Peptide Design Based on an Antibody Complementarity-Determining Region (CDR): Construction of Porphyrin-Binding Peptides and Their Affinity Maturation by a Combinatorial Method

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Abstract: We have utilized sequence information from an antiheme monoclonal antibody to develop novel porphyrin-binding peptides. Several peptides which have an intramolecular disulfide bond in different positions and different chain lengths were prepared. The affinities of peptides for *meso*-tetrakis(4-carboxyphenyl)porphyrin were increased by an appropriate conformational restraint using a disulfide bond. Detailed studies with a representative 12-peptide, **12C4**, whose length was reduced from 20 residues of the complementarity-determining region (CDR), indicated that both the hydrophobic and electrostatic interactions were essential factors in the peptide–porphyrin binding. Moreover, two-dimensional ^{1}H NMR spectroscopy revealed the conformation of the peptide and the critical residues for the porphyrin-binding. According to the obtained results, a further minimized 9-peptide, **9L**, was successfully redesigned with a sequence capable of forming a β-turn instead of a disulfide bond. Furthermore, affinity maturation studies of **9L** were performed by using a combinatorial approach such as the spot-synthesis method. Peptides with an improved affinity for porphyrins were prepared by systematic amino acid replacement. Thus, the design of peptides targeted to porphyrins was demonstrated by the combination of antibody information and the rationally designed combinatorial method.

Keywords: antibodies · combinatorial chemistry · molecular recognition · peptides · porphyrinoids

Introduction

Every life process is a manifestation of the specific and finely tuned interactions of biological molecules with complex structures. One example, par excellence, is found in the antigen recognition by antibodies. Their binding sites for specific antigens are mainly composed of amino acid residues located in six so-called hypervariable loops or complementarity-determining regions (CDRs), three each from the heavy- and light-chain variable regions.[1] These CDRs fold into loop structures that are stabilized by the β-sheet framework of the variable domains of antibodies. The main-chain conformations of CDRs have been classified into a small family referred to as "canonical structures".[2] Consequently, once the sequences of antibodies have been determined, the CDRs can be systematically identified and their conformations are predictable.[2d, 3]

These well-studied antigen-binding sites of antibody molecules hold considerable promise as model systems for the design of an antibody-like recognition motif, particularly that by antibody-derived small peptides.[4] Small peptides that can avoid disadvantages of a full antibody molecule such as complexity, instability, and immunoreactivity have a great application potential. Crystal structures of antigen–antibody complexes and/or antibody modeling studies have shown that, in many cases, only a few residues in CDRs contribute to antigen binding directly.[1b] This implies that smaller antigen-binding peptides can be designed by isolating these residues in CDRs. There are several examples of the utilization of CDR-derived peptides for the inhibition of receptor–ligand interactions or cell adhesion, and so on.[5] This strategy to design peptides may also be useful in the development of peptides that bind small molecules such as co-factors or functional chromophores.

The family of porphyrins, important co-factors, have various functions which have been utilized in artificial systems as well as in nature.[6] For well-controlled utilization of the functions, construction of a binding moiety for porphyrins might be particularly beneficial. In recent years, a number of

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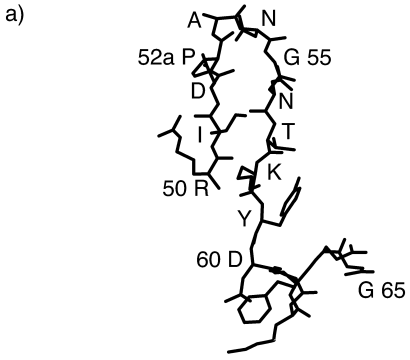
de novo designed polypeptides that conjugate with porphyrin molecules as a model of hemeprotein functions were reported.[7] There are also some examples of monoclonal antibodies for porphyrins used for incorporation of porphyrins into their binding sites to control the function as a peroxidase-like catalyst.[8]

In order to develop a novel porphyrin-binding motif, we have designed and synthesized a set of peptides based on the CDR structure of an antiheme monoclonal antibody (mAb).[9] Their porphyrin-binding properties have been examined by spectroscopic studies using various porphyrins including *meso*-tetrakis(4-carboxyphenyl)porphyrin (TCPP). Furthermore, the conformations of the porphyrin-binding peptide were investigated by using two-dimensional ^{1}H NMR spectroscopy and molecular dynamics (MD) simulations. The NMR studies also allowed the critical components of the peptide for the porphyrin-binding to be determined and a further minimized peptide was redesigned. In addition, the affinity maturation of the designed peptide by amino acid substitution was performed by a combinatorial strategy using the spot-synthesis method.[10] Thus, we have demonstrated a useful strategy to develop small peptides capable of binding a functional molecule.



b)

20C4	Py-GRIC^{Acm}PANGCTKYDPKFQGKA-NH$_2$
20C6	Py-GRCDPANGNCKYDPKFQGKA-NH$_2$
20C8	Py-GCIDPANGNTCYDPKFQGKA-NH$_2$
20L	Py-C^{Acm}RIDPANGNTKYDPKFQGKA-NH$_2$
12C4	Py-GRIC^{Acm}PANGC^{Acm}TKY-NH$_2$
12L	Py-GRIC^{Acm}PANGC^{Acm}TKY-NH$_2$
12Lscram	Py-GAKNINYPRDTG-NH$_2$
PyG	Py-G-NH$_2$

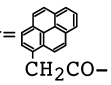
Py=  CH_2CO-

Figure 1. a) Modeled structure of CDRH-2 of 2H5. b) Structures of synthesized peptides derived from CDRH-2 of 2H5.

Results and Discussion

Peptide design and synthesis: The antiheme mAb, 2H5 (IgM), was prepared by Uda et al. by immunization of the Fe^{III}-protoporphyrin IX–hIgG complex.[11] It was reported that mAb 2H5 has a strong affinity for not only Fe^{III}-protoporphyrin IX but also for TCPP.[12] In this mAb, the heavy chain, especially heavy chain CDR-2 (CDRH-2), plays a critical role in the antigen binding, this was elucidated from binding assays using the heavy- and light-chain antibody or CDR-derived peptides.[12] Therefore, the amino acid sequence of CDRH-2 of 2H5 was selected as a starting template for the construction of novel porphyrin-binding peptides. Since the crystal structure of 2H5 is unknown, we examined the conformations of four antibodies (1BBD, 1DVF, 1HKL, 1PLG) from the Protein Data Bank (PDB) which have CDRH-2 sequences similar to 2H5. Because their backbone conformations of CDRH-2 were very similar to each other, molecular modeling studies of CDRH-2 of 2H5 with energy minimization were carried out using InsightII/Discover programs (Biosym Technologies Inc.) based on CDRH-2 conformations of four antibodies. The conformation of CDRH-2 of 2H5 was modeled as β-strand–turn–β-strand (β-hairpin), which is classified as the canonical structure 2 of H2 (Figure 1a).[2d]

According to the above information, we designed and synthesized a set of peptides derived from the CDRH-2 structure of 2H5 (Figure 1b).[9] On the basis of the 20-mer sequence of CDRH-2 (**20L**), an intramolecular disulfide bond was introduced at several positions to restrict the conformation of the modeled structure (**20C4**, **20C6**, **20C8**). The shorter peptides, **12C4** and **12L**, were designed to examine the necessity of the C-terminal tail in the modeled structure. All the peptides were modified with a pyrene (Py) moiety to detect the porphyrin-binding by spectroscopic measurements and to improve the binding efficacy by making a hydrophobic basement like β-sheets in an antibody. As reference compounds, **PyG**, which has no peptide chain except Gly, and **12Lscram**, which has a randomized sequence with the same amino acid composition, were also prepared.

The peptides were synthesized by the solid-phase method using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry.[13] After coupling the amino acids, 1-pyreneacetic acid was introduced to the N-terminus. Cyclic peptides were obtained by oxidizing Cys residues to an intramolecular disulfide bond. Synthetic peptides were purified with reversed-phase HPLC (RP-HPLC) and identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS).

Binding studies with TCPP: The binding properties of the model peptides with porphyrins were investigated by fluorescence and absorption spectroscopies, the results have been briefly reported in a previous communication.[9] TCPP was used as a representative porphyrin because of its relatively good solubility in the buffer. The results suggested that the designed peptides strongly interacted with TCPP, while the reference compounds, **PyG** and **12Lscram**, could not bind TCPP effectively. The binding constants (K_a) of the peptides with TCPP were calculated from the decrease of absorbance of TCPP at 415 nm upon the addition of peptides by means of a single-site binding equation[14] (Table 1). The best fit was obtained using an equation which assumed a 1:1 binding.[9]

Peptide **20C4** bound TCPP most effectively ($K_a = 4.5 \times 10^6\,\text{M}^{-1}$). This implied that restriction of the peptide conformation by introduction of a disulfide bond caused an increase

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Table 1. Binding constants (K_a) of peptides with TCPP.[a][9]

Compounds	Sequence	K_a [$\times 10^{-5}$ M^{-1}]
20C4	Py-GRI*C*PANG*C*TKYDPKFQGKA-NH_2	45 ± 4
20C6	Py-GR*C*DPANGN*C*KYDPKFQGKA-NH_2	7.8 ± 0.3
20C8	Py-G*C*IDPANGNT*C*YDPKFQGKA-NH_2	0.37 ± 0.06
20L	Py-CRIDPANGNTKYDPKFQGKA-NH_2	4.9 ± 0.1
12C4	Py-GRI*C*PANG*C*TKY-NH_2	6.8 ± 0.2
12L	Py-GRICPANGCTKY-NH_2	4.8 ± 0.2
12Lscram	Py-GAKNINYPRDTG-NH_2	0.86 ± 0.08
PyG	Py-G-NH_2	n.d.[b]

[a] Calculated from the absorbance change of TCPP (1.5 μM) at 415 nm in 20 mM Tris–HCl (pH 7.4) at 25 °C. [b] n.d. = not detected. *C* indicates intramolecular disulfide-bonded cysteine residues.

in the binding ability. In contrast, the inefficient binding of **20C8** was possibly owing to the loss of amino acids important to the binding. Thus, the shorter 12-peptide, **12C4**, was designed and found to bind TCPP with a relatively high affinity ($K_a = 6.8 \times 10^5$ M^{-1}), suggesting that the residues of the N-terminus, and not the C-terminus, mainly bind the porphyrin. In addition, the affinity of **12L** was almost the same as that of **20L**, which also indicates that the 12-mer has enough length for porphyrin-binding. However, **12L** was slightly inferior to **12C4**, suggesting that the conformational restriction of the peptide by a disulfide linkage contributed to improvement of the binding affinity. According to the obtained results, the peptide **12C4**, which has a rather high ability for porphyrin-binding, was used for further studies.

Interaction with various porphyrins: To obtain further information on the peptide–porphyrin interaction, fluorescence quenching studies of **12C4** with various porphyrins or other quenchers were performed. The fluorescence quenching of a pyrene moiety in **12C4** by the anionic porphyrins such as TCPP, *meso*-tetrakis(4-sulfonatophenyl)porphyrin (TPPS), and coproporphyrin I (CP) showed similar upward Stern–Volmer plots regardless of the existence of a central metal ion (Figure 2). This could be ascribed to static quenching as well as dynamic quenching caused by the peptide binding to these porphyrins.[15] In contrast, quenching by the cationic porphyrin, *meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) obeyed a linear Stern–Volmer relationship, and this indicates that TMPyP could not interact with the peptide effectively. The structural characteristic common to these compounds is a porphyrin ring. Nevertheless, the anionic porphyrins did interact with the peptide but the cationic one did not. That is, the side chain anion of porphyrins is a requisite for the peptide–porphyrin interaction. This result, coupled with the fact that **12C4** has positively charged residues (Arg, Lys), indicates that the electrostatic interactions are important for the porphyrin-binding. This is consistent with the results that the peptide **20C8** without Arg and Lys residues had a faint affinity for TCPP. The introduction of iron into TCPP as a central metal appeared to improve the binding ability. FeCP, however, showed a weaker quenching than CP without iron. Although the reason for the different effects of iron introduction is not clear, the different aggregation between the porphyrins with or without iron may affect the quenching efficiency.

On the other hand, the anionic quenchers for pyrene such as dinitrophenylacetic acid and anthracenecarboxylic acid also showed linear Stern–Volmer plots in the quenching of the peptide (Figure 2). These compounds have both an aromatic ring and carboxyl groups (negative charge), but they are not enough to interact with the peptide. This means that the porphyrin ring is important for the recognition by the peptide. Previously, the binding constants of the peptides with TCPP were shown to decrease remarkably with increasing percentage volume of methanol as a solvent[9] and suggest that the hydrophobic interactions also contribute to the porphyrin-binding of the peptide. All these results taken together, indicate that both the porphyrin ring and its side chain anions are essential for recognition by the peptide.

Conformational analysis of 12C4: In order to reveal the critical factors of the peptide for the effective porphyrin-binding, the conformation of the peptide was examined by circular dichroism (CD) and ^{1}H NMR spectroscopies. There was no concentration effect on the CD spectra (2.4–130 μM) in the buffer. Also no significant change in the chemical shifts or the line widths was observed in the 1D ^{1}H NMR spectra (0.02–1.3 mM). These results indicate that the peptide does not aggregate in the concentration range analyzed here. The far-UV CD spectrum of **12C4** in the buffer (pH 7.4) showed a maximum at 225 nm and a minimum at 200 nm, and suggests the presence of a certain population of structured conformations (Figure 3). There was no significant change in spectrum shape and intensity at pH 4.0, indicating that acidic pH requirement for the measurements of ^{1}H NMR spectra in H_2O may not affect the conformation of the peptide. On the other hand, the CD spectrum was changed by the addition of TCPP at pH 7.4 (Figure 3), which suggests that some conformational changes of the peptide may occur through the TCPP binding.

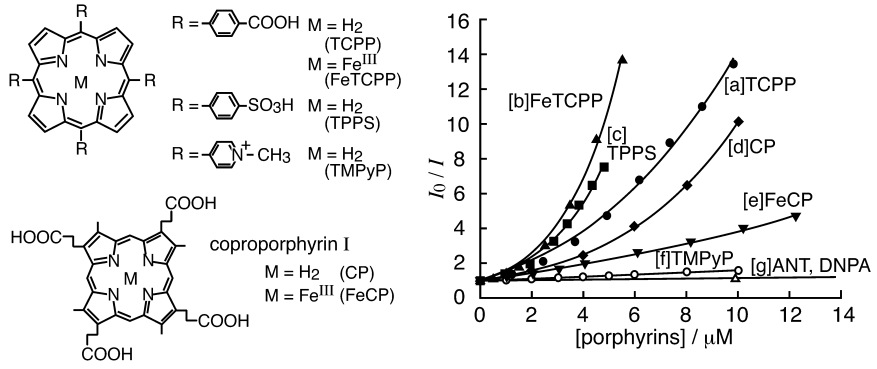

Figure 2. Stern–Volmer plots for the quenching of **12C4** by [a] TCPP, [b] FeTCPP, [c] TPPS, [d] coproporphyrin I (CP), [e] Fe^{III}-coproporphyrin I (FeCP), [f] TMPyP, [g] dinitrophenylacetic acid (DNPA) and/or anthracenecarboxylic acid (ANT). [**12C4**] = 3.7 μM (a–f), 5.0 μM (g), λex = 276 nm (a–f), 345 nm (for DNPA), 275 nm (for ANT), λem = 377 nm, in 20 mM Tris–HCl (pH 7.4) at 25 °C.

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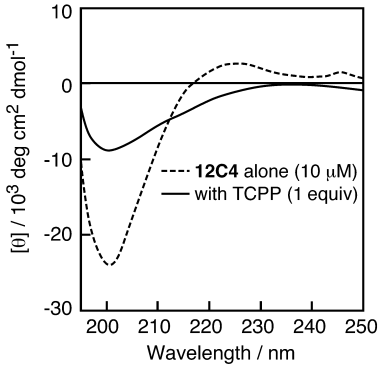

Figure 3. CD spectra of **12C4** with and without TCPP. [**12C4**] = 10 μM, [TCPP] = 0 (broken line) or 10 μM (solid line), in 20 mM Tris-HCl (pH 7.4) at 25 °C.

1D ^{1}H NMR, TOCSY, and ROESY spectra of **12C4** were collected in aqueous solutions (30 % D_2O/H_2O or D_2O, pH 5). Proton resonances were assigned by following the standard procedures using TOCSY and ROESY experiments.[16] It has been commonly accepted that chemical shift values for αH resonances are dependent on the peptide conformations.[17] In the present case (Figure 4), negative

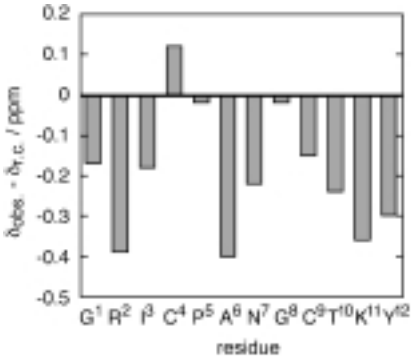

Figure 4. Chemical shift deviations of αH from random coil values.[17b]

(upfield) shifts from tabulated random coil values[17b], which are associated with residues found in α-helix or turn conformations, were mainly observed. These tendencies to deviate to negative regions indicated that the peptide formed a packed structure rather than an extended β-strand structure, although their values might have been affected by the existence of the aromatic ring of the pyrene moiety. Furthermore, spin–spin coupling constants, $^3J_{H,N\alpha}$, were determined from HN peaks of the 1D spectrum in H_2O/D_2O. In general, relatively large (> 8.0 Hz) or small (< 5.5 Hz) values of $^3J_{H,N\alpha}$ correlate with an extended or a turn structure, respectively.[18] The observed value for Ala^6 was remarkably small (3.75 Hz), and suggests that Ala^6 is in a turn position as expected. The values of other residues were in the range of 5.6–7.8 Hz, and were not correlated to a specific conformation.

The observation of NOEs between nonsequential residues also supported the formation of an expected turn structure in the peptide, while some of the NOE interactions were hard to estimate due to peak overlap. However, five long-range NOEs between Arg^{2}αH–Tyr^{12}NH (strong), Arg^{2}εNH–Tyr^{12}-αH, pyreneArH–Tyr^{12}2,6H, pyreneArH–Tyr^{12}3,5H (medium), and Ile^{3}αH–Tyr^{12}3,5H (weak) were clearly observed, and suggests that these residues were brought close together by the disulfide linkage and that the hydrophobic groups including pyrene, Ile^3, and Tyr^{12} were gathered to make a cluster.

NOE and $^3J_{H,N\alpha}$-derived distance and dihedral angle constraints were input as restraints in the MD simulations to predict the solution conformations of **12C4**. For the family of calculated structures (total 50 structures), the root-mean-squared deviations estimated for backbone atoms is 0.79 ± 0.06 Å and for heavy atoms is 1.18 ± 0.08 Å. One of the structures calculated for **12C4** is shown in Figure 5a. A turn

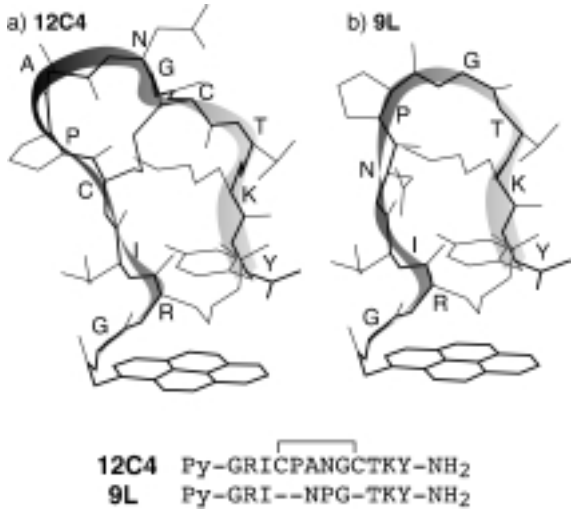

Figure 5. a) One of the best structures of **12C4** obtained by the MD simulations. b) Modeled structure of **9L** designed according to the **12C4** structure. The peptide backbone and the pyrene moiety are showed in bold lines.

structure was obtained and this structure did not contradict the small value of $^3J_{H,N\alpha}$ for Ala^6. Overall, the calculated structure, however, looked like a bent structure rather than an extended β-strand structure like a modeled structure in Figure 1a. This bent structure may be affected by the existence of the pyrene moiety as a conformational scaffold. These results are consistent with the tendency of upfield shifts of αH resonances and also suggests a nonextended conformation.

Porphyrin-binding study by ^{1}H NMR spectroscopy: Because of the limited solubility of porphyrins in water, it was impossible to measure the ^{1}H NMR spectrum of an equimolar solution of peptide–porphyrins in water. Some spectral changes, however, were observed in the ^{1}H NMR spectra of **12C4** upon the addition of TCPP (0.06 equiv) in D_2O, at pH 8. In the 1D spectra, peaks of CH_3 protons in Ile^3 and aromatic protons in the pyrene moiety were remarkably broadened in comparison with other signals (Figure 6a, b). In the ROESY spectra, intraresidual NOE cross peaks of αH–βH in Arg^2, and αH–βH, αH–γH, and αH–δH in Ile^3 were remarkably decreased (Figure 6c, d).

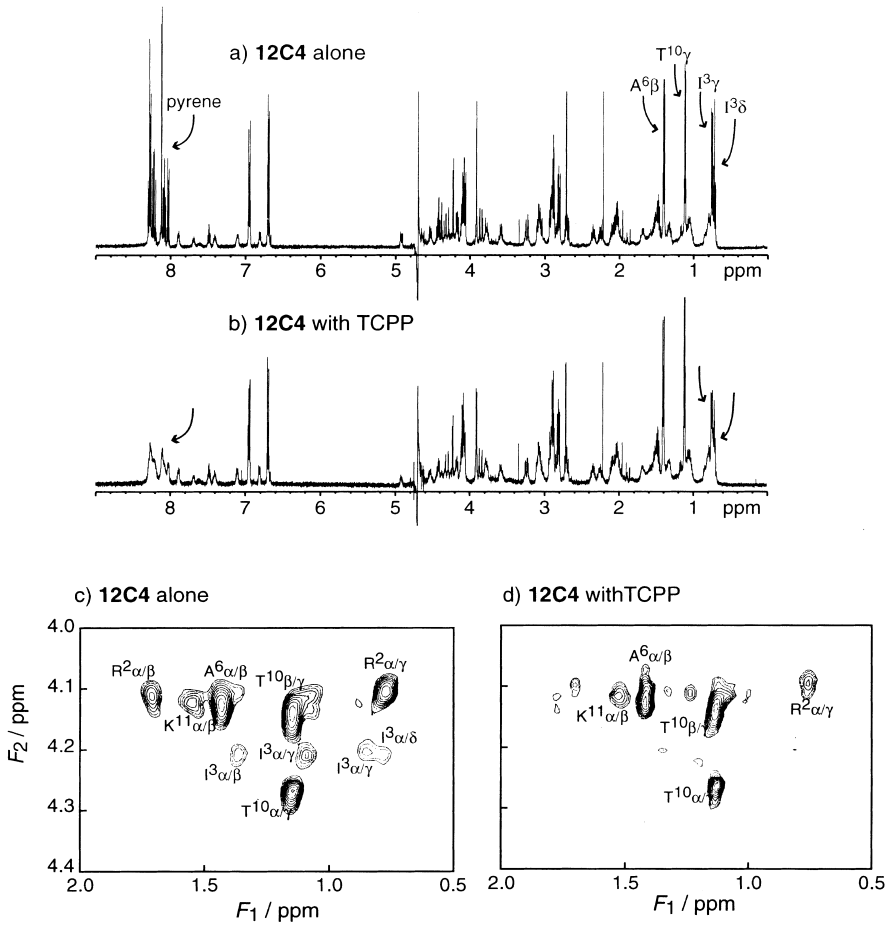

Figure 6. a) b) 1D and c) d) ROESY ^{1}H HMR spectra of **12C4** with and without TCPP. [**12C4**] = 0.5 mM, [TCPP] = 0 (a,c) or 30 μM (b,d), in D_2O (pH 8) at 30 °C.

These results strongly suggest that the pyrene moiety and the Ile and Arg residues interact directly with the porphyrin, and are consistent with the suggestion in the previous sections that both the electrostatic and hydrophobic interactions contribute to the peptide–porphyrin interaction. The Arg and/or Lys residues provide positive charges, while the pyrene moiety and the Ile residue are important for the formation of the hydrophobic core. These moieties, the pyrene group and the Ile, Arg, and Lys residues, are positioned in both ends of the peptide, not in the tip of the loop. Thus, the close proximity of these moieties in the peptide formed by a disulfide linkage are the main factors for porphyrin-binding. Additionally, the CD study suggested some conformational changes of the peptide through the porphyrin-binding. The conformational changes may help the porphyrin-binding.

Redesign of a further minimized peptide: Taking all the obtained results above into consideration, the critical parts of the peptide for the porphyrin-binding were indicated. Thus, we have attempted to redesign a simplified 9-residue peptide, **9L**, in which the key residues for porphyrin-binding of **12C4** were incorporated by a turn sequence such as Asn–Pro–Gly–Thr without the need to introduce an intramolecular disulfide bond. A modeled structure of **9L** based on the structure of **12C4** is shown in Figure 5b.

The porphyrin-binding ability of **9L** was examined by spectroscopic studies using TCPP. The fluorescence of the pyrene moiety in **9L** was remarkably quenched by the addition of TCPP. The absorbance at the Soret band of TCPP was decreased by the addition of **9L**. Both results indicate that the minimized peptide **9L** without a disulfide linkage can effectively interact with porphyrin. The binding constant of **9L** was calculated as $1.4 \times 10^6\,M^{-1}$ which was determined by the variation of the absorption spectra of TCPP. This value was larger than for **12C4** ($6.8 \times 10^5\,M^{-1}$). Thus, a much-downsized peptide was successfully constructed.

Screening of mutated peptides on cellulose membranes: In order to mature the porphyrin-binding affinity of the peptide, a combinatorial approach using the spot-synthesis method[10] on cellulose membranes was employed for the synthesis of several sets of mutated peptides based on the **9L** sequence. Each peptide was synthesized as an individual spot on a cellulose paper, and peptides on spots showed a high purity (> 92 %) on HPLC after cleavage from the membrane.[10c] The binding affinity of immobilized peptides to porphyrin was evaluated by using relative color intensity of spots (the parent sequence = 1.0) by binding of porphyrins (Figure 7a). For this study, we used Fe^{III}–TCPP as a target porphyrin because of solubility and good color contrast. In preliminary experiments, we ascertained that the pyrene moiety at the N-terminus was not necessary to estimate the affinity of the peptides for porphyrin on the cellulose membrane. The lack of the pyrene moiety did not affect the binding affinity on the membrane, possibly owing to immobilization on the polymer support. Therefore, we synthesized mutated peptides without the pyrene moiety on the membrane.

At first, Ala scanning of the **9L** sequence was performed to elucidate the importance of each residue. The results are quantitatively shown in Figure 7a, b. The Ala replacement of Ile^3 or Thr^7 led to increased binding, while the replacement of Arg^2, Lys^8, or Pro^5 caused a weaker binding. Although the results in the previous sections indicated that the Ile residue was one of the determinant residues for the porphyrin-binding, the Ala replacement of Ile^3 caused a rise in the affinity of the peptide. This result suggests that the mutation of Ile^3 critically influences the binding affinity of the peptide. In contrast, the results of the substitution of the Arg or Lys

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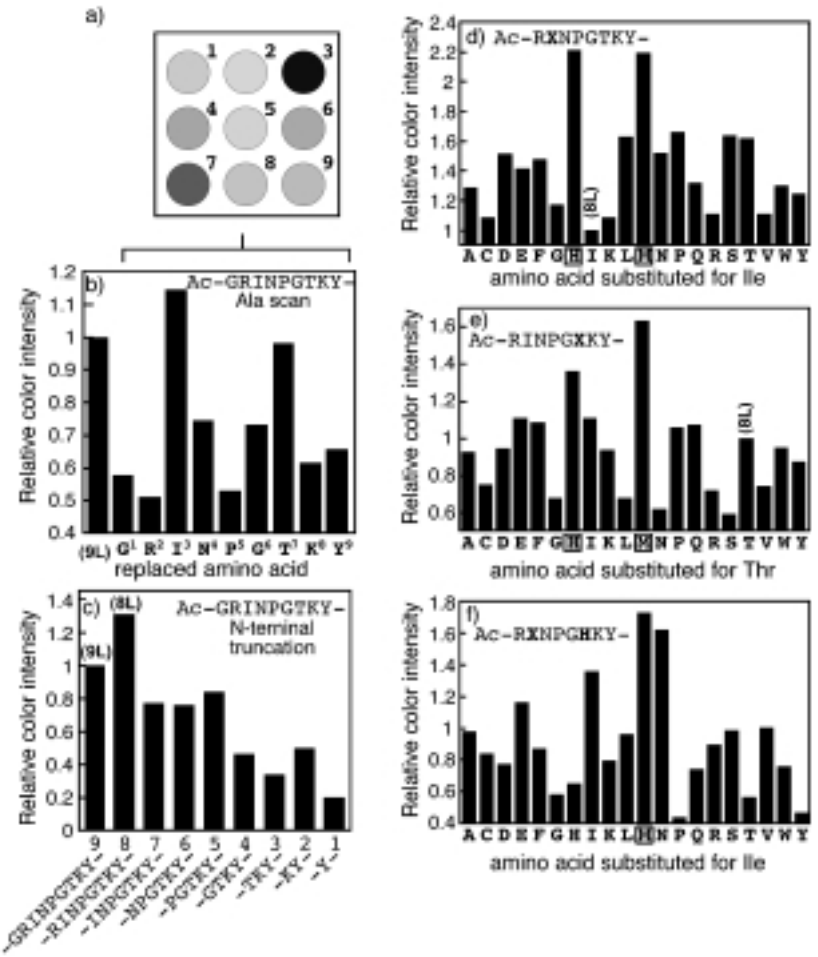

Figure 7. FeTCPP binding to peptides bound on the cellulose membrane. a) Schematic drawing of a piece of paper. The depth of color of each spot was expressed by gray scale. b)–f) Results of b) Ala scanning. c) N-terminus truncation. d) Ile^2 and e) Thr^6 mutation of the **8L** sequence. f) Ile^2 mutation of [His^6]**8L**. Values represent relative color intensity (**9L** (b,c) or **8L** (d–f) = 1.0).

residues support the finding that these residues play a critical role in the porphyrin-binding. The Pro residue is possibly important for the conformational stability, especially for the turn structure.

Additionally, the results of N-terminus truncation of **9L** (Figure 7c) revealed that the peptides shorter than five residues almost lost their binding ability for the porphyrin, and indicated that this sequence is essential for the porphyrin-binding. Interestingly, the 8-mer peptide **8L** without Gly^1 showed a higher affinity than the 9-mer peptide, **9L**, suggesting that the shorter 8-mer peptide could be used as a parent sequence to screen the mutated peptide with higher affinity.

According to these results, two sets of 8-mer peptides, in which Ile^2 or Thr^6 was replaced by 20 individual natural amino acids (Cys was used in its acetamidomethyl (Acm)-protected form), were synthesized and assayed in the same way (Figure 7d,e). In both cases, replacement by a His or Met residue led to increased affinity compared with the parent sequence. These results suggest that the introduction of residues which can be ligands of metal porphyrins such as His or Met causes an increase in the binding affinity of the peptides for the porphyrin. On the other hand, the replacements by the positively charged residues such as Arg and/or Lys did not clearly affect their binding affinity in any case, and suggests that the further addition of a positive charge is not effective.

Figure 7f shows the results for the mutations of Ile^2 in the peptide [His^6]**8L** by 20 individual amino acids. The peptides in which Ile^2 was replaced by Met or Asn showed a particularly high affinity, whereas changing to His did not modify the binding.

On the basis of the information obtained by the combinatorial approach with the spot-synthesis method, two peptides, **9L-IH** and **9L-MH**, were synthesized by the batch method and their affinity to TCPP in the solution phase was examined. Their binding constants for TCPP were calculated using the single-site binding equation by the same method. As expected, the binding constants of these peptides for TCPP were almost two or four times larger than the value for **9L** (Table 2). Thus, maturated peptides with an improved affinity in a smaller size were successfully obtained.

Table 2. Binding constants (K_a) of maturated peptides with TCPP.[a]

Compounds	Sequence	K_a [$\times 10^{-6}$ M^{-1}]
9L	Py-GRINPGTKY-NH_2	1.4 ± 0.2
9L-IH	Py-GRINPGHKY-NH_2	4.4 ± 0.3
9L-MH	Py-GRMNPGHKY-NH_2	2.4 ± 0.4

[a] Calculated from the absorbance change of TCPP (1.5 μM) at 415 nm in 20 mM Tris–HCl (pH 7.4) at 25 °C.

Conclusion

In this study, we have accomplished the design, synthesis, binding study, and optimization of the binding affinity of the porphyrin-binding peptides from antibody CDR-derived peptides as a design scaffold. At first, several peptides with different chain length and an intramolecular disulfide bond in different positions were prepared to compare their affinity for a porphyrin. From the porphyrin-binding studies by spectroscopic measurements, it has been revealed that the affinities for TCPP of the peptides are increased by the addition of a conformational restriction by an intramolecular disulfide bond. Furthermore, the detailed studies using the peptide,

12C4, have revealed that both the hydrophobic and electrostatic interactions are essential factors in the peptide–porphyrin interaction. Additionally, the NMR studies provided the structural properties and the critical components of the peptide necessary for porphyrin-binding. A further minimized peptide, **9L**, was redesigned by the careful investigation of the above results. In the next step, the affinity maturation from the minimized sequence, **9L**, has been accomplished using the spot-synthesis method. Systematic amino acid replacements with this combinatorial method made it possible to obtain peptides with an improved affinity for porphyrins. In this way, we were able to obtain novel porphyrin-binding peptides.

For the construction of a small recognition motif for specific molecules, the advantage of using an antigen binding site of antibodies as a design scaffold is clear; its binding sites are easily predictable and they certainly have some structural or sequential factors to bind a ligand. The design strategy, such as protein surface mimics, has generally been used for the development of inhibitors of protein–protein interactions.[19] This strategy has provided some good results, but some uncertainties remain in the selection of the target sequences because of the difficulties associated with the prediction of the actual binding region unless the three-dimensional structure of the complex has been determined. On the contrary, the binding sites of antibodies are in limited regions, mostly in six CDR loops. Although the peptides with the same sequence as an antibody CDR would have a limited affinity in comparison with an intact antibody, such peptides must include some factors which bind a targeted molecule. Moreover, we can put artificial modifications into a CDR structure in the design of peptides; for instance, in this case, we introduced an intramolecular disulfide linkage for conformational restriction and a pyrene moiety as an auxiliary binding site.

The combination of this rational design approach with a combinatorial technology potentially provides maturated peptides with a higher affinity in a smaller size. Another useful strategy in the search for compounds which bind to a target molecule is a random combinatorial approach. In particular, a peptide library offers the possibility of the acquisition of peptides with targeted affinities. At present, many kinds of synthetic procedures for peptide libraries with a higher diversity have been established.[20] A random library approach, however, needs a large number of compounds and does not always meet a consensus in principles of molecular recognition. The rationalized design of functional peptides starting from an antibody CDR has the possibility of reducing the wastefulness involved in the random approach. This strategy can now be applied to the tailor-made design of peptides for a targeted compound including small and macromolecules.

Experimental Section

General: All chemicals and solvents were of reagent or HPLC grade. Amino acid derivatives were purchased from Watanabe Chemical Co. (Hiroshima, Japan). RP-HPLC system was composed of a Hitachi D7500 Chromato-Integrator, a L7400 UV-VIS Detector using a Wakosil 5C18 (4.6×150 mm) for analysis or a YMC ODS A323 (10×250 mm) for purification with a linear gradient of acetonitrile/0.1% trifluoroacetic acid (TFA). MALDI-TOFMS was performed on a Shimadzu KOMPACT MALDI III mass spectrometer with 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix.

Synthesis of isolated peptides: All peptides were synthesized by the solid-phase method by means of Fmoc chemistry[13] on Rink amide resin[21] using benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)[22] as a coupling reagent. Side chain protections were as follows: acetamidomethyl (Acm) or triphenylmethyl (Trt) for Cys; *tert*-butyl (*t*Bu) for Asp, Glu, Thr, Tyr, Ser; Trt for Asn, Gln, His; *tert*-butyloxycarbonyl (Boc) for Lys; 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) for Arg. After coupling all amino acids, 1-pyreneacetic acid was introduced to the N-terminus. The resin and the protecting group except Acm were removed by treatment of 1M trimethylsilyl bromide/thioanisole in *m*-cresol/ethanedithiol/trifluoroacetic acid (TFA; 1/5/75) at 0°C for 1 h.[23] Cyclic peptides were obtained by oxidizing Cys residues to an intramolecular disulfide[24] after removing the Acm group using $AgBF_4$ (20 equiv) in the presence of anisole (10 equiv) in TFA.[25] The peptides were purified by RP-HPLC and characterized by MALDI-TOFMS: **20L** *m/z*: 2537.1 ($[M+H]^+$ calcd. 2536.9); **20C4** 2395.0 (2394.8); **20C6** 2409.9 (2409.7); **20C8** 2339.8 (2339.6); **12C4** 1523.4 (1522.9); **12L** 1667.9 (1667.1); **12Lscram** 1548.2 (1547.7); **9L** 1247.7 (1247.4); **9L-IH** 1282.9 (1283.5); **9L-MH** 1301.2 (1301.5).

Spectroscopic measurements: All measurements were performed at 25°C, typically in Tris–HCl buffer (20mM, pH 7.4). Fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer 850 or a Shimadzu RF-5300PC spectrofluorophotometer with a thermoregulator with a quartz cell of 4.0 mm pathlength. UV/Vis absorption spectra were measured on a Shimadzu UV-3100 spectrophotometer with a quartz cell of 10 mm pathlength. Circular dichroism spectroscopy was performed on a Jasco J-720WI spectropolarimeter with a quartz cell of 4 mm (for peptide concentrations of 2.4–10μM) or 1 mm (30–130μM) pathlength.

NMR spectroscopy: ^{1}H NMR spectra were recorded on a Varian UNITY INOVA 500 (500 MHz) or a UNITY plus 400 (400 MHz) spectrometer for the measurements in H_2O/D_2O (7:3), and a VXR-500S (500 MHz) spectrometer in D_2O at 25°C or 30°C. The solution pH was adjusted with small additions of DCl or NaOD using a glass electrode uncorrected for isotope effects. The water signal was presaturated. The chemical shifts were obtained relative to the peak of HDO ($\delta = 4.7$). Solutions of the peptide at concentrations of 0.02–1.3mM were used for assessing aggregation, 1.3mM for the conformational analysis, and 0.5mM for the porphyrin-binding studies. Mixing times were 50 ms for TOCSY and 200 or 300 ms for ROESY spectra. Two-dimensional spectra were analyzed by using Vnmr Ver. 4.2 or 5.3 software.

MD simulation: The MD simulations were carried out using InsigntII/Discover programs (Biosym Technologies Inc.) with the CVFF force field with a cutoff distance of 13 Å. The initial coordinate for **12C4** was built based on the crystal structure of CDRH-2 of an antibody (PDB; 1BBD). An intramolecular disulfide bond was not made in the initial coordinate but a limit distance of 1.8–3.0 Å was set between γS of two Cys residues. A total of 73 upper limit distance restraints derived from NOEs were classified as strong, medium, and weak for 2.5, 4.0, and 5.0 Å, respectively. The dihedral angle of the amide bonds (ω) was set to *trans* for all residues except Pro and to *cis*/*trans* for Pro. The dihedral angle ϕ of Ala^6 was set to $-60°$. The MD simulations were carried out using a step size of 1 fs. For the first 7.5 ps the temperature was maintained at 1000 K. The restraining functions were applied with force constants of 5.0 kcal mol^{-1} Å^{-2} (for distances) or 5.0 kcal rad^{-2} (for dihedral angles). Then, the temperature was reduced stepwise until 298 K with a decrement of 50 K, and 1 ps simulation was performed at each temperature. When the temperature was below 298 K, the simulation was resumed at 298 K for 15 ps with force constants of 2.5 kcal mol^{-1} Å^{-2} or 2.5 kcal rad^{-2}. A final structure was saved after minimization, and then repeat simulations were started from 1000 K. A total of 50 structures were saved and their convergencies were examined. From these structures, five of the lowest energy structures were extracted and a disulfide bond was created for these structures.

Synthesis of peptides on a cellulose membrane: The technique used is essentially identical to the spot-synthesis method described by Frank.[10] Cellulose membrane (Whatman 1Chr) was derivatized with *N*,*N*-diisopropylcarbodiimide (DIC)-activated Fmoc-βAla. A spacer residue, another βAla residue, was coupled to the derivatized paper. The designed peptides

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were then synthesized using Fmoc-protected amino acid 1-hydroxybenzotriazole (HOBt) esters dissolved in *N*-methylpyrrolidone (NMP, 0.3 M) by spotting (1 μL each). Fmoc-protected amino acid HOBt esters were prepared with Fmoc-amino acid, DIC (1.2 equiv) and HOBt (1.5 equiv) in NMP at room temperature for 60 min. Coupling efficiency was monitored by bromophenol blue staining. The protecting groups for the side chains were the same as described above except 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. Cys was used in its Acm-protected form. After coupling of all amino acids, the N-termini were acetylated by treatment with acetic anhydride/diisopropylethylamine/*N*,*N*-dimethylformamide (2/1/97) and the side chain protecting groups were cleaved by treatment with phenol/triisopropylsilane/water/TFA/dichloromethane (1/3/2/44/50) at room temperature for 2 h.

Binding experiments with cellulose-bound peptides: The peptide-bound cellulose membrane was washed three times with 20 mM Tris–HCl buffer (pH 7.4) for 10 min, followed by treatment with FeTCPP solution (ca. 30 μM in the buffer) for 20–30 min. After washing by the buffer (three times), the paper was dried under vacuum and the color intensity of each spot was estimated by the software; Photoshop (Adobe Systems Inc.).

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