N160C QBP-GS1-E12 Adhered to Unmodified Hydrophobic Surfaces. After fixation of IANBD-labeled N160C QBP-GS1-E12 to an unmodified microplate in accordance with the above procedure, glutamine titration was performed by monitoring fluorescence signals in response to glutamine binding events on the microplate (Figure 5). Fitting of the observed binding curve to the isotherm (1) yielded the dissociation constant $K_d = 2.4$ μM. It is remarkable that the K_d value is nearly consistent with that when IANBD-labeled N160C QBP-GS1-E12 was not adhered to microplate surfaces (2.1 μM). This result illustrates that the intrinsic binding activity of the QBP unit is successfully maintained even after immobilization of the conjugate at the surface. Probably, the adhesion mediated by the E12 domain could make possible interferences between the QBP unit and solid surfaces much smaller. Furthermore, in the addition of structurally similar amino acids, asparagine and glutamic acid, the increased tendency in fluorescence emission intensity was absolutely not observed (Figure 5). The observation reveals plainly that the specificity of QBP for glutamine is conserved in the simple adhesion of the conjugate by E12. Taken together, these results demonstrate that IANBD-labeled N160C QBP-GS1-E12 adheres automatically to the hydrophobic surface via the E12 rather than QBP unit, and then QBP displayed at the

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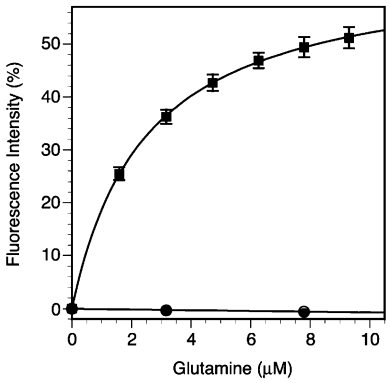

Figure 5. Binding of amino acids to IANBD-labeled N160C QBP-GS1-E12 adhered to unmodified surfaces of the hydrophobic microplate (■, glutamine; ●, asparagine; ○, glutamic acid). The binding curve is the average of three independent measurements (λ_{ex} = 485 nm, λ_{em} = 530 nm).

surface can perform smoothly the conformational movement upon ligand binding. It is therefore obvious that the fusion of independently designed hydrophobic polypeptide E12 can transform a fluorophore-labeled QBP into a reagentless optical sensing device with a self-adhering capability on unmodified hydrophobic surfaces.

Summary and Conclusion

The site-directed mutagenesis was performed to generate the mutant N160C QBP, in which the Asn residue at position 160 was replaced by a Cys residue. The N160C QBP was then modified with the three sulfhydryl-reactive fluorescent reagents to afford IAANS-, acrylodan-, and IANBD-labeled N160C QBPs. All the labeled mutants exhibited the increased tendencies in fluorescence intensity upon glutamine binding. By assorted use of these conjugates, the glutamine concentration in the range from 0.1 to 50 μM can be determined correctly. The results reflect that the appropriate site-specific mutation of QBP and chemical coupling of environmentally sensitive fluorophores can produce the useful reagentless fluorescence biosensors in homogeneous solution.

We also designed and constructed the novel QBPs, which are connected to the fluorophore IANBD as a signal transducer and the artificial hydrophobic polypeptide E12 with an adhesion ability to unmodified hydrophobic surfaces. In ELISA and fluorescence experiments on the multiwell hydrophobic microplate coated with the conjugates, the IANBD-labeled N160C QBP-GS1-E12 showed not only the sufficient reproducibility of adhesion onto the surfaces but also a good correlation between glutamine concentrations and fluorescence changes attributable to the binding event. Based on the data, the system using the multiwell microplate treated with this sensing device and plate reader makes it possible to determine wide-range glutamine concentrations in many samples all at once.

This study is the first example of the construction of genetically and chemically engineered proteins as reagentless optical sensing devices with a self-adhering capability to unmodified hydrophobic surfaces. Additionally, we claim that the hydrophobic interaction of E12 with solid surfaces is more useful in preparing analytical microplates/arrays/chips[10,12] where any other proteins and polypeptides are required to be adhered in rows. Because the function of E12 as a surface-linking domain is not adapted only to the nature of QBP, it therefore can be generalized to structurally similar amino acid- or sugar-binding proteins[38−41] as candidates for the sensing device. Soon, the technique that simplified protein immobilization by hydrophobic interactions would be applied to easy-to-use fabrications of protein microarrays and peptide chips for ligand binding assays and protein−protein interaction analyses. [10−15]

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Supporting Information Available: Compound characterization data and additional micrographs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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