Full-length paper

Peptide arrays with designed α-helical structures for characterization of proteins from FRET fingerprint patterns

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Summary

A practical high-throughput protein detection system is described, based on synthetic peptide arrays consisting of designed α-helical peptides, detected by fluorescence resonance energy transfer (FRET). Initially a model α-helical peptide known to interact with a structured protein, calmodulin, was selected to establish the strategy for high-throughput detection. In comparison to peptides with a single probe, a much higher FRET response has been observed with two fluorescent probes (7-diethylaminocoumarin-3-carboxylic acid and 5(6)-carboxy-fluorescein) at both termini of the synthetic peptides. To establish a reproducible high-throughput detection system, peptides were also immobilized onto a solid surface for detection of the target proteins. A small library of 112 different peptides was constructed, based on a model of the α-helical peptide with systematic replacement of residues carrying specific charges and/or hydrophobicities. The library was used to effectively characterize various proteins, giving their own 'protein fingerprint' patterns. The resulting 'protein fingerprints' correlate with the recognition properties of the proteins. The present microarray with designed synthetic peptides as the capturing agents is promising for the development of protein detection chips.

Abbreviations: AEDANS, *N*-(acetamidoethyl)-1-naphthylamine-5-sulfonic acid; CaM, calmodulin; DABCYL, 4-dimethyl-aminoazobenzene-4'-carboxylic acid; DAC, 7-diethylaminocoumarin-3-carboxylic acid; FAM, 5(6)-carboxyfluorescein; FRET, fluorescence resonance energy transfer; β-LG, β-lactoglobulin; PKA, protein kinase A; S-100, S-100 proteins; TAD, activated dextran

Introduction

The genome information of human and various other organisms has recently been extensively elucidated. In the postgenome era, a vast amount of knowledge based on these genomes will be applied in various fields; in particular proteomics will be one of the most important areas in bio-medical research. A number of technologies have been developed to investigate cellular events. For example, oligonucleotide arrays [1] provide information on changes in mRNA expression levels in response to a variety of physiological stimuli [2]. The protein expression levels, however, often do not correlate with mRNA levels [3]. Proteins thus need to be analyzed directly in order to understand their functions, structures, interactions and regulation in various types of cells. For the elucidation of protein structures, functions and/or interactions, technologies such as two-dimensional gel electrophoresis [4], chromatographic separation methods or surface plasmon resonance methods in conjunction with mass spectrometry [5–7] have been developed and are now widely used. These technologies, however, do not allow sufficient throughput and the progress of proteomics studies thus depends on the development of high-throughput technologies for direct analysis of protein functions and interactions.

Protein chips are expected to be one of these high throughput technologies. Since the pioneering demonstration by MacBeath et al. [8] of the feasibility of immobilizing proteins on a conventional glass slide, protein chips have become a significant research area in biotechnology [9-14]. Because of the complex structures and interactions of proteins in comparison to DNA or RNA, various difficulties have still to be overcome before protein chips can become useful as microarrays. There are four basic requirements to develop practical protein chips [14]: (1) Capturing agents that are immobilized on the array must respond to target

proteins; (2) surface immobilization methods must be developed; (3) effective detection methods must be employed; (4) industrial arrays must be manufactured at a competitive price. Although a number of protein chip systems have been developed, few systems satisfying all these four points have been built accomplished.

From this point of view, designed peptide libraries with suitable secondary structures are promising candidates for protein capturing agents for the following reasons: (1) Peptides with secondary structures can mimic protein-protein interactions; not only amino acids but also various functional moieties such as carbohydrates and co-factors can be employed as building blocks in the designed peptides; (2) peptides can be immobilized at defined points through their functional groups; (3) probes for protein detection, such as fluorophores, can be introduced into peptides at any required position; (4) peptides are more easily designed and synthesized than antibodies or recombinant proteins. We have recently constructed a designed peptide library with a loop structure using these criteria [15]. However, loop peptide libraries are not sufficient the detection of many proteins. Here, we describe the construction of a protein detection system using a peptide library based on an α-helix structure, which is a typical secondary structure, appropriate for characterizing a variety of proteins. Initially, fluorescence detection methods such as fluorescence resonance energy transfer (FRET) were examined in solution and on a solid surface using as a model fluorescent α-helical peptides that interact with calmodulin (CaM). An α-helical peptide library with changing charges and/or hydrophobicities was then constructed based on a model peptide, and various proteins were characterized. The present concept – the structure-designed peptide arrays using a fingerprint method – is a promising route to high-throughput protein chips.

Materials and methods

General remarks

All chemicals and solvents were of reagent or HPLC grade and were used without further purification. Protein samples were purchased from Sigma-Aldrich Japan. HPLC was performed with Hitachi L7000 or Shimadzu LC2010C equipment using a Wakosil 5C18 or a YMC-Pack ODS-A (4.6 x 150 mm) for analysis, and a YMC ODS A323 (10 x 250 mm) for preparative purification with a linear gradient of acetonitrile / 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min^{-1} for analyses and 3.0 ml/min^{-1} for preparative separation, respectively. MALDI-TOFMS was measured on a Shimadzu KOMPACT MALDI III with 3,5-dimethoxy-4-hydroxycinnamic acid as matrix. Amino acid analysis was carried out using a Wakopak WS-PTC column (4.0 x 200 mm; Wako Pure Chemical Industries) after hydrolysis in 6 M HCl at 110 °C for 24 h in a sealed tube followed by phenyl isothiocyanate labeling.
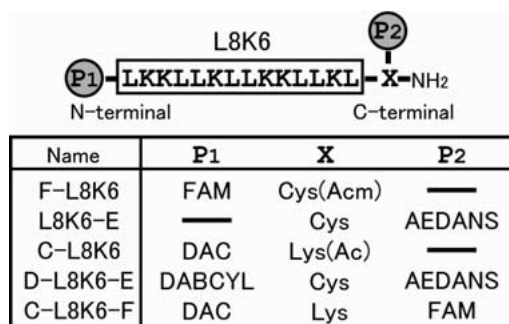

Figure 1. Structures of α-helical peptides with various fluorescent probes.

Synthesis of individual peptides with various fluorophores

Peptides were synthesized by means of Fmoc chemistry [16] on Rink amide resin with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as a coupling reagent. Side chains were protected as follows: acetamidomethyl (Acm) or triphenylmethyl (Trt) for Cys; t-butyloxycarbonyl (Boc) or 4-methyltrityl (Mtt) for Lys. All fluorescent probes except N-(acetamidoethyl)-1-naphthylamine-5-sulfonic acid (AEDANS) were introduced on the resin after assembly of all amino acids. In the case of 5(6)-carboxyfluorescein (FAM), 5(6)-carboxyfluorescein succinimidyl ester (FAM-SE, 3 eq., Molecular Probes, Inc.) was attached to the N-terminus or the ε-amino group of Lys. The free ε-amino group of Lys was generated by removal of the Mtt group by treatment with dichloromethane (DCM) / triisopropylsilane (TIS) / trifluoroacetic acid (TFA) (94 / 5 / 1, v/v). In the case of coumarin, 7-diethylaminocoumarin-3-carboxylic acid (DAC, 3 eq., Fluka) was coupled to the N-terminus of the peptides using HBTU (3 eq.), 1-hydroxybenzotriazole (HOBt, 3 eq.) and diisopropylethylamine (DIEA, 6 eq.). In the case of AEDANS, N-(iodoacetamidoethyl)-1-naphthylamine-5-sulfonic acid (IAEDANS, 2eq., Research Organics) was coupled to the thiol group of Cys peptides in 100 mM Tris-HCl (pH 7.4) after cleavage by 1 h treatment with TFA / m-cresol / ethanedithiol / thioanisole (40 / 1 / 3 / 3, v/v). In the case of DABCYL of D-L8K6-E, 4-dimethylaminoazobenzene- 4'-carboxylic acid (DABCYL, 3 eq.) was coupled to the N-terminus of peptides using the HBTU-HOBt method on the resin before the introduction of AEDANS to the peptide. After introduction of the fluorescent moiety (except for AEDANS), the resin and all the protecting groups except Acm were removed by the TFA method. The peptides were purified by RP-HPLC and characterized by MALDI-TOFMS: L8K6-E, m/z 2102.2 ([M+H]$^+$ calcd. 2101.8); F-L8K6, m/z 2224.8 ([M+H]$^+$ calcd. 2224.8); C-L8K6, m/z 2105.5 ([M+H]$^+$ calcd. 2105.8); D-L8K6-E, m/z 2355.5 ([M+H]$^+$ calcd. 2353.1); C-L8K6-F, m/z 2424.9 ([M+H]$^+$ calcd. 2422.1). Peptide abbreviations are shown in Figure 1.

Synthesis of a peptide library

The designed peptide library was synthesized on Tenta-Gel S RAM resin by a combination of automatic (Advanced ChemTech Model 348 MPS) and manual syntheses. The methods for side chain protection were the same as those described in the previous section, and additionally: *t*-butyl (tBu) for Glu and Ser; Trt for Gln; and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. Initially, Fmoc-Lys(Mtt)-Gly-resin was synthesized from Fmoc-AA-OH (5 eq.) by the manual HBTU-HOBt method. After removal of the Mtt group by treatment with DCM / TIS / TFA (18 / 1 / 1, v/v), the fluorescein moiety was introduced with FAM-SE (3 eq.) and DIEA (2 eq.). Ac-Cys-Gly-K(Mtt)- [various sequences]-Lys(FAM)-Gly-resins were then synthesized using the synthesizer with Fmoc-AA-OH (10 eq.) by the HBTU-HOBt method. After assembly of all peptides, the Mtt group was removed by treatment with DCM / TIS / TFA (94 / 5 / 1, v/v) and the coumarin moiety was introduced using DAC (3 eq.) with the HBTU-HOBt method. The peptides with fluorescent probes were cleaved from the resin and side chain protections were removed by treatment with TFA / TIS / H_2O (40 / 1 / 1, v/v) for 2 h. The peptides were precipitated from diethyl ether and collected by centrifugation. The crude peptides were quickly purified by gel permeation chromatography using Sephadex LH-20 (Amersham Biosciences KK) swelled with methanol. The purified peptides were dissolved in methanol or trifluoroethanol (TFE) at ca. 1 mM, and stored at 4 °C. In order to estimate the concentration of the stock solution of the library peptides, absorbance at 450 nm of a diluted solution of peptides was compared with that of a standard peptide, L8K6, the concentration of which was determined by amino acid analysis (ε=13200). The absorbance was measured on a Benchmark Multiplate Reader (Bio-Rad Laboratories) with a 450 nm filter using microtiter plates (Assay Plate, IWAKI) in 50 mM KH_2PO_4 containing 5 M guanidine hydrochloride (pH 9.0).

Spectroscopic measurements

The fluorescence measurements were performed in 20 mM Tris-HCl containing 0.1 mM $CaCl_2$, 150 mM NaCl, 20 mM PEG2000 (pH 7.4). Fluorescence spectra were recorded on a Hitachi F-2500 fluorescence spectrophotometer with a thermoregulator using a quartz cell with 10 mm pathlength at 25 °C. Fluorescence intensities of the fluorescent-labeled peptides in microtiter plates were measured by the Twinkle LB970 apparatus (Berthold) at 30 °C. 440 nm and 535 nm filters were used for excitation and emission, respectively. Circular dichroism (CD) measurements were performed in 20 % TFE, 20 mM Tris-HCl containing 0.5 mM $CaCl_2$ (pH 7.4). CD spectroscopy was performed on a Jasco J-720WI spectropolarimeter with a thermoregulator using a quartz cell with 1 mm pathlength at 25 °C. Molecular ellipticities represented mean residual values calculated by the number of residues of the core 14 amino acids.

Peptide immobilization onto 96-well polystyrene plates

The 'hydrocoating' technique used here is essentially identical to that previously described [15, 17]. 2,2,2-Trifluoroethanesulfonyl (tresyl) activated dextran (TAD) was prepared from dextran (MW 74,000) with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride). TAD-modified plates were also prepared by the method described in [17]. Briefly, a solution of poly-L-lysine (MW 70,000–150,000, 0.01 mg/ml) in carbonate buffer (0.1 M, pH 9.6) was added to polystyrene microtiter plates (150 μl per well), and the plates were incubated overnight at 4 °C. The plates were washed and TAD (0.5 mg/ml) in phosphate buffer (10 mM phosphate, 150 mM NaCl, pH 7.2) was added to the plates (150 μl/well) and incubated for 2 h at 4 °C. To the TAD-modified plates, 1,4-diaminobutane or diethylene glycol bis(3-aminopropyl) ether (10 mM in the carbonate buffer) was added (100 μl/well) and incubated for 2 h at room temperature. After washing and blocking with 0.1 M 2-aminoethanol, bromoacetic acid (BrAcOH) was introduced to the amino-functionalized plates using its anhydride, which was prepared by mixing BrAcOH and N, N'-diisopropylcarbodiimide (DIC) in N-methylpyrrolidone (NMP) for 1 h. The fluorescent-labeled peptides (10-30 μM in 100 mM Tris HCl, pH 8.0) were added to the bromoacetic acid-modified plate (100 μl/well) and incubated for 8-10 h at room temperature. After washing with phosphate buffer containing 1% Triton X-100 (washing buffer) followed by water, the plate was dried with nitrogen and stored at 4 °C or used for the protein binding assay as described below.

Protein detection assay using microtiter plates

All measurements were performed in 20 mM Tris-HCl containing 0.1 mM $CaCl_2$, 150 mM NaCl, and 20 mM PEG2000 (pH 7.4) (assay buffer). In the case of solution assay, each solution of peptides (2.0 μM, 50 μl/well) and proteins (1.0 mg/ml, 50 μl/well) was added to the plates (total 100 μl/well), and the fluorescence intensities (I) were immediately measured using a microplate reader at 30 °C. The intensities of peptide alone (I_0) were also measured with peptide samples at 1.0 μM (100 μl). In the case of immobilized peptides, the protein solutions were prepared at 500 μg/ml. The solutions were added to the peptide-bound plates (100 μl/well) and incubated for 24 h at room temperature. The fluorescence intensities (I) were measured using a microplate reader. After washing with the washing buffer and water, the assay buffer was added onto the plates (100 μl/well), and fluorescence intensities (I_0) were measured. The peptide response to the proteins was expressed using intensity ratio, I/I_0, for both solution and immobilized assays.

Data treatment for color scale 'protein fingerprints'

Data treatment for 'protein fingerprints' used here was essentially identical to that reported previously [15, 18]. The file format used was portable-pixel-map (.ppm). Each grid position was first assigned three whole numbers corresponding to RGB color codes from (0, 0, 0) (full black, minimum value) to (255, 0, 0) (red) to (255, 255, 0) (yellow, maximum value), which correspond to all the fluorescence change rates (*I/Io*) divided into 511 levels. A grid with X columns and Y lines was thus coded with 3X columns and Y lines of integers between 0 and 255. The numbers of the grid were saved as a comma-separated-value (.csv) file. This file was then opened in a text editor (Notepad) and the following three (or four) lines were inserted at the top of the file:

> P3
> # (optional line with identifier)
> X Y
> 255

where X = number of columns in the image table and Y = number of lines in the image table. The file was then saved from the text editor in the portable-pixelmap format by simply adding '.ppm' to the filename. This file was opened by graphic viewer software such as Irfan view, resized and saved in other formats such as bitmap file format (.bmp).

Results

Design and synthesis of model peptides

In order to establish the synthetic and assay procedures, a model peptide-protein system was selected to give a designed peptide with an α-helical structure bound to a known protein. Calmodulin (CaM), chosen as model protein, consists of 148 amino acids with two EF-hands and binds to cationic amphiphilic α-helical peptides in the presence of Ca^{2+} [19, 20]. The model peptide, LK2 (L8K6), was a basic amphiphilic α-helical peptide with the sequence LKKLLKLLKKLLKL [21].

Using this model system, fluorescent probes were chosen for the detection of proteins. The structures of peptides with various fluorescent probes are shown in Figure 1. The peptide with a single probe at the C-terminus was L8K6-E (L8K6 with AEDANS). The peptides with a single probe at the N-terminus were F-L8K6 (L8K6 with FAM) and C-L8K6 (L8K6 with DAC). The peptides that have probes at both termini were D-L8K6-E (L8K6 with DABCYL and AEDANS), C-L8K6-F (L8K6 with FAM and DAC). The combinations of FAM-DAC and DABCYL-AEDANS were selected to use the fluorescence resonance energy transfer (FRET) function since DAC and AEDANS have fluorescence emissions at wavelengths near the absorption peaks of FAM and DABCYL, respectively (ca. 490 nm). The FRET of the combination of DABCYL-AEDANS is a quenching system, since DABCYL is not a fluorophore.
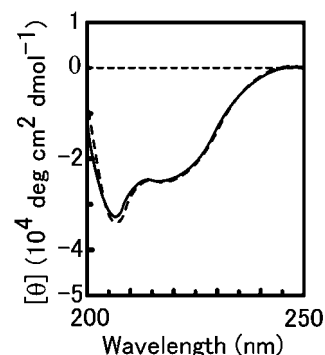

Figure 2. Circular dichroism spectra of designed α-helical peptides. D-L8K6-E (—), C-L8K6-F (---). [Peptide] = 20 μM in 20% TFE, 20 mM Tris-HCl, 0.5 mM $CaCl_2$ (pH 7.4) at 25 °C.

The designed peptides based on L8K6 were synthesized by standard solid-phase Fmoc chemistry [16]. After assembly of all the amino acids, various fluorescent probes were incorporated into the N-terminus or both termini of the peptidyl resin. In the case of the peptides with two probes, fluorophores were introduced on the resin by the orthogonal protecting strategy or one of them was introduced in solution after cleavage.

Structural analysis using circular dichroism spectroscopy

In order to estimate the structural features of the peptides, the far-UV CD spectra of designed peptides were measured, as shown in Figure 2. All peptides displayed negative minima in ellipticities at 208 nm and 222 nm, which are characteristics of an α-helical structure [22]. These results suggest that the peptides themselves adopted an α-helical structure in aqueous solution, although it was difficult to obtain information about the conformation of the peptides complexed with CaM because of strong signals from the protein.

Detection of CaM using model peptides with various fluorophores in solution

To compare the responses of the peptides with a variety of fluorophores by addition of the target protein, the fluorescence spectrum of these peptides and the change upon addition of CaM were measured in the buffer solution (Figure 3). In the case of an FAM-labeled peptide, F-L8K6, a ca. 6 fold increase in the fluorescence intensity was observed on addition of CaM. From the fluorescence changes, the binding constant of F-L8K6 with CaM was calculated as 3.7×10^6 M^{-1} by means of a single site binding equation [23]. The DAC-labeled peptide, C-L8K6, did not show any remarkable increase in the fluorescence intensity (ca. 3 fold). The AEDANS-labeled peptide, L8K6-E, was not suitable for detection of CaM because its fluorescent increment was very low on addition of CaM. In the cases of C-L8K6-F and D-L8K6-E, which have two fluorescent moieties using the FRET technique, C-L8K6-F showed the most remarkable fluorescence increase (ca. 13 fold) among all the peptides
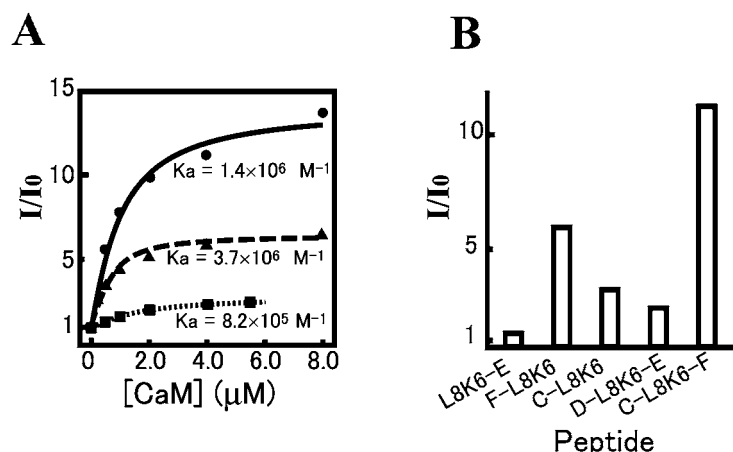
Figure 3. Fluorescence changes of α-helical peptides upon addition of CaM in 20 mM Tris-HCl, 0.1 mM CaCl$_2$, 150 mM NaCl, 20 mM PEG2000 (pH 7.4) at 25 °C. (**A**) Fluorescence titration curves of C-L8K6-F (λex. 440 nm, λem. 522 nm) (——), F-L8K6 (λex. 490 nm, λem. 530 nm) (——), and D-L8K6-E (λex. 338 nm, λem. 480 nm) (---) upon addition of CaM. [Peptide] = 0.5-0.8 μM. (**B**) Fluorescence increases of L8K6-E (λex. 338 nm, λem. 494 nm), F-L8K6, C-L8K6 (λex. 440 nm, λem. 482 nm), D-L8K6-E, and C-L8K6-F upon addition of CaM (4.0 μM).

upon addition of CaM. The binding constant of C-L8K6-F with CaM was calculated to be 1.4×10^6 M^{-1}. Only a small fluorescence increment (ca. 2.5 times) of the quenching FRET system, D-L8K6-E, was observed.

These results suggest that the target protein, CaM, was detectable at high sensitivity using labeled peptides such as F-L8K6 and C-L8K6-F. In particular, C-L8K6-F was the most suitable for detection of CaM as it acquired a high fluorescent intensity and the most remarkable fluorescence increase. The FRET system with coumarin and fluorescein was therefore selected and used in further experiments.

Immobilization of model peptides for detection of model proteins

Detection of the protein using immobilized peptides on a solid surface was also attempted in order to develop a high-throughput detection system. In this experiment, a 96-well microtiter plate was used as solid support. The 'hydrocoating' technique [17] was used to immobilize peptides onto the plate [15]. 'Hydrocoating' is a technique for the covalent immobilization of biomolecules in highly hydrophilic surroundings produced by dextran coating. The method is outlined in Figure 4A. A polymer surface (polystyrene) of a microtiter plate was first coated with poly-L-lysine to produce an amino-functionalized surface. TAD was attached covalently to the surface leaving a sufficient number of active groups for secondary binding of other molecules having amino or thiol groups. In this case, further modifications of the surface by diamine and bromoacetic acid were carried out for the selective attachment of peptides using the reaction between a thiol group and alkyl halide. Detailed protocols are described in the experimental section.

Using this method, eight different peptides labeled with fluorescein and coumarin moieties were immobilized onto a microtiter plate via a free thiol group of the Cys peptide side

chain. Figure 4B shows the sequences of the eight peptides. On the basis of the L8K6 sequence, eight Leu residues were replaced systematically with Ala (with respect to hydrophobicity) or six Lys residues were replaced systematically with Glu (change in charge).

The fluorescence changes of the immobilized peptides after addition of the protein were measured using a microplate reader. The CaM solution was added to each well containing the immobilized peptides, incubated and the fluorescence intensities (I) were then measured with a microplate reader. After removal of solutions and washing with the washing buffer and water, the assay buffer was added to the wells, and the fluorescence intensities of immobilized peptides were measured as their initial intensities (I_0). The responses to the addition of the proteins were evaluated using the fluorescent intensity (I/I_0). As shown in Figure 4C, L8K6 immobilized on the 96-well plate gave ca. 1.6 times increments of fluorescence intensity on addition of CaM. The eight peptides afforded different increases in fluorescence intensity. The peptides with the more positive and the more hydrophobic residues showed higher responses to CaM, which indicates that CaM prefers to bind a cationic amphiphilic α-helical peptide, which is compatible with the reported features of CaM binding peptide [19, 21]. In addition, as shown in Figure 4D, all peptides gave almost no response upon addition of insulin. These results therefore imply that a protein can be characterized by patterns in the fluorescence response of library peptides.

Design of an α-helical peptide library for detection of various proteins

A small library consisting of 112 designed α-helical peptides with various charges and/or hydrophobicities was constructed to detect a variety of proteins as well as CaM. The strategy for the construction of an α-helical peptide library
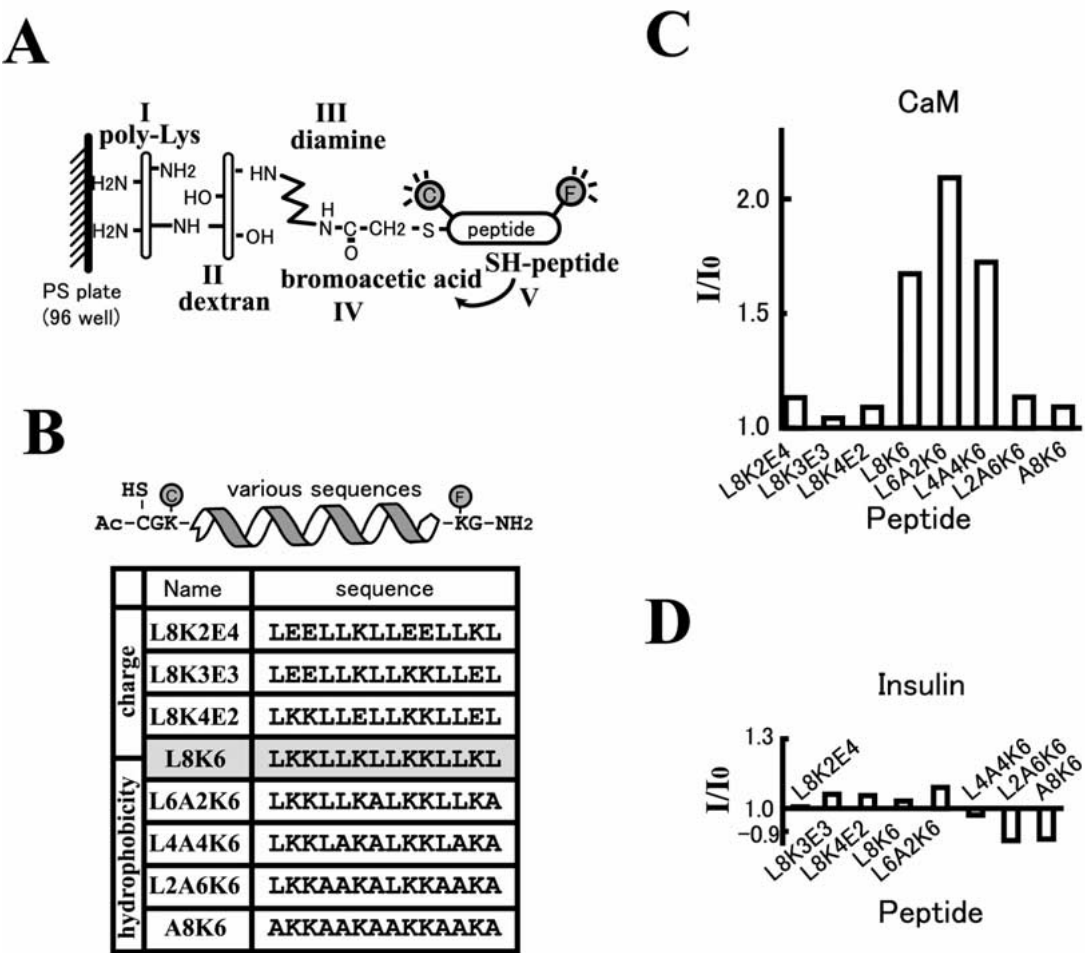
Figure 4. Fluorescence measurements of immobilized peptides on addition of CaM or insulin. (**A**) Outline of the method for immobilization of peptides onto a microtiter plate [15, 17]. (**B**) Sequences of eight immobilized peptides labeled with the fluorescein and coumarin moieties. Fluorescence increases of immobilized peptides (**C**) upon addition of CaM (10 μM) and (**D**) upon addition of insulin (10 μM) in 20 mM Tris-HCl, 0.1 mM $CaCl_2$, 150 mM NaCl, 20 mM PEG2000 (pH 7.4) at 30 °C, λex. 450 nm, λem. 530 nm.

is shown in Figure 5A. On the basis of the L8K6 sequence, the eight Leu residues were replaced systematically with Ala or Phe, and the six Lys residues were also replaced systematically with Glu (K-E series), Ser (K-S series), or Gln (K-Q series). Furthermore, instead of the six Lys residues, six Arg residues were employed and similarly replaced with Glu (R-E series). Figure 5B shows all species of the library peptides. The column heading and the row heading represent, respectively, hydrophobicity (A or F substituted for L), and charge (E, S or Q substituted for K and E substituted for R). Each cell displays the abbreviation of the synthesized peptide in this library. The library thus consists of four series of designed peptides, each of which had 28 systematic peptides, to give a total of 112 α-helical peptides in the library.

Preparation of library peptides

The designed peptide library (112 peptides) was constructed in a parallel solid-phase synthesis by a combination of manual and automatic procedures. Fmoc-K(FAM)-G-resin was first synthesized manually. Fmoc-K(Mtt)-G-

resin was prepared and the 4-methyltrityl (Mtt) group of the Lys residue was removed. The fluorescein moiety was introduced using the succinimidyl ester of 5(6)-carboxyfluorescein to the side chain of the Lys residue. Remaining residues, including the core sequences of the library position, were assembled on a peptide synthesizer. The Mtt group of the Lys residue near the N-terminus was then removed manually. The coumarin moiety was introduced into the side chain of the Lys residue by the same method as the manual synthesis of peptides. After cleavage, the peptides were quickly purified by gel permeation chromatography.

Characterization of CaM using α-helical peptide library

CaM was characterized using the α-helical peptide library in solution. The assay buffer containing peptide alone and the buffer containing peptide and CaM were added to each well, and the fluorescence intensities were measured (I_0 and I, respectively). The responses to the addition of the proteins were evaluated using the I/I_0 values calculated as an incre-
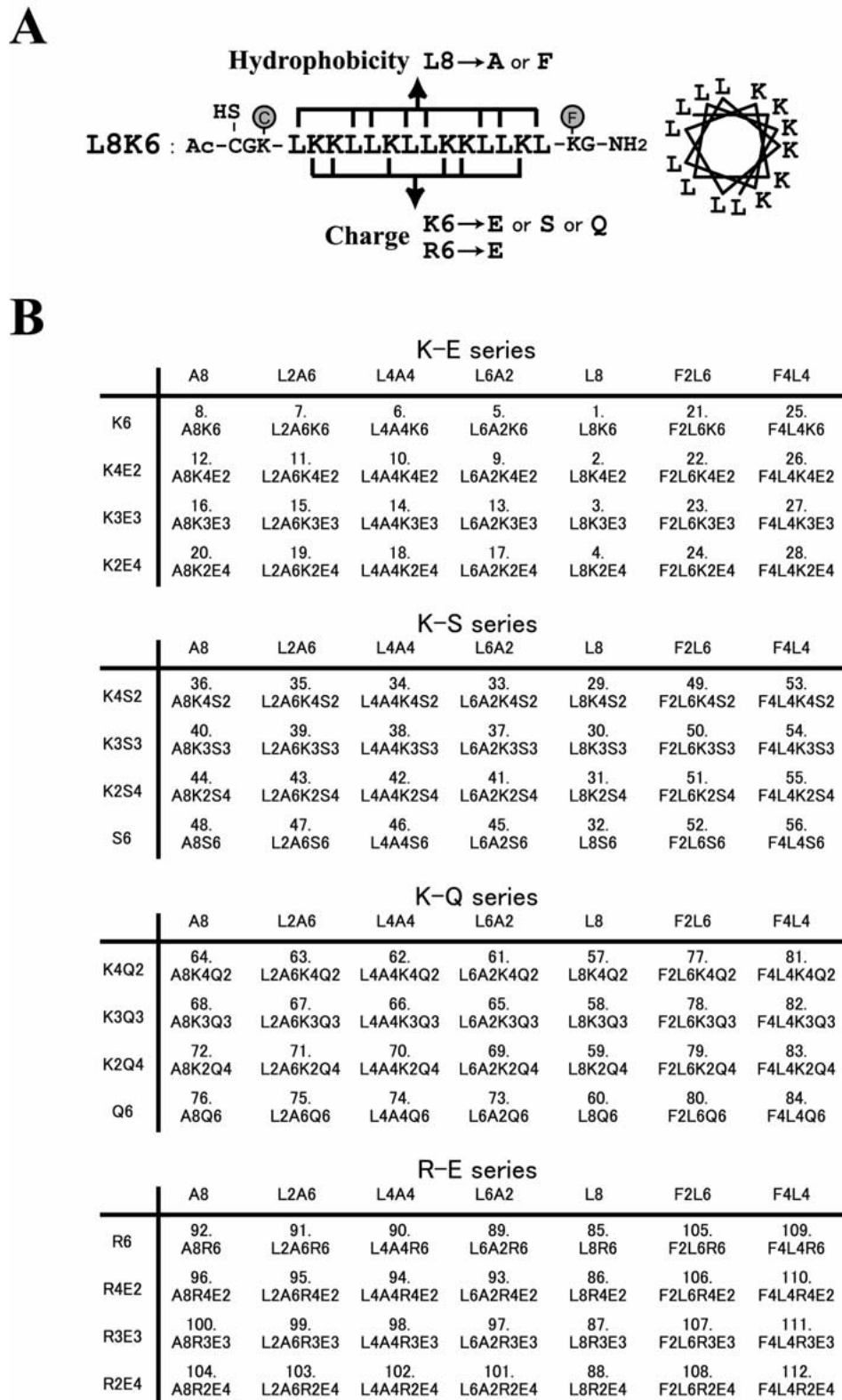
A

Hydrophobicity L8→A or F

HS ⓒ
L8K6 : Ac-CGK- LKKLLKLLKKLLKL -KG-NH2 Ⓕ

Charge K6→E or S or Q
R6→E

B

K-E series

	A8	L2A6	L4A4	L6A2	L8	F2L6	F4L4
K6	8. A8K6	7. L2A6K6	6. L4A4K6	5. L6A2K6	1. L8K6	21. F2L6K6	25. F4L4K6
K4E2	12. A8K4E2	11. L2A6K4E2	10. L4A4K4E2	9. L6A2K4E2	2. L8K4E2	22. F2L6K4E2	26. F4L4K4E2
K3E3	16. A8K3E3	15. L2A6K3E3	14. L4A4K3E3	13. L6A2K3E3	3. L8K3E3	23. F2L6K3E3	27. F4L4K3E3
K2E4	20. A8K2E4	19. L2A6K2E4	18. L4A4K2E4	17. L6A2K2E4	4. L8K2E4	24. F2L6K2E4	28. F4L4K2E4

K-S series

	A8	L2A6	L4A4	L6A2	L8	F2L6	F4L4
K4S2	36. A8K4S2	35. L2A6K4S2	34. L4A4K4S2	33. L6A2K4S2	29. L8K4S2	49. F2L6K4S2	53. F4L4K4S2
K3S3	40. A8K3S3	39. L2A6K3S3	38. L4A4K3S3	37. L6A2K3S3	30. L8K3S3	50. F2L6K3S3	54. F4L4K3S3
K2S4	44. A8K2S4	43. L2A6K2S4	42. L4A4K2S4	41. L6A2K2S4	31. L8K2S4	51. F2L6K2S4	55. F4L4K2S4
S6	48. A8S6	47. L2A6S6	46. L4A4S6	45. L6A2S6	32. L8S6	52. F2L6S6	56. F4L4S6

K-Q series

	A8	L2A6	L4A4	L6A2	L8	F2L6	F4L4
K4Q2	64. A8K4Q2	63. L2A6K4Q2	62. L4A4K4Q2	61. L6A2K4Q2	57. L8K4Q2	77. F2L6K4Q2	81. F4L4K4Q2
K3Q3	68. A8K3Q3	67. L2A6K3Q3	66. L4A4K3Q3	65. L6A2K3Q3	58. L8K3Q3	78. F2L6K3Q3	82. F4L4K3Q3
K2Q4	72. A8K2Q4	71. L2A6K2Q4	70. L4A4K2Q4	69. L6A2K2Q4	59. L8K2Q4	79. F2L6K2Q4	83. F4L4K2Q4
Q6	76. A8Q6	75. L2A6Q6	74. L4A4Q6	73. L6A2Q6	60. L8Q6	80. F2L6Q6	84. F4L4Q6

R-E series

	A8	L2A6	L4A4	L6A2	L8	F2L6	F4L4
R6	92. A8R6	91. L2A6R6	90. L4A4R6	89. L6A2R6	85. L8R6	105. F2L6R6	109. F4L4R6
R4E2	96. A8R4E2	95. L2A6R4E2	94. L4A4R4E2	93. L6A2R4E2	86. L8R4E2	106. F2L6R4E2	110. F4L4R4E2
R3E3	100. A8R3E3	99. L2A6R3E3	98. L4A4R3E3	97. L6A2R3E3	87. L8R3E3	107. F2L6R3E3	111. F4L4R3E3
R2E4	104. A8R2E4	103. L2A6R2E4	102. L4A4R2E4	101. L6A2R2E4	88. L8R2E4	108. F2L6R2E4	112. F4L4R2E4

Figure 5. (**A**) Strategy for construction of the α-helical peptide library. (**B**) Numbers and names of peptides in the α-helical peptide library.

ment of the fluorescence intensity with protein against that without protein. The peptides showed a sequence-dependent resp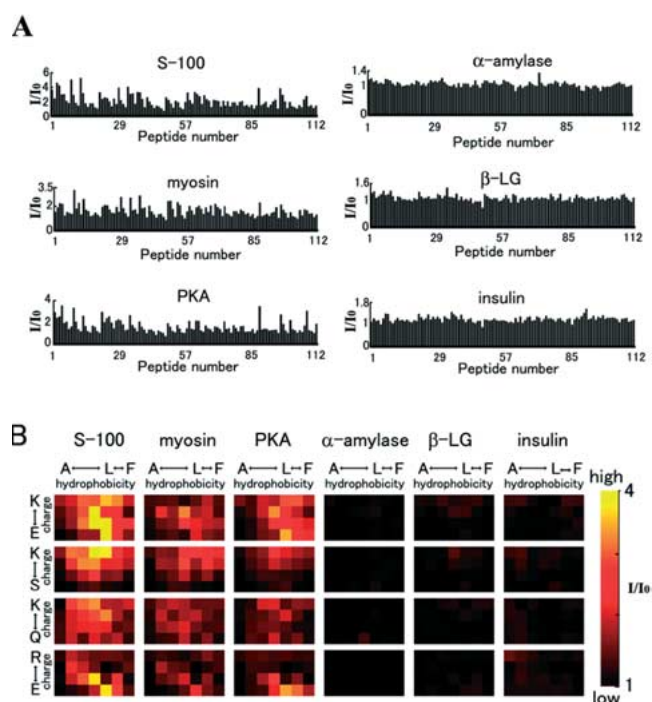onse to CaM addition (Figure 6A). Some showed higher fluorescent responses (3-4 fold), and some gave responses less than 2 fold. To understand the binding properties of CaM more easily, the pattern of these fluorescence responses
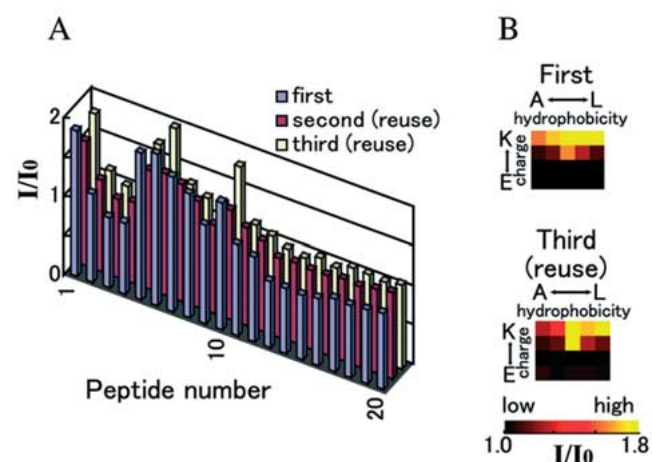
A

B

C
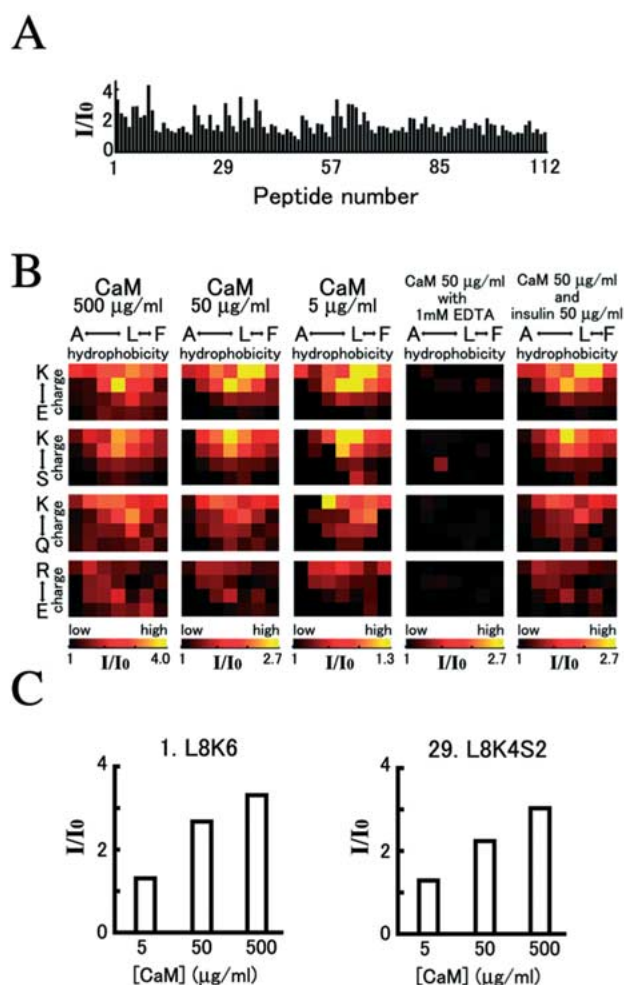
Figure 6. (**A**) Fluorescence responsive pattern of the α-helical peptide library on addition of 500 μg/ml CaM in 20 mM Tris-HCl, 0.1 mM CaCl$_2$, 150 mM NaCl, 20 mM PEG2000 (pH 7.4) at 30 °C, λex. 440 nm, λem. 535 nm. [Peptide] = 1.0 μM. (**B**) The 'protein fingerprints' of CaM (500 μg/ml, 50 μg/ml, 5 μg/ml), CaM (50 μg/ml with 1 mM EDTA) and a mixture (CaM 50 μg/ml and insulin 50 μg/ml) in 20 mM Tris-HCl, 0.1 mM CaCl$_2$, 150 mM NaCl, 20 mM PEG2000, (pH 7.4) at 30 °C. [Peptide] = 1.0 μM. (**C**) Fluorescence intensities of L8K6 (No.1) and L8K4S2 (No.29) on addition of CaM (500 μg/ml, 50 μg/ml, 5 μg/ml). [Peptide] = 1.0 μM.

A

B

Figure 7. (**A**) Fluorescence responses of the α-helical peptide library on addition of 500 μg/ml of various proteins in 20 mM Tris-HCl, 0.1 mM CaCl$_2$, 150 mM NaCl, 20 mM PEG2000 (pH 7.4) at 30 °C, λex. 440 nm, λem. 535 nm. [Peptide] = 1.0 μM. (**B**) 'Protein fingerprints' of various proteins. S-100: S-100 proteins, PKA: protein kinase A, β-LG: β-lactoglobulin.

A

B

Figure 9. (**A**) Reuse of the immobilized α-helical peptide library on addition of 500 μg/ml CaM in 20 mM Tris-HCl, 0.1 mM CaCl$_2$, 150 mM NaCl, 20 mM PEG2000 (pH 7.4) at 30 °C, λex. 440 nm, λem. 535 nm. (**B**) 'Protein fingerprints' of CaM using the immobilized α-helical peptide library after the first and the third usage.

was converted into a color image [15], regarded as a 'protein fingerprint' as shown in Figure 6B. The color images correspond to four series of peptides (Figure 5B), and the color of each cell displays the response of each peptide against CaM. The cells in the top right-hand portion of each series were colored yellow or red, except for the R-E series. According to these results, CaM obviously prefers to bind cationic amphiphilic peptides with Leu residues. A protein fingerprint of CaM in the buffer solution including 1 mM EDTA was also obtained (Figure 6B). This 'protein fingerprint' was almost dark in contrast to that of CaM (50 μg/ml) with free Ca^{2+} in the assay buffer solution. This indicated that in the absence of Ca^{2+}, CaM can bind few peptides due to a structural change of CaM itself, and that the fingerprint depends strictly on the protein tertiary structure. Clearly the

α-helical peptide library has the capability of recognizing protein tertiary structure.

Furthermore, CaM at various concentrations showed the same relative responses (Figure 6B), although the fluorescence intensities were different. The fluorescence increments of typical peptides (No. 1 and 29) in the library are shown in Figure 6C, indicating that the fluorescence intensities for CaM are dose-dependent. These results imply that the
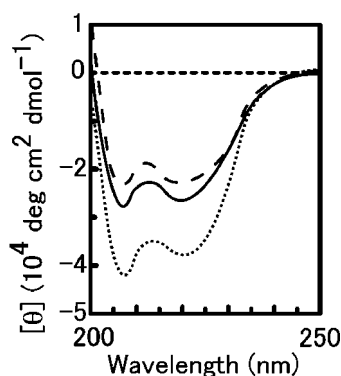
Figure 8. Circular dichroism spectra of designed α-helical peptides. No. 37 L6A2K3S3 (—), No. 81 F4L4K4Q2 (——), No. 98 L4A4R3E3 (---). [Peptide] = 5.0 μM in 20% TFE, 20 mM Tris-HCl , 0.5 mM CaCl$_2$ (pH 7.4) at 25 °C.

concentration of CaM could be evaluated from the information of both the protein fingerprints and the fluorescence intensities.

Characterization of various proteins using α-helical peptide library

A variety of proteins could also be characterized using the α-helical peptide library. Representative protein samples were selected as follows; CaM, S-100 proteins (S-100), myosin as model proteins containing EF hand motifs, protein kinase A (PKA) as a model of kinase, α-amylase as a model of glycosidase, β-lactoglobulin (β-LG) as a model of proteins abundant in β-strand structures, insulin as a model of small proteins abundant in α-helix structures. The responses upon addition of the proteins were evaluated using the I/I_0 values. Figure 7A shows the responses of each peptide against various proteins. The responses to CaM, S-100, myosin or PKA showed characteristic patterns for each protein with 3-5 fold increments for some peptides. α-Amylase, β-LG and insulin showed weak response patterns.

In order to compare these patterns easily, color image 'protein fingerprints' are shown in Figure 7B. Protein fingerprints are clearly characteristic and are able to identify each protein individually. It is noteworthy that remarkable differences occur for the protein fingerprints of CaM, S-100 and myosin, even though these proteins contain EF hand α-helical motifs.

The far-UV CD spectra of typical peptides in the library (Nos. 1, 37, 81, 98) were measured, as shown in Figure 8 (No. 1 in Figure 2). All the peptides displayed negative minima in ellipticities at 208 nm and 222 nm, which are characteristic of α-helical structure [22]. These results indicate that the peptides themselves assume an almost α-helical structure in aqueous solution.

Furthermore, the protein fingerprint of a mixture (50 μg/ml CaM and 50 μg/ml insulin) was obtained (Figure 6B). The protein fingerprint of the mixture was almost the same as that of 50 μg/ml CaM alone as insulin gave weak response

patterns. Therefore, CaM was also characterized using the α-helical peptide library in the mixed solution. This indicates that the target protein can be characterized from its 'protein fingerprint' in mixtures that contain the target protein and other proteins giving weak response patterns.

Characterization of CaM using immobilized peptide library

Furthermore, CaM could be characterized using the immobilized α-helical peptide library (No. 1-20) on a 96-well microtiter plate in the same manner as described above. Upon addition of CaM, the peptides showed different responses depending on their sequences (Figure 9A). The CaM solution was added to each well containing an immobilized peptide and the increase in the fluorescence intensity (I) was then measured with a microplate reader. After removal of the solutions and washing with the washing buffer and water, the assay buffer alone was added to the wells and the fluorescence intensity of each immobilized peptide alone was measured (I_0). The responses to the addition of proteins were evaluated using the I/I_0 values. Some peptides showed characteristic responses with fluorescence increase (ca. 1.5-2 fold) and the 'protein fingerprint' was obtained, as shown in Figure 9B. Substantially similar results to those in the solution assay were obtained, although this protein fingerprint has some differences and low responses (low fluorescence increases). In addition, the 'protein fingerprint' of the first assay was comparable to that of the repeated assay using the same array. This implies that the α-helical peptide array could be reused several times.

Discussion

We have described the successful construction of a protein detection system using structurally designed and synthetic α-helical peptides with a FRET function. To establish the synthetic and detection methods we selected a model peptide, L8K6, which is a cationic amphiphilic α-helical peptide that binds to calmodulin. The designed peptides could be synthesized with fluorescent probes by introducing them on the resin. The increments in the fluorescence intensity of the peptides with various fluorophores were examined by addition of calmodulin in aqueous solution. In comparison to the peptides with a single probe, a relatively higher FRET response could be observed between two probes at both termini. This indicates that transition from rather flexible to rigid structures affects the higher responses. It was also found that fluorescein was an effective fluorophore in this system because of its high fluorescence intensity. In addition, the target protein was detectable with the peptides immobilized on a solid support.

Subsequently, an α-helical peptide library with systematically changed charges and/or hydrophobicities was constructed for peptide microarrays. When the various proteins were added, peptides showed different fluorescent responses

depending on their sequences. As a result, each protein acquired its individual fluorescence patterns. The pattern of these responses could be regarded as a 'protein fingerprint', which was able to establish the identity of the proteins. The present technology using the 'protein fingerprints' with the designed peptide library can be used to characterize various proteins. This α-helical peptide library can recognize tertiary structures of proteins and discriminate some proteins with structural similarity, such as α-helical motifs, using their 'protein fingerprints'. This study also implies that concentrations of a protein could be estimated from both the 'protein fingerprints' and fluorescence intensities. As we had initially expected, the α-helical synthetic peptide array could be reused in the solid phase assay. Furthermore, these 'protein fingerprints' indicate binding features of the proteins, as the α-helical library was designed with various charge variation and/or hydrophobicities by systematic substitutions within the peptide sequences.

The study has demonstrated that a novel protein detection system can be developed using 'protein fingerprints' and a well-designed peptide library. Further improvement of the detection sensitivity and the number of peptides together with miniaturization of the array and/or robotic handling will provide practical protein chips. Designed peptide microarrays including the α-helical peptide library can be more easily manufactured than protein and antibody microarrays [15]. The peptide arrays will be applicable not only for exploratory scanning of proteins but also for high throughput/sensitive detection and analyses.

Acknowledgments

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