Design of FAD-binding Peptide Using a Combinatorial α-Helix Peptide Library

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We constructed a resin-bound peptide library in which the peptide was designed to take an α-helix structure, and *p*-boronobenzoic acid was conjugated on the ε-amino group of the Lys side-chain placed at the middle of the helix. Screening this library allowed selection of a peptide that can bind flavin adenine dinucleotide with high affinity and selectivity.

Flavin functionalities play important roles in nature as cofactors of flavoproteins.[1] In the field of de novo protein design, much effort has been devoted to the construction of artificial polypeptides that incorporate flavin molecules, but most of these systems feature covalent attachment of flavin chromophore to the peptide.[2,3] Recently, Butterfield et al. designed a β-hairpin peptide that binds flavin mononucleotide (FMN) via noncovalent interaction.[4] However, in order to establish an artificial flavoprotein having multiple functions, another binding motif toward flavin functionalities needs to be developed. In light of the above, we have attempted to develop a peptide that can bind to a flavin adenine dinucleotide (FAD) by a combinatorial chemistry using an α-helix scaffold.

In this study, we used an α-helical peptide library, H_2N-**X** E A **Y** **Z** R E **K(BB)** A A R E A A A R A-βAla-NH-resin (BB; *p*-boronobenzoyl). The library peptide was designed based on the sequence reported by Marqusee and Baldwin.[5] This peptide has three sets of Glu–Arg pairs to form salt bridges in the side-chains of the α-helical form. We employed the Lys residue in place of the original Ala at the 8th-position in order to combine the BB group selectively on its side-chain. It is known that boronic acid forms complexes with a variety of sugar molecules in an aqueous solution.[6] Therefore, we expected that the BB group could provide a suitable binding site for either the ribitol or ribose unit of FAD. Each **X**, **Y**, or **Z** position in the sequence had one of 19 amino acids (all standard amino acids except for Cys), giving a library with 6859 ($=19^3$) different compounds. In the designed α-helical structure, the **X**, **Y**, and **Z** residues and the BB moiety on the Lys[8] side-chain are arranged on the same side of the helix (Figure 1). The peptide library was synthesized by the Fmoc solid-phase method on an amino PEG resin (Fmoc, fluoren-9-ylmethoxycarbonyl).[9] For the screening of the library, 10 μM of FAD was incubated with sufficient resin at 25 °C in 50 mM $NaHCO_3$/100 mM NaCl buffer (pH 10.0). After washing the beads with the buffer to remove unbound FAD, the FAD bound to peptides on the beads were detected using fluorescence emission at around 510 nm. The screening procedure identified a total of 3 active beads, and their peptide sequences were determined by the Edman method using a protein sequencer (Table 1). The screening results suggested a preference for aromatic residues in the **Y** and **Z** positions, with all sequences containing Trp or Tyr at either of both of these loci. In these peptides, a π–π stacking interaction between the aromatic side-chain and the isoalloxazine ring might contribute to the formation of peptide-FAD conjugate. Such an interaction has been observed in natural flavoproteins. In addition, Butterfield et al. have shown in their model β-hairpin peptide, the importance of the stacking interaction between FMN and Trp residues.[4] Although, the natural flavoenzymes often have aromatic residues for flavin interaction in the loop structure of the flavin binding site, such residues may also be strong contributors to FAD-binding in the designed α-helical peptide.

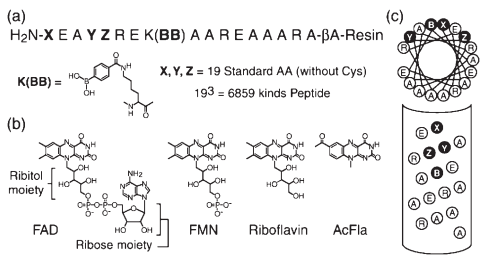

Figure 1. (a) Chemical structure of the resin-bound peptide library; (b) Chemical structure of FAD, FMN, riboflavin, and AcFla; (c) Helix wheel and net drawings of the 17-peptide. The letter B denotes K(BB).

Table 1. Residues at X, Y, and Z positions selected from the library

Peptide	X	Y	Z
1	Val	**Trp**	Met
2	Ser	**Trp**	**Trp**
3	Asn	Ala	**Tyr**

In order to clarify the FAD-binding behavior, a resin-free peptide having one of the selected sequences (Rho–VWM–BB) was resynthesized by a standard solid-phase method (Figure 2). The peptide Rho–VWM–BB has a 5(6)-carboxytetramethylrhodamine at the *N*-terminus as a spectroscopic probe. As control compounds, the Rho–AAA–BB, which has Ala residues at the **X**, **Y**, and **Z** loci, and Rho–VWM–Ac, in which Lys[8] was modified with the acetyl group instead of the BB group, were also prepared.[8]

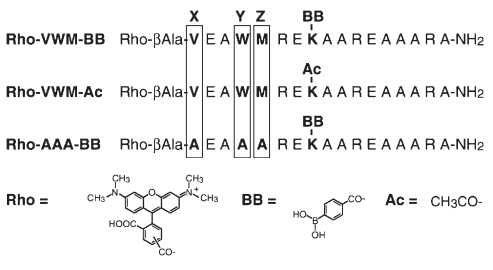

Figure 2. Chemical structure of the peptides used in the binding study.

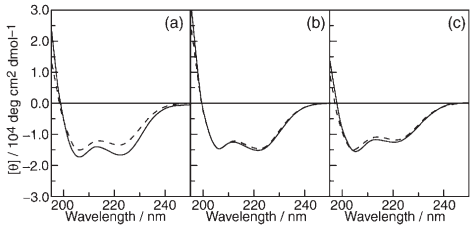

Figure 3. CD spectra of Rho–VWM–BB (a), Rho–VWM–Ac (b), and Rho–AAA–BB (c) in the presence (——) and absence (- - -) of FAD in a buffer (pH 10) at 25 °C. [Peptide] = 10 μM and [FAD] = 10 μM.

In the buffer, the peptides showed circular dichroism (CD) spectra typical for an α-helical structure both in the absence and presence of FAD (Figure 3). Interestingly, the addition of FAD (1.0 equiv.) increased the helical content of Rho–VWM–BB from 43% to 53%.[9] The peptides Rho–AAA–BB and Rho–VWM–Ac did not show notable changes in their CD spectra by the addition of FAD. These results suggest that Rho–VWM–BB bound FAD and that the increase in α-helicity took place via the FAD-binding.

The FAD-binding behavior was further characterized through titration of the peptides with FAD, monitored using UV–vis spectroscopy (Figure 4). Upon the addition of FAD, the absorbance due to the Rho chromophore at 558 nm decreased and the peak maximum shifted to a longer wavelength. Fitting the absorbance change data as a function of FAD concentration to a 1:1 binding equation gave an affinity constant (K_a) of $7.0 \times 10^5\,M^{-1}$ (Figure 4b and Table 2). The affinity constants of control peptides with FAD were smaller ($K_a = 0.23 \times 10^5\,M^{-1}$ for Rho–AAA–BB and $K_a = 0.10 \times 10^5\,M^{-1}$ for Rho–VWM–Ac, respectively). This implies that the Val, Trp, and Met residues and the BB moiety contributed to the effective FAD-binding. Meanwhile, the Rho–VWM–BB did not bind FAD in 6 M guanidine hydrochroride (GuHCl) solution. This suggests that the α-helical structure and the consequent arrangement of Val, Met, Trp, and Lys(BB) on the peptide were necessary for the FAD-binding. On the basis of the difference in affinity constants for FAD, FMN, riboflavin, and 7-acetyl-10-methylisoalloxazine (AcFla) with Rho–VWM–BB, the interaction of boronic acid in the peptide with the ribose portion of FAD is feasible (Table 2). In contrast, the peptide Rho–AAA–BB showed no preference for FAD. These results mean that the Val, Trp, and Met residues found by the screening were responsible for the observed selectivity.

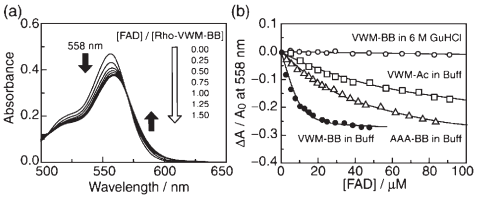

Figure 4. (a) UV–vis spectra of Rho–VWM–BB with increasing concentrations of FAD in a buffer (pH 10) at 25 °C. [Peptide] = 10 μM; (b) Plots of absorbance change at 558 nm as a function of FAD concentration at 25 °C. [Peptide] = 10 μM. Rho–VWM–BB (●), Rho–AAA–BB (△), and Rho–VWM–Ac (□) in a buffer (pH 10), Rho–VWM–BB (○) in 6 M GuHCl solution.

Table 2. Binding constants of peptides with FAD, FMN, riboflavin, and AcFla in 50 mM $NaHCO_3$/100 mM NaCl buffer (pH10) at 25 °C

Peptide	$K_a/10^5\,M^{-1}$			
	FAD	FMN	Riboflavin	AcFla
Rho–VWM–BB	7.0	0.19	1.0	<0.01
Rho–AAA–BB	0.23	0.01	0.39	<0.01
Rho–VWM–Ac	0.10	N.D.[a]	N.D.[a]	N.D.[a]

[a]Not determined.

In conclusion, we successfully obtained an artificial FAD-binding peptide by combining the rational α-helix design, the nonnatural functional unit, and the combinatorial chemistry. The method developed here will be available for the establishment of artificial flavoproteins having minimal requirements for the function.

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References and Notes

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7 The ε-amino group of Lys[8] was protected with the methyltrityl (Mtt) group to introduce the BB moiety. The **X**, **Y**, and **Z** residues were randomized using 19 amino acids according to the previously reported split and mix method. After the stepwise elongation of the peptide chain, the Mtt group was selectively deprotected by washing the resin with 1% trifluoroacetic acid/5% triisopropylsilane/dichroromethane (2 min × 5) without cleaving other protecting groups. Then, *p*-boronobenzoic acid was coupled with the Lys[8] side-chain using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole hydrate, and diisopropylethylamine as coupling reagents.
8 Peptides were identified by MALDI-TOFMS and amino acid analysis. Analytical size-exclusion chromatography using natural globular proteins as standards suggest that the peptides existed as monomeric forms under the experimental conditions.
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