Effects of amino acids substitution of hydrophobic residues on haem-binding properties of designed two-α-helix peptides

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We have designed and synthesized a series of amphiphilic two-α-helix peptides, which bound Fe^{III}-mesoporphyrin (haem) through a ligation of two His residues. In the designed structure, amino acid residues arranged around the axial ligands were systematically substituted by hydrophobic Phe, Ile, Leu, Val and Ala residues, in order to know how their hydrophobic and/or steric differences influenced the interaction between the peptides and haem. The binding constants of the peptides with haem, which were estimated from UV–VIS measurements, were significantly correlated with the hydrophobicity at the haem-binding site. Size-exclusion chromatography demonstrated that a tetrameric assembly of peptides was induced cooperatively by the haem-binding. Furthermore, computer-modeling studies suggested that van der Waals contacts between the haem and the side-chains of amino acids around His were important for effective haem-binding. Especially, a Phe residue introduced at an appropriate position contributed to effective haem-binding *via* the edge-to-face interaction between the aromatic Phe side-chain and the porphyrin ring. In addition to the haem-binding properties of the peptides, the catalytic activity of the haem bound to peptides, which was similar to that of peroxidase, varied significantly depending on the amino acid composition at the haem-binding site. The results obtained in this study demonstrated that the amino acid composition and arrangement at the haem-binding site affected the haem-binding properties of the artificially designed two-α-helix peptides and the catalytic activity of the haem bound to the peptides.

Introduction

A variety of haemproteins display diverse functions in nature, such as oxygen carrier/storage, electron transfer, redox catalysis[1,2] and transmission of information,[3] though they employ a simple iron porphyrin as a common cofactor. Each specific function of haemproteins is regulated by the environments around a porphyrin molecule formed by polypeptide chains.[4] In the protein three-dimensional (3D) structure, amino acids arranged around an active site interact with the haem *via* ligation to a central ion, hydrophobic interaction and/or ionic interaction. All these interactions define the chemical, physical and functional properties of the iron porphyrin. Recently, in order to develop a structural model system which has more native-like properties and can remove the complexity of the natural counterpart, considerable effort has been devoted to the construction of *de novo* designed polypeptide 3D structures conjugated with porphyrin molecules *via* chelation[5–11] or covalent linkage[12–16] with peptides. Because these artificial haem-conjugated proteins and peptides are characterized as having well defined 3D structure with relatively simple amino acid compositions, they will enable us to understand the structural roles of polypeptides in the function of proteins.

In order to establish an artificial haemprotein that meets minimal requirements for the function, we have also attempted to design and synthesize two-α-helix peptides,[10] which bind iron-mesoporphyrin IX (haem) through a ligation of His residues. However, little is known yet about the factors that determine the interaction between the designed peptides and haem. In previous communications,[10c,d] we examined the peptide–haem interaction using three peptides H2α17-LL, H2α17-II and H2α17-VV. The peptides have an identical amino acid sequence except for their hydrophobic residues which are arranged around the axial ligand His to construct a haem-binding site. H2α17-LL and H2α17-II bound the haem effectively in the buffer and haem-binding induced the association of peptide molecules from a monomeric to a tetrameric form. However, H2α17-VV could not bind the haem under the same conditions. This result implied that amino acid disposition at the haem-binding site is a significant contributor to the peptide–haem interaction.[10d] In this study, in order to obtain more detailed information about the factors that determine the interaction between peptide and haem, we have prepared a series of two-α-helix peptides H2α17-XY (X, Y = F, L, I, V and A) (Fig. 1) and examine the effects of their sequences on the binding constant, the secondary structure and the molecular association state. Furthermore, the catalytic activity of the haem–peptide conjugates, which resembles that of peroxidase, is examined in order to know how the haem activity is regulated by the polypeptide binding.

Results and discussion

Design and synthesis of peptides H2α17-XY

The design of a series of peptides H2α17-XY was based on one of the template peptides H2α17-LL, which took an α-helix structure in the buffer both with and without bound haem.[10c] The design of H2α17-LL was described in the previous communication.[10c] The amino acid sequences of H2α17-XY are identical to that of H2α17-LL except for their hydrophobic residues, X and Y (Fig. 1). The peptides H2α17-XY have the amino acid sequence, Ac–A A E A **X Y** K A **H** A E **Y X** A K A A–GGG C–NH_2, where X is the amino acid residue at the 5th and 13th positions and Y is the amino acid residue at the 6th and 12th positions. The peptides H2α17-XY were designed to fold into a parallel two-α-helix structure *via* the disulfide linkage of the Cys^{21} residues and to bind a haem between the helices *via*

(*a*)

Ac-A A E A **X Y** K A **H** A E **Y X** A K A A-GGG C-NH_2

Ac-A A E A **X Y** K A **H** A E **Y X** A K A A-GGG C-NH_2

Peptide	X	Y	Peptide	X	Y
H2α17-FF	Phe	Phe	H2α17-VV	Val	Val
H2α17-FL	Phe	Leu	H2α17-VL	Val	Leu
H2α17-LF	Leu	Phe	H2α17-LV	Leu	Val
H2α17-I I	Ile	Ile	H2α17-AA	Ala	Ala
H2α17-IL	Ile	Leu	H2α17-AL	Ala	Leu
H2α17-LI	Leu	Ile	H2α17-LA	Leu	Ala
H2α17-LL	Leu	Leu			

(*b*)

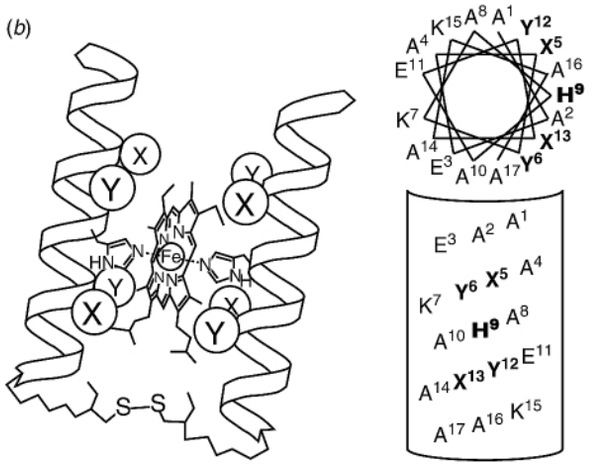

Fig. 1 Structure of the designed peptide, H2α17-XY. (*a*) Amino acid sequence of H2α17-XY; (*b*) Schematic illustration of two-α-helix peptide structure bound to the haem and helix wheel and net drawings of the 17-peptide.

ligation with two His^{9} residues. The amphiphilic α-helical structure was stabilized by two sets of E–K salt bridges.[17,18] In the designed α-helix structure, four hydrophobic residues (X and Y) per helix were arranged around the His^{9} to make a hydrophobic haem-binding site and a haem would be deployed parallel to the helices. The Y residues are directed to the outside of the two-α-helix bundle structure relative to the residues X, as shown in the helical wheel drawing (Fig. 1). To probe hydrophobic and steric interactions at the binding site, Phe, Ile, Leu, Val and Ala were used as residues X and Y.

Peptides were synthesized by the Fmoc solid-phase method (Fmoc, fluoren-9-ylmethoxycarbonyl)[19] using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. The disulfide bond at *C*-terminals was formed in trifluoroacetic acid (TFA) by using dimethyl sulfoxide (DMSO) (10%) as an oxidant in the presence of anisole.[20] The peptides were purified by semi-preparative reversed-phase HPLC (RP-HPLC) to give a high purity (>98% on analytical RP-HPLC). The peptides were identified by amino acid analysis and matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). The amino acid analysis was also utilized for determination of the peptide concentration of the stock solutions.

Estimation of hydrophobicity at the haem-binding site

A lot of hydrophobicity scales have been developed in several laboratories, as described in a review by Nakai *et al.*[21] However, the ranking order of hydrophobicity of amino acids derived by various methods is significantly different. The diversity of the ranking order in each hydrophobicity scale seems to arise from the diverse definitions of hydrophobicity.

Table 1 Relative hydrophobicity of peptides, H2α17-XY, determined by reversed-phase HPLC

Peptide H2α17-XY	Residue X	Residue Y	Retention time/ min	Relative hydro-phobicity
H2α17-FF	Phe	Phe	36.02	5.00
H2α17-LF	Leu	Phe	36.00	4.99
H2α17-FL	Phe	Leu	35.79	4.85
H2α17-IL	Ile	Leu	34.99	4.31
H2α17-II	Ile	Ile	34.65	4.08
H2α17-LL	Leu	Leu	34.53	4.00
H2α17-LI	Leu	Ile	34.29	3.84
H2α17-VL	Val	Leu	32.31	2.51
H2α17-LV	Leu	Val	31.51	1.97
H2α17-VV	Val	Val	28.41	−0.12
H2α17-LA	Leu	Ala	27.69	−0.60
H2α17-AL	Ala	Leu	27.12	−0.99
H2α17-AA	Ala	Ala	21.15	−5.00

Since the retention time of a given peptide in RP-HPLC is based on the relative strength of hydrophobic interactions between the peptide molecule and the nonpolar stationary phase, the hydrophobic molecules interact more strongly with the hydrophobic stationary phase, with the result being a relatively longer retention time. Liu and Deber recently presented a set of hydrophobicity scales derived from RP-HPLC, which was based on the retention times of 20 Ala-based peptides.[22] They showed that the trans-membrane helical segments in natural membrane proteins could be well predicted by using their hydrophobicity scale.

In the present work, because the peptides H2α17-XY have an identical amino acid sequence except for X and Y residues, the hydrophobicity at the haem-binding site can be estimated by the retention time of each peptide. Peptide retention times were determined by RP-HPLC on a YMC-Pack C4 column (4.6 × 150 mm) using a linear gradient of 10–50% acetonitrile (ACN)–0.1% TFA (40 min). The experimentally measured retention times of peptides were converted to a relative hydrophobicity index for the corresponding X and Y residue pairs using a method similar to that of Liu and Deber.[22] Eqn. (1) was used in

$$H = 10 \times \Delta tR_{XY-AA}/\Delta tR_{FF-AA} - 5.00 \qquad (1)$$

the data conversion, where ΔtR_{XY-AA} is the retention time difference between the peptide H2α17-XY and the most hydrophilic H2α17-AA, and ΔtR_{FF-AA} is the retention time difference between H2α17-FF and H2α17-AA. The resulting values are listed in Table 1.

The rank order of hydrophobicity of amino acids, derived from the retention times of the H2α17-XY series, was given as F > I ≥ L > V > A. This rank order was almost coincident with the results reported by Liu and Deber.[22] The good agreement between the two cases provided evidence that HPLC retention times represent a reliable method for evaluating the relative hydrophobic character of the substituted X and Y residues at the haem-binding site.

It is noteworthy that the retention times of H2α17-XL and those of H2α17-LY (X = Y; Phe, Ile, Val or Ala) were slightly different. For example, the retention times of H2α17-FL, -LI, -LV, and -AL were shorter than those of counterparts H2α17-LF, -IL, -VL and -LA, respectively. The latter peptides would interact more strongly with the hydrophobic stationary phase than the former peptides. These results indicate that the residue X and the residue Y at the haem-binding site are not equivalent with respect to the interaction with the hydrophobic face of the column. The difference might affect the interaction between the hydrophobic porphyrin plane and peptides.

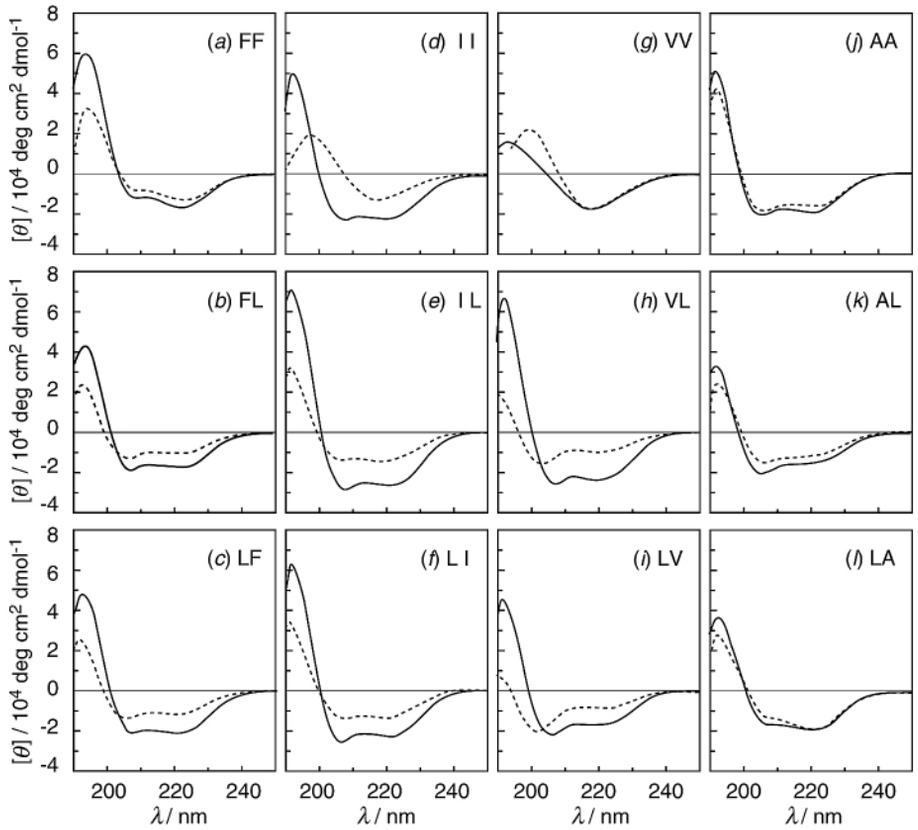

Fig. 2 CD spectra of H2α17-FF (*a*), H2α17-FL (*b*), H2α17-LF (*c*), H2α17-II (*d*), H2α17-IL (*e*), H2α17-LI (*f*), H2α17-VV (*g*), H2α17-VL (*h*), H2α17-LV (*i*), H2α17-AA (*j*), H2α17-AL (*k*) and H2α17-LA (*l*) in the absence (----) and presence (——) of haem (1.0 equiv.) in 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) containing 1% TFE at 25 °C. [peptide] = 1.0×10^{-5} mol dm^{-3}.

Far-UV CD study of peptides

Circular dichroism (CD) spectra of the peptides H2α17-XY were measured in 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4). Most of the peptides including H2α17-LL showed an α-helical CD in the absence or presence of haem (Fig. 2). The results for H2α17-LL, H2α17-II and H2α17-VV were also described in previous communications.[10c,d] In the absence of haem, the peptides H2α17-II and H2α17-VV took a β-sheet structure in the buffer due to the introduction of four Ile or four Val residues per strand, respectively.[10d] The α-helix contents of peptides were estimated from the ellipticity at 222 nm and are summarized in Table 2.[23] There was no correlation between the α-helix contents and $\langle P_\alpha \rangle$ values estimated by the Chou–Fasman parameter.[24] The α-helix propensity predicted by a statistical method (*i.e.* Chou–Fasman) mainly represents relatively short-range interactions. It is considered that the stabilization of the α-helix of H2α17-XY in the absence of haem was attained by relatively long-range interactions, such as intramolecular hydrophobic interaction between the amphiphilic α-helical segments.

Most of the H2α17-XY peptides showed lower α-helix contents than that of the template peptide H2α17-LL (54%). Especially, Val containing peptides H2α17-VL and H2α17-LV showed the lowest value among the peptides (27%). These results were mainly caused by the introduction of two β-branched amino acids into the middle of α-helix segments. The β-branched amino acids are considered to be α-helix breakers, because their side-chain torsional angle χ_1 is severely restricted when they are forced to reside within a helix.[25,26] The peptides H2α17-AA and -AL showed lower α-helix contents, probably because the hydrophobic interaction between amphiphilic helical segments was smaller than that of H2α17-LL.

In the presence of haem, CD spectra of the peptides H2α17-XY were also measured in the buffer (Fig. 2, Table 2). Except for the peptide H2α17-VV, all peptides showed a typical

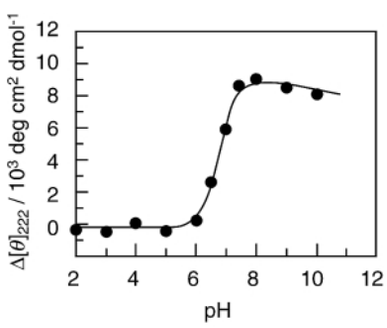

Fig. 3 α-Helix improvement of H2α17-LL by the addition of haem (1.0 equiv.) in H_2O at pH ranging from 2.0 to 10.0 at 25 °C. [peptide] = 1.0×10^{-5} mol dm^{-3} and [haem] = 1.0×10^{-5} mol dm^{-3}.

α-helical pattern with double negative maxima at around 208 and 222 nm. Interestingly, the peptide H2α17-II took an α-helix structure in the presence of haem, although the peptide took a β-sheet structure without haem in the buffer.[10d] On the other hand, H2α17-VV took a β-sheet structure even in the presence of haem, because this peptide could not bind the haem in these conditions as was confirmed by UV–VIS titration.[10d] With respect to other peptides, the α-helicities of the peptides were increased by the addition of haem (1.0 equiv.) (Fig. 2). The addition of an excess amount of cyanide ion [2.5×10^{-2} mol dm^{-3}, 2.5×10^3 equiv.] inhibited the coordination of His ligands, resulting in a decrease in the α-helicity to the level observed without the haem. Furthermore, there was no significant change in the CD spectra on the addition of haem at acidic pH (2.0–6.0) (Fig. 3). Because the pK_a of imidazole is *ca.* 6.4, the pH effect is attributed to the protonation of His side chains such that they cannot act as a ligand. Therefore, we concluded that the increase in α-helicity of the peptides took place *via* the haem-binding by ligation with His residues.

Table 2 α-Helix contents[a] of the peptides in the absence and presence of haem

Peptide H2α17-XY	Without haem		With haem		Relative hydrophobicity	$\langle P_\alpha \rangle_{XY}$[b]
	$-[\theta]_{222}$/deg cm^2 dmol^{-1}	α-Helicity (%)	$-[\theta]_{222}$/deg cm^2 dmol^{-1}	α-Helicity (%)		
H2α17-FF	12 700	40	16 800	53	5.00	1.13
H2α17-LF	11 080	35	20 580	65	4.99	1.17
H2α17-FL	11 400	36	18 720	60	4.85	1.17
H2α17-IL	14 100	45	26 900	85	4.31	1.15
H2α17-II	[15 100 at 218 nm (β-strand)]		22 500	71	4.08	1.08
H2α17-LL	17 100	54	26 800	85	4.00	1.21
H2α17-LI	13 200	42	22 700	72	3.84	1.08
H2α17-VL	8530	27	22 800	72	2.51	1.14
H2α17-LV	8550	27	16 800	53	1.97	1.14
H2α17-VV	[12 000 at 218 nm (β-strand)]		12 000 at 218 nm (β-strand)]		−0.12	1.06
H2α17-LA	20 520	65	20 400	65	−0.60	1.32
H2α17-AL	13 800	44	19 200	51	−0.99	1.32
H2α17-AA	16 100	51	19 500	62	−5.00	1.42

[a] α-Helix contents were estimated from molecular ellipticity at 222 nm according to the equation of Scholtz *et al.* [Ref. 23], [peptide] = 1.0×10^{-5} mol dm^{-3}, [haem] = 1.0×10^{-5} mol dm^{-3}. [b] $\langle P_\alpha \rangle_{XY} = (P_\alpha$ of X residue + P_α of Y residue)/2.

As described above, although β-branched amino acids opposed a helix formation due to the loss of side-chain entropy, the Ile or Val containing peptides with bound haem showed high α-helix contents comparable to that of a template peptide H2α17-LL. These results indicate that the hydrophobic interaction between the apolar side chains on helical segments and the hydrophobic porphyrin periphery is enough to overcome the α-helix destabilization by the introduction of β-branched amino acids. The increase in α-helicities of H2α17-VL and H2α17-IL on the addition of haem was larger than that of their counterparts H2α17-LV and H2α17-LI, respectively, although the amino acid compositions of the former peptides were identical to those of the latter peptides. The HPLC based relative hydrophobicities of H2α17-VL and H2α17-IL are larger than those of H2α17-LV and H2α17-LI, respectively. Possibly, because H2α17-VL and H2α17-IL could make a more hydrophobic haem-binding site relative to H2α17-LV and H2α17-LI, the former might interact with the haem more strongly, resulting in a higher increase of α-helicities. It is noteworthy that the α-helix improvements of H2α17-AA, H2α17-AL and H2α17-LA on the addition of haem were smaller (<10%) than those of the other peptides. As described in the section on UV–VIS titration, these peptides could not bind the haem effectively in these conditions. These results also provide evidence that the increase in α-helicity of the peptides took place *via* haem-binding by ligation with His residues.

UV–VIS titration of the haem with peptides

In order to further characterize the interaction between the haem and the peptides, UV–VIS titration of the haem with a series of peptides was carried out in the buffer. The results for H2α17-LL, H2α17-II and H2α17-VV were also described in previous papers.[10c,d] As representatives in this work, the results for H2α17-FL and H2α17-AA are shown in Fig. 4. In the buffer, with increasing concentration of the peptide H2α17-FL, increases of the Soret band at 405 nm and the α/β band near 530 nm, and a decrease of the band around 355 nm were observed [Fig. 4(*a*)]. Except for the peptides H2α17-VV, H2α17-AL, H2α17-LA and H2α17-AA, all peptides showed a similar spectral change. That is, with the addition of peptides, the Fe^{III} in haem was converted from the high-spin to predominantly the low-spin form with an isosbestic point at around 390 nm.[27] The spectral features of the haem in the presence of the peptides were similar to those of natural cytochromes with six-coordinate iron(III). Fe^{II}-haem–peptide conjugates were also obtained by adding minimum amounts of sodium dithionite directly to the solution of Fe^{III}-haem–peptide conjugate in the buffer. UV–VIS Spectra of the Fe^{II} complex showed the expected red shift of the Soret band to 413 nm and the prominent splitting of the β- and α-bands (520 and 550 nm) [Fig. 4(*b*) and Table 3]. The UV–VIS spectrum of the Fe^{II}-haem in the presence of peptide also resembled those of natural cytochromes with six-coordinate iron(II). These results indicated that the peptides could bind the haem in the buffer *via* the ligation of two His residues. On the other hand, addition of H2α17-VV, H2α17-AA, H2α17-AL and H2α17-LA caused little increase of the Soret band, suggesting that the peptides could not bind the haem effectively in this concentration range [Fig. 4(*c*)].

As demonstrated in previous papers[10c,d] and in this paper by size-exclusion chromatography (data shown later) and sedimentation equilibrium studies, the peptides associated to a tetrameric form from a monomer upon the haem-binding. This unique property of our designed peptides implies a complicated equilibrium in the haem-binding process. However, size-exclusion chromatography showed that there was no intermediate between the monomeric state and the tetrameric state. Thus, we assumed that the haem-binding and the association was a cooperative process. That is, the tetrameric assembly coincided with the haem-binding. In addition, the plots of absorbance change in the Soret band as a function of peptide concentration showed the bending point to be at the 1:1 [peptide]:[haem] ratio, suggesting that all the peptides bind the haem with 1:1 stoichiometry. It should be noted that there is a large difference between our case and the four haem-binding 4α-helix polypeptide (the dimer of two-helix peptide) reported by Dutton *et al.*[5b] Their polypeptide was designed to have four binding sites for a haem in the initial dimeric structure. On the other hand, our peptides with one haem-binding site are in a monomeric form in the absence of haem. For these reasons, we analyzed the reaction as the haem–peptide interaction assuming 1:1 binding.

The binding constants (K_a) determined from the absorbance change at the wavelength of the Soret band using a single site binding equation,[28] were in the order of 10^6–10^7 dm^3 mol^{-1} (Table 3). The absorbance changes in the Soret band caused by the addition of H2α17-VV, H2α17-AA, H2α17-AL and H2α17-LA are too small to determine the binding constants by UV–VIS titration. These peptides needed a concentration of the order of *ca.* 10^{-3} mol dm^{-3} for the haem binding. For these peptides, the relative hydrophobicities at the haem-binding site estimated by RP-HPLC are much smaller than those of the other peptides (<−0.12) (Table 1). The extent of hydrophobic interaction of the binding site in these peptides with the hydrophobic haem surface may provide an explanation for the haem-

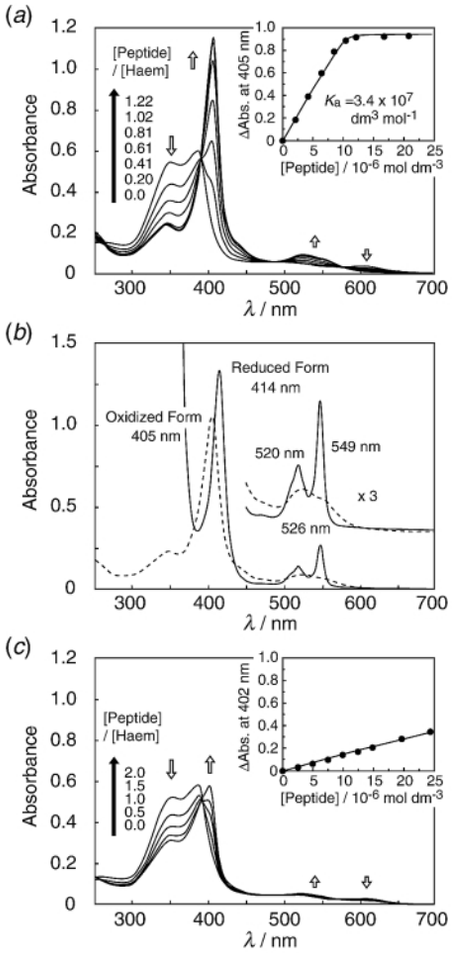

Fig. 4 (*a*) UV–VIS spectra of haem with increasing concentration of H2α17-FL in the buffer, pH 7.4 at 25 °C. The TFE solution of the peptide was added to the buffer solution of haem (final TFE content; 1.5%). [haem] = 1.0×10^{-5} mol dm^{-3}. Inset: Plots of absorbance at the Soret band of haem as a function of H2α17-FL concentration; (*b*) UV–VIS spectra of Fe^{III} (-----) and Fe^{II} (———) haem in the presence of H2α17-FL in the buffer, pH 7.4 at 25 °C. Haem was reduced by adding the minimum amount of solid sodium dithionite directly to the sample. [haem] = 1.0×10^{-5} mol dm^{-3} and [peptide] = 1.2×10^{-5} mol dm^{-3}; (*c*) UV–VIS spectra of haem with increasing concentration of H2α17-AA in the buffer, pH 7.4 at 25 °C. The TFE solution of the peptide was added to the buffer solution of haem (final TFE content; 2.0%). [haem] = 1.0×10^{-5} mol dm^{-3}. Inset: Plots of absorbance at the Soret band of haem as a function of H2α17-AA concentration.

binding constants of the peptides. The importance of the hydrophobic effect for the formation of peptide–haem conjugates can be seen in Fig. 5. A good correlation was obtained when comparing the free energy of haem binding for the peptides, except H2α17-FF and H2α17-FL, with the relative hydrophobicity values estimated by RP-HPLC. A similar result has recently been reported by Huffman *et al.*[11] They showed a linear correlation between the free energy of haem binding and the hydrophobicity of the haem-binding site, using designed 1α-helix peptides. These results demonstrated that the hydrophobicity of the side chain in contact with the haem was a primary determinant for effective haem-binding by the designed α-helix peptides.

It is noteworthy that the binding constant of H2α17-XL and that of H2α17-LY (X = Y; Phe, Ile, Val) are slightly different. For example, the binding constants of H2α17-FL, -VL and -IL were larger than those of counterparts H2α17-LF, -LV and -LI, respectively. These results indicate that the residue X and the residue Y at the haem-binding site are not equivalent with respect to the interaction with the hydrophobic face of the haem as mentioned for the CD study. The residues X and His are separated by three amino acids (His and X position *i* and

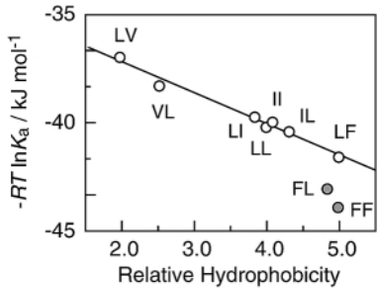

Fig. 5 Plots of free energy for formation of peptide–haem conjugates *vs*. the relative hydrophobicity estimated from RP-HPLC retention time.

i ± 4). On the other hand, the residues Y and His are separated by two amino acids (His and Y are *i* and *i* ± 3). The difference in the binding constant appeared to be attributed, in part, to the difference in spatial arrangement of X and Y residues relative to the His residue.

It should be noted that the points of H2α17-FF and H2α17-FL deviated markedly from the correlation line in Fig. 5. For these peptides, edge-to-face interactions between the aromatic side chain of the Phe residues and the porphyrin plane may contribute to the formation of peptide–haem conjugates. An edge-to-face interaction between the haem and the aromatic side chain has been observed in natural haemproteins, such as *Aplysia limacina* myoglobin[29] and cytochrome b_5.[30] As an example of the artificial peptide–haem conjugate, Williamson and Benson recently reported that tryptophan residues in a peptide-sandwiched iron-porphyrin provided helix stabilization *via* the edge-to-face interaction.[16d] It has been reported that an edge-to-face orientation between two aromatic groups is energetically favorable.[31] In the peptides H2α17-FF and H2α17-FL, two Phe residues per helix are located at *i* ± 4 (X position) relative to the axial ligand His[9] residue. This His–Phe *i* ± 4 arrangement was identical to that of *Aplysia limacina* myoglobin.[29] In contrast, the binding constant of the peptide H2α17-LF, in which two Phe residues in a helix are located at *i* ± 3 (Y position) relative to the His[9] residue, was comparable to that of the other peptides, and the plot was on the line in Fig. 5. These results implied that the *i* and *i* ± 4 positioning of Phe–His was critical to the edge-to-face interaction between the Phe side chain and the porphyrin ring, providing stabilization of peptide–haem conjugates. The UV–VIS titration data in this study indicated that the amino acid composition and arrangement at the haem-binding site was important for effective haem-binding by the two-α-helix peptides.

Molecular modeling study of the peptides

In order to estimate the steric interaction between the haem and amino acid residues arranged around the His residues, simple molecular models of the peptides were created. Using the Insight II molecular modeling program (SGI), an α-helical ($\varphi = -58°$; $\psi = -47°$) 17 residue peptide having the sequence Ac-A A E A **X Y** K A H A E **Y X** A K A A-OH was created with energetically reasonable side chain torsional angles. Haem ligation in natural haemproteins always occurs through the N^{ε} rather than the N^{δ} of His due to the unfavorable steric interaction between the His C^{β} methylene group and the haem plane.[32] Thus, the Fe–His bond was formed through N^{ε} of the His side chain in the model structures. As a combination of His side-chain torsional angles, $\chi_1 = -60°$ and $\chi_2 = 90°$ were selected. This χ_1/χ_2 combination is observed in most haemproteins, such as erythrocruorin, cytochrome c_3, haemoglobin and myoglobin, in which the axial ligand His occurs in a helical region of the protein.[32] The created two α-helical segments and the haem molecule were arranged with the following prerequisites: (i) the helix axis and haem plane angle was *ca*. 0° and two helical segments were deployed

Table 3 UV–VIS spectral data of the haem bound to the peptides and binding constants (K_a) of the peptides with the haem in the buffer

Peptide H2α17-XY	Relative hydrophobicity	UV–VIS Soret, λ_{max}/nm		UV–VIS α/β, λ_{max}/nm		Binding constant/ 10^7 dm^3 mol^{-1}
		Ferric	Ferrous	Ferric	Ferrous	
H2α17-FF	5.00	406	415	528	521/550	4.7
H2α17-LF	4.99	406	414	526	519/547	1.9
H2α17-FL	4.85	405	414	526	520/549	3.4
H2α17-IL	4.31	406	414	528	521/547	1.2
H2α17-II	4.08	406	414	528	520/548	1.0
H2α17-LL	4.00	405	413	528	520/547	1.1
H2α17-LI	3.84	405	413	527	520/549	0.9
H2α17-VL	2.51	406	414	528	521/547	0.5
H2α17-LV	1.97	405	413	525	520/529	0.3

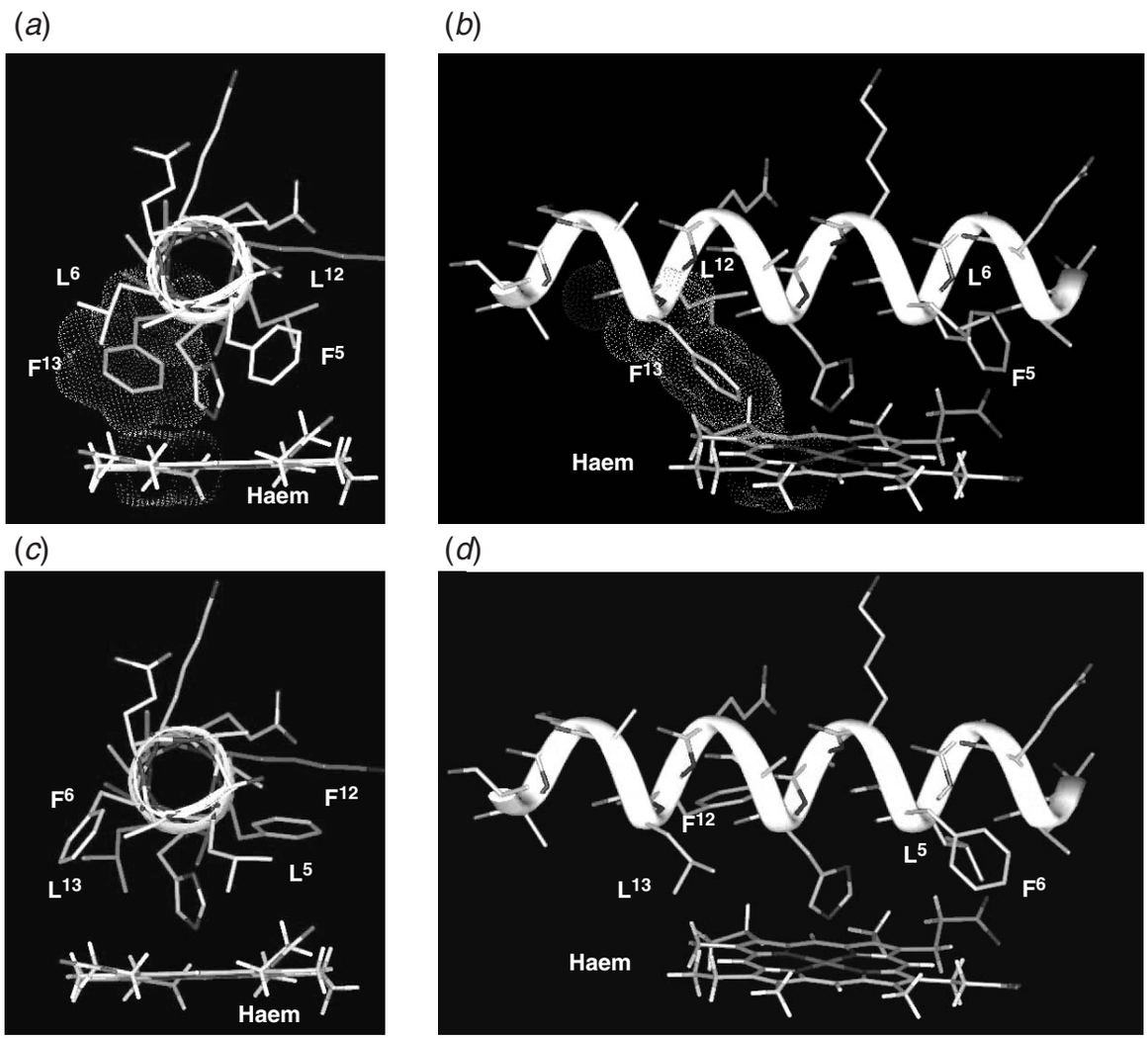

Fig. 6 Possible structures of peptide H2α17-FL (*a*, *b*) and H2α17-LF (*c*, *d*) with haem constructed by molecular modeling. Only one helix is shown.

nearly parallel; (ii) the bond length between the Fe in haem and the N^{ε} of His is fixed at 2.0 Å, which is the average value of the Fe–His bond length in natural haemproteins.[32] Next, two helical segments were connected *via* the –Gly–Gly–Gly–Cys–NH_2 linker at *C*-terminals. It has been confirmed that the length of linker was sufficiently long to prevent strain and distortion of the haem-binding two-α-helix structure. Finally, hydrophobic amino acid residues X and Y in the template model structure were systematically replaced by Phe, Ile, Leu, Val and Ala, with the result that 13 molecular models could be created.

Although the structural models were constructed using a simple method, the models gave good information about the steric interaction between the haem and the side-chains of the amino acids, X and Y. For Phe-containing peptides, hydrophobic residues positioned at X and Y could make van der Waals contacts with the haem. Especially, in the model structures of H2α17-FF and H2α17-FL, the phenyl rings of Phe^{13}, which were located at position $i + 4$ (X), could be nearly perpendicular to the haem plane and could make van der Waals contacts with the haem [Fig. 6(*a*),(*b*)]. That is, energetically favorable edge-to-face interactions between aromatic Phe side-chains at the X position and the haem were possible in the structures of H2α17-FF and H2α17-FL. On the other hand, in the model structure of H2α17-LF, in which the phenyl rings of Phe^6 and Phe^{12} were located at position $i \pm 3$ (Y), the edge-to-

face interactions could not be expected since the side-chains of Phe[6] and Phe[12] are slightly apart from the haem plane [Fig. 6(*c*)(*d*)]. These results seemed to support the contribution of the edge-to-face interactions to the higher binding constants of H2α17-FF and H2α17-FL.

For Ile- and Leu-containing peptides, hydrophobic residues X and Y could also make van der Waals contacts with the haem. However, for the Val- and Ala-containing peptides, Val and Ala residues introduced at the X or Y positions could not be in contact with the haem due to their small side-chains. Especially, the model structures of H2α17-VV and H2α17-AA suggested that an effective van der Waals contact could not be found. These results are in accord with the binding constants of the peptides, which are estimated from the UV–VIS titration. The smaller binding constant of these peptides appeared to result from the lack of these steric interactions between the side-chain and the haem. The computer modeling studies implied that the steric interactions between the haem and the side-chain of amino acids around the His were also important for the effective haem-binding.

Size-exclusion chromatography of peptides

To examine apparent molecular weights of the peptides in aqueous solution, the peptide samples (1.0×10^{-5} mol dm^{-3}) were passed through a Pharmacia Superdex 75HR size-exclusion column (Fig. 7). The unique results for H2α17-LL and H2α17-II described in previous communications[10c,d] were similar to the results for H2α17-IL. H2α17-AA, H2α17-AL and H2α17-LA were not examined, because the UV–VIS titration revealed that the peptides could not bind the haem effectively in the 10^{-6} mol dm^{-3} concentration range. The hydrophobic H2α17-FF, H2α17-FL and H2α17-LF gave no peak on the column in the absence and presence of haem, suggesting that the peptide could not be eluted due to the hydrophobic interaction between the peptide and column. The other peptides gave a single peak on chromatograms in the absence of haem. The elution volumes of the peptides were almost identical to that of a template peptide H2α17-LL, which was in a monomeric form elucidated by sedimentation equilibrium analysis.[10c]

When the buffer solution of a peptide containing the haem (1.2 equiv.) was chromatographed, the haem was co-eluted with the peptide in a sharp peak at a higher molecular weight, suggesting that the haem-binding changed the self-association state of the peptide in a regulated manner (Fig. 7). The elution volumes of the haem–peptide conjugates were coincident with those of haem-H2α17-LL and haem-H2α17-II conjugates, which form a tetrameric assembly under the conditions.[10c,d] That is, the haem-binding induced molecular association of the peptides to a tetrameric form from a monomeric (*i.e.*, the peptide–haem assembly was made up of eight α-helices and four haem molecules). This result suggested that the quaternary (4D) structure of the artificially designed peptide could be regulated by the haem binding. Although hydrophobic residues were different, the molecular association state of the peptides was identical to those of H2α17-LL and H2α17-II.

It should be noted that H2α17-VL and H2α17-LV gave two peaks on the size-exclusion chromatograms. The peak at a higher molecular weight was derived from the tetrameric haem–peptide assembly and the peak at a lower molecular weight was derived from the monomeric peptide, in which the haem was stripped in the column. It has been reported that hydrophobic molecules, such as haem, are tightly adsorbed to Superdex.[5a,6a,8] On the chromatogram of H2α17-VL, the peak area of the tetrameric peptide–haem conjugate was significantly larger than that of the monomeric haem-free peptide [Fig. 7(*h*)]. On the other hand, in the case of H2α17-LV, the peak area of the tetrameric peptide–haem conjugate was significantly smaller than that of monomeric haem-free peptide [Fig. 7(*k*)]. This

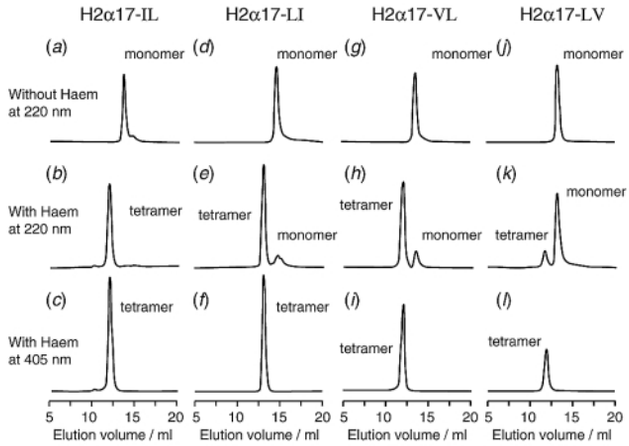

Fig. 7 Size-exclusion chromatograms of H2α17-IL (*a–c*), H2α17-LI (*d–f*), H2α17-VL (*g–i*) and H2α17-LV (*j–l*): (*a*,*d*,*g*,*j*) Peptide (1.0×10^{-5} mol dm^{-3}) in the absence of haem, detection at 220 nm; (*b*,*e*,*h*,*k*) Peptide (1.0×10^{-5} mol dm^{-3}) in the presence of haem (1.0×10^{-5} mol dm^{-3}, 1.0 equiv.), detection at 220 nm; (*c*,*f*,*i*,*l*) Peptide (1.0×10^{-5} mol dm^{-3}) in the presence of haem (1.0×10^{-5} mol dm^{-3}, 1.0 equiv.), detection at 405 nm. Column, Superdex 75 HR 10/30 (10 × 300 mm), 0.1 mol dm^{-3} NaCl–5.0×10^{-2} mol dm^{-3} Tris HCl buffer, pH 7.4 at 25 °C; flow rate, 0.5 cm^3 min^{-1}.

result is in accord with the binding constant estimated from UV–VIS titration. The elution profiles [Fig. 7(*b*),(*e*),(*h*),(*k*)] were parallel to the order of the binding constants. These results indicate that the arrangement of hydrophobic residues at the haem binding site is critical for tight haem-binding.

CD spectra of the haem bound to peptides

The CD spectra of haem bound to the peptides were investigated in the Soret band region (300–500 nm) in 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) (Fig. 8, Table 4). The haem bound to the peptides, except H2α17-VV and Ala-introducing peptides, showed induced CD peaks in the Soret region. The result for H2α17-LL described previously[10c] was almost the same as that for H2α17-II. Since the haem group is a symmetrical chromophore, the haem itself should exhibit no inherent optical activity. Thus, the obtained CD feature confirmed that the haem was chirally localized and oriented in the two-α-helix peptides.

The haem bound to the Ile- and Val-containing peptides, except H2α17-VV, showed a strong induced CD peak in the Soret region, which was split into a negative peak at a longer wavelength and a positive peak at a shorter wavelength [Fig. 8(*d*)–(*f*),(*h*),(*i*)]. The crossover wavelengths were coincident with those of the Soret peak top and the intensities of both positive and negative peaks were almost equal, indicating that the induced Soret-CD were derived from the exciton coupling with haem–haem interactions.[33] The split Cotton effect, *i.e.* exciton-coupling, is more evidence supporting the tetrameric self-association of peptide–haem conjugates, and indicates that the haem groups are highly oriented in close positions in the self-assembled peptides. Among the Ile- and Val-containing peptides, the most hydrophobic H2α17-IL showed the largest Soret-CD peaks. On the other hand, the most hydrophilic H2α17-LV showed the smallest Soret-CD peaks. These results implied that the mutual orientation and distance of haem molecules in the assembled peptide varied depending on the hydrophobicity and/or arrangement of hydrophobic residues around the haem.[33,34]

The haem in the presence of the Phe-containing peptides showed more complex CD spectra. The CD spectra of haem bound to H2α17-FF and H2α17-LF were characterized by a negative peak at around 410 nm, a positive peak at around 400 nm and a negative peak at around 390 nm [Fig. 8(*a*),(*c*) and

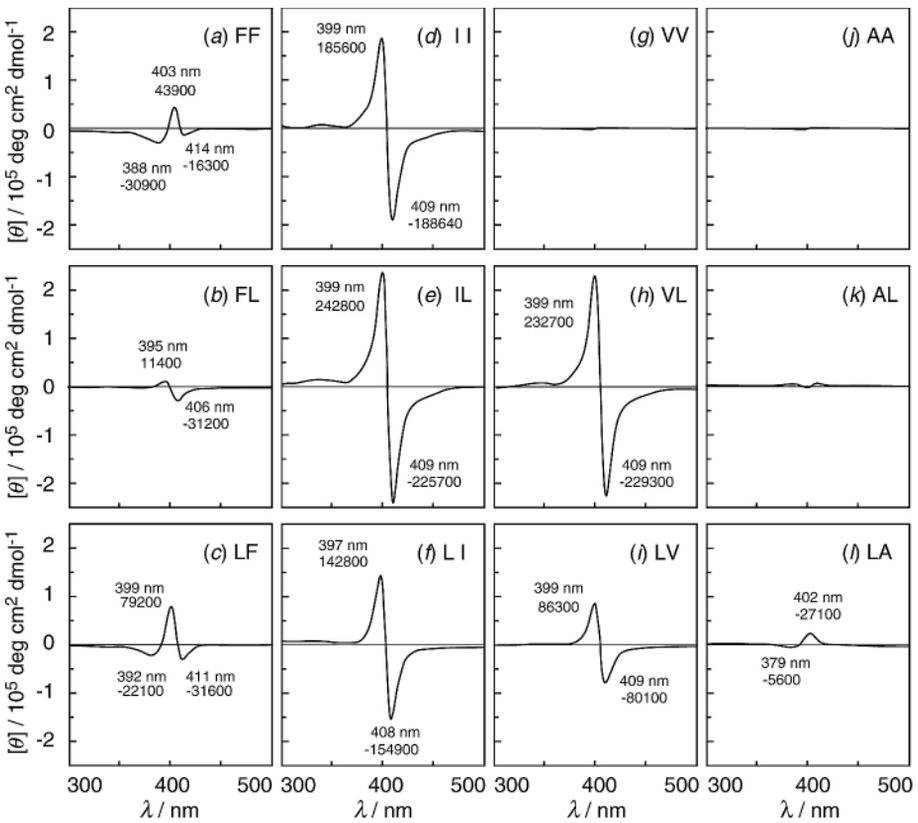

Fig. 8 CD spectra of haem in the presence of H2α17-FF (*a*), H2α17-FL (*b*), H2α17-LF (*c*), H2α17-II (*d*), H2α17-IL (*e*), H2α17-LI (*f*), H2α17-VV (*g*), H2α17-VL (*h*), H2α17-LV (*i*), H2α17-AA (*j*), H2α17-AL (*k*) and H2α17-LA (*l*) in 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) containing 1.2% TFE at 25 °C. [haem] = 1.0×10^{-5} mol dm^{-3}, [peptide] = 1.2×10^{-5} mol dm^{-3}.

Table 4 Soret CD intensity of the haem bound to peptides[a]

Peptide H2α17-XY	Soret CD intensity/deg cm^2 dmol^{-1} (λ, peak top/nm)		
H2α17-FF	−16 300 (414)	43 900 (403)	−30 900 (388)
H2α17-LF	−31 600 (411)	79 200 (399)	−22 100 (392)
H2α17-FL	−31 200 (406)	11 400 (395)	
H2α17-IL	−225 700 (409)	242 800 (399)	
H2α17-II	−188 640 (409)	185 600 (399)	
H2α17-LL	−194 000 (411)	178 000 (400)	
H2α17-LI	−154 900 (408)	142 800 (397)	
H2α17-VL	−229 300 (409)	232 700 (409)	
H2α17-LV	−80 100 (409)	86 300 (399)	
H2α17-VV	No peak		
H2α17-LA	27 100 (402)	−5600 (379)	
H2α17-AL	Weak peak		
H2α17-AA	No peak		

[a] [haem] = 1.0×10^{-5} mol dm^{-3}, [peptide] = 1.2×10^{-5} mol dm^{-3}.

Table 4]. On the other hand, the CD spectrum of haem bound to H2α17-FL was characterized by a strong negative peak at a longer wavelength (406 nm) and a weak positive peak at a shorter wavelength (385 nm) [Fig. 8(*b*)]. These complex features might arise *via* coupling interaction of the haem with the Phe side-chain. According to Hsu and Woody, coupled interactions between B_x and B_y of the haem and π–π* transitions of aromatic amino acids are the primary contributors to the Soret CD band.[35] It was difficult to interpret the Soret CD of haem bound to Phe-containing peptides, due to the lack of information about the relative geometry of the haem and the peptide, and the molecular association state of the peptide–haem conjugates.

The CD spectra of haem in the presence of H2α17-VV, H2α17-AA, H2α17-AL and H2α17-LA were also investigated in the buffer [Fig. 8(*g*),(*j*)–(*l*)]. The haem in the presence of the peptides showed no or small induced CD peaks in the Soret region. This result suggested that the peptides could not bind the haem effectively in this concentration range.

Peroxidase-like activities of the haem bound to peptides

In order to know how the haem catalytic activity was regulated by the binding of polypeptides, we examined the catalytic activity of the haem bound to the peptides in 0.1 mol dm^{-3} Tris HCl buffer, pH 7.4, using an oxidation reaction of *o*-methoxyphenol to its tetramer, tetraguaiacol, which is one of the typical reactions catalyzed by peroxidase or monooxygenase in the family of haemenzymes.[36] In the design of haem-conjugated peptides, haem was fixed in the polypeptide 3D structure by bis-His coordination, similar to natural *b*-type haemproteins. Thus, as the peptide binds the haem more tightly, the haem reacts less readily with a substrate, such as hydrogen peroxide. Therefore, we thought that the peroxidase activity was a good indicator to estimate the rigidity of haem-binding by peptide.[10a,b] The determined initial rates are summarized in Fig. 9. The initial rate, *v*, of the reaction in the presence of peptide H2α17-FF (0.49×10^{-6} mol dm^{-3} min^{-1}) was suppressed to a relative activity of 9.6 [the relative activity, 100, haem only in the absence of peptide (5.1×10^{-6} mol dm^{-3} min^{-1})]. The reaction rates of haem bound to peptides H2α17-FL, H2α17-LF, H2α17-II, H2α17-IL, H2α17-LI and H2α17-LL were also diminished to (0.15–0.68) $\times 10^{-6}$ mol dm^{-3} min^{-1} (the relative activity, 2.9–13). These *o*-methoxyphenol oxidation activities were comparable to that of natural electron transfer protein, cytochrome *c* (0.32×10^{-6} mol dm^{-3} min^{-1}, 6.0).[37] These results suggested that the haem was tightly fixed in the polypeptide 3D structure at a level similar to that of the natural bis-ligated haemproteins. In contrast, the reaction was more weakly suppressed by the addition of H2α17-VL (2.12×10^{-6} mol dm^{-3} min^{-1}, 42) and

H2α17-LV (4.66×10^{-6} mol dm^{-3} min^{-1}, 91). Because nearly all of the haem (>95%) bound the peptide under the reaction conditions according to the binding constant, the moderate suppression of haem reactivity by H2α17-VL and H2α17-LV appeared to reflect a looser coordination by the axial ligands relative to the other peptides. As described above, the smaller K_a (H2α17-VL; $K_a = 0.5 \times 10^7$ dm^3 mol^{-1}, H2α17-LV; $K_a = 0.3 \times 10^7$ dm^3 mol^{-1}) was correlated to the loosened coordination by the axial ligands in the peptides. This result implied that interactions between the haem and peptides were determined by both the composition and arrangement of hydrophobic residues around the axial ligand His. On the other hand, the peptides H2α17-AA, H2α17-AL and H2α17-LA, which did not bind the haem in the concentration range, did not influence the reaction rate of haem at all (the relative activity, 100 for H2α17-AA, 106 for H2α17-AL and 103 for H2α17-LA).

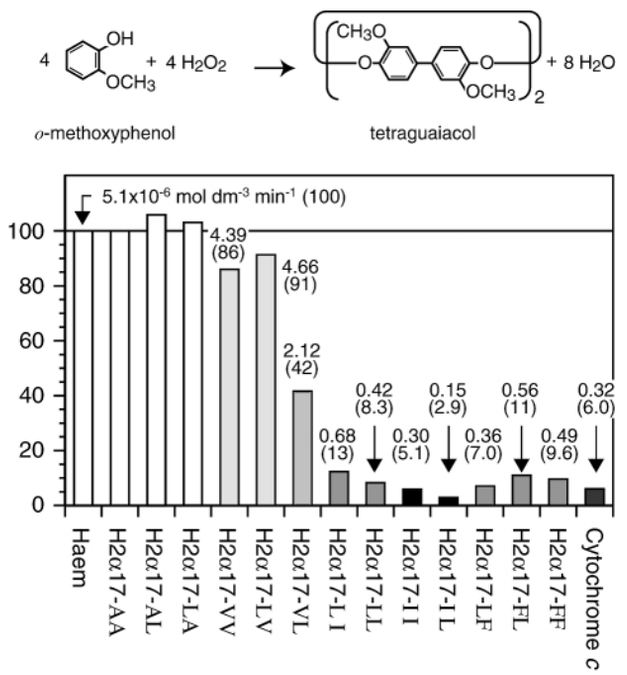

Fig. 9 Initial rates of tetraguaiacol formation catalyzed by the haem in the presence of peptides in 0.1 mol dm^{-3} Tris HCl buffer, pH 7.4, at 25 °C. [haem] = 0.5×10^{-5} mol dm^{-3}, [peptide] = 1.0×10^{-5} mol dm^{-3}, [*o*-methoxyphenol] = 1.0×10^{-2} mol dm^{-3}, [H_2O_2] = 0.5×10^{-3} mol dm^{-3}.

Conclusions

It has been demonstrated that a series of two-α-helix peptides, which bound a haem through a ligation of two His axial ligands, were successfully designed and synthesized. By using the peptides, the steric and hydrophobic interactions between the haem and the amino acid residues arranged around the His were systematically evaluated. The interactions between the haem and the peptides were controlled by several factors. At the simplest level, the binding constant of the peptide with the haem was related to the relative hydrophobicity around the His residue, which was estimated from the retention time of each peptide on RP-HPLC. The good correlation between two experimental data (*i.e.* binding constant estimated from the UV–VIS titration *vs*. relative hydrophobicity derived from the RP-HPLC) was observed for a series of peptides. This result indicated that the hydrophobic interaction at the haem-binding site was a determinant stabilizing the peptide–haem conjugates. Furthermore, simple molecular modeling studies suggested that the van der Waals contacts between the side-chain of the amino acid residues and the haem were also an important contributor for effective haem-binding. In the model structures of the peptides, which showed a strong interaction with the haem ($K_a > ca.\ 1.0 \times 10^7$ dm^3 mol^{-1}), the amino acid residues constructing a haem binding site were sufficiently large to make van der Waals contacts with the haem. Especially, H2α17-FF and H2α17-FL, in which the phenyl rings of Phe[13] could be nearly perpendicular to the haem plane, showed higher binding constants among the peptides. These results implied that there was a contribution from the edge-to-face interactions between the aromatic side chain and the haem to the stability of the peptide–haem conjugate. On the other hand, the binding constants of H2α17-AA and H2α17-VV were significantly smaller than those of other peptides (K_a was roughly estimated as *ca.* ~10^3 dm^3 mol^{-1}). In the modeling structures of the peptides, the side chains of Ala and Val residues were too small to make van der Waals contacts with the haem, indicating that the size of the amino acid side chain at the haem-binding site was important for effective haem-binding.

The differences in the binding ability for the haem, arising from the amino acid composition and the arrangement around the axial ligand His, were also significantly reflected by the peroxidase activity of haem. That is, the peptide possessing a stronger binding ability (larger K_a) more effectively prevented susceptibility to the oxidant, H_2O_2 (Fig. 10). On the other hand, the haem bound to the peptide possessing a weaker binding ability (smaller K_a), such as H2α17-VL and H2α17-LV, maintained the haem reactivity under the conditions, where nearly

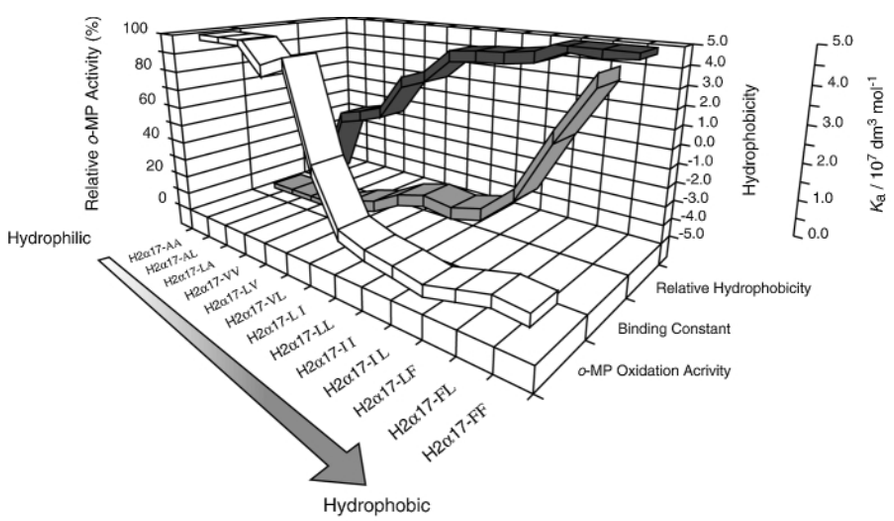

Fig. 10 Relationship between the hydrophobicity derived from RP-HPLC of peptides (dark gray), binding constants of peptides for the haem (gray) and catalytic activities of haem bound to the peptides (white).

all of the haem (>95%) bound to the peptides. The higher haem reactivity of the haem bound to H2α17-VL and H2α17-LV appeared to reflect a looser coordination by the axial ligands relative to the other peptides. These results indicate the possibility that the functional property of the haem could be controlled by the amino acid composition and arrangement of the amino acids around the axial ligand in the designed two-α-helix structure. Although detailed examination of the 3D structure of haem–peptide conjugates will be needed, further systematic substitution of amino acids forming the haem-binding site, including the axial ligands, will lead to the design of artificial haem proteins with a variety of specific functions with minimum structural elements.

Experimental

Peptide synthesis

The synthesis of H2α17-LL, H2α17-II and H2α17-VV was described briefly in previous communications.[10c,d] Ac–Ala–Ala–Glu(OBu^t)–Ala–X–Y–Lys(Boc)–Ala–His(Tr)–Ala–Glu(OBu^t)–Y–X–Ala–Lys(Boc)–Ala–Ala–Gly–Gly–Gly–Cys(Tr)–NH–Rink amide resin (X, Y = Ala, Phe, Ile, Leu or Val; Bu^t, *tert*-butyl; Boc, *tert*-butoxycarbonyl; Tr, trityl) was synthesized by the Fmoc solid-phase method[19] using Fmoc protected amino acid derivatives (6.0 equiv.), DIC (6.0 equiv.) and HOBt (6.0 equiv.) on Rink amide resin (Advanced Chemtech) (0.5×10^{-4} mol scale) in an Advanced Chemtech BenchMark model 348 multiple peptide synthesizer. The protecting groups and the resin were removed by stirring the dried resin for 2 h at 25 °C with TFA–*m*-cresol–ethanedithiol–thioanisole (85 : 6 : 6 : 2). The solvent was evaporated and the residues were solidified with diethyl ether on an ice-bath to give crude peptides. Crude peptides were purified by RP-HPLC on a YMC ODS A-323 column (10 × 250 mm) using a linear gradient of ACN–0.1% TFA (1.0% min^{-1}) to give the product with a single peak on analytical HPLC [Wakosil 5C18, 4.6 × 150 mm with a linear gradient of ACN–0.1% TFA (1.0% min^{-1})].

To form a disulfide bond at the *C*-terminal, each peptide (20 mg, 2.0×10^{-5} mol) was dissolved in TFA (1.8 cm^3) and then anisole and DMSO (0.2 cm^3) were added to the solution (10% DMSO–TFA).[20] The peptide solution was stirred at room temperature for 1 h. After the reaction was stopped by the addition of diethyl ether, peptides were solidified with ether. The crude peptides were purified by RP-HPLC to give the product with a single peak by analytical HPLC. The peptides were identified by MALDI-TOFMS and amino acid analysis; H2α17-FF, *m/z* 4284.4 [(M + H)$^+$] (Calc. = 4283.8); H2α17-FL, *m/z* 4147.1 [(M + H)$^+$] (Calc. = 4147.8); H2α17-LF, *m/z* 4147.6 [(M + H)$^+$] (Calc. = 4147.8); H2α17-IL, *m/z* 4010.9 [(M + H)$^+$] (Calc. = 4011.7); H2α17-LI, *m/z* 4011.8 [(M + H)$^+$] (Calc. = 4011.7); H2α17-VL, *m/z* 3955.6 [(M + H)$^+$] (Calc. = 3955.4); H2α17-LV, *m/z* 3957.7 [(M + H)$^+$] (Calc. = 3955.4); H2α17-AA, *m/z* 3673.5 [(M + H)$^+$] (Calc. = 3675.0); H2α17-AL, *m/z* 3843.0 [(M + H)$^+$] (Calc. = 3843.4); and H2α17-LA, *m/z* 3844.0 [(M + H)$^+$] (Calc. = 3843.4).

The peptides were dissolved in trifluoroethanol (TFE) in a peptide concentration of 1.0×10^{-3} mol dm^{-3} and the solution was used as a stock solution for measurements. It has been confirmed that there is little effect of small amounts of TFE (<2.0%) on the measurements of CD or UV–VIS titration of the haem with the peptides.

Estimation of relative hydrophobicity

The retention time of each H2α17-XY peptide was determined on a YMC-Pack C4 reversed-phase column (4.6 × 150 mm) using a linear gradient of 10–50% ACN–0.1% TFA (40 min) at 30 °C. The wavelength for the detection was 220 nm. The RP-HPLC was performed by employing two SHIMADZU LC-10ACvp pumps equipped with a SHIMADZU-M10Avp photodiode array UV–VIS detector and a SHIMADZU-CTO-10Avp column oven. Equal amounts of each peptide dissolved in TFE were injected into the column and eluted at a flow rate of 1.0 cm^3 min^{-1}.

CD measurements

CD spectra were recorded on a J-720 spectropolarimeter using a quartz cell with a 1.0 mm pathlength in the amide region (190–250 nm) and 10 mm in the Soret region (300–500 nm), respectively. Peptides were dissolved in 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) in peptide concentration of 1.0×10^{-5} mol dm^{-3} (TFE concentration, 1.0%). For the measurements of haem bound to the peptide, the haem and the peptide were dissolved in 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) in the haem and peptide concentrations of 1.0×10^{-5} mol dm^{-3} and 1.2×10^{-5} mol dm^{-3}, respectively (TFE concentration, 1.2%).

UV–VIS measurements

UV–VIS spectra were recorded on a SHIMADZU UV-3100 spectrophotometer using a quartz cell with a 10 mm pathlength. Fe^{III}-mesoporphyrin was reduced by adding an excess amount of solid sodium dithionite. Fe^{III}-mesoporphyrin in the buffer was titrated with peptides in increments of about 0.2 equiv. After each addition of peptide, samples were equilibrated for 30 min at 25 °C, then UV–VIS spectra (250–700 nm) were measured (TFE concentration, 0–2.0%). The increase in absorbance at the peak top of the Soret band with increasing peptide concentration was corrected for dilution and fitted by a single site binding equation[28] using KaleidaGraph (Synergy Software).

Size-exclusion chromatography

For analytical size-exclusion HPLC, a Superdex 75HR column (10 × 300 mm) (Pharmacia Biotech) was used and the chromatographic conditions were as follows: eluent, 0.1 mol dm^{-3} NaCl–5.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4); flow rate 0.5 cm^3 min^{-1}. The wavelength for the detection was 220 nm (without haem) and 405 nm (with bound haem). The template peptide H2α17-LL with and without haem was used as a standard. As described in the previous communication, analytical ultracentrifugation indicated that H2α17-LL existed as a monomeric and tetrameric form in the absence and presence of haem, respectively.[10c] The peptide sample was dissolved in the buffer (1.0×10^{-5} mol dm^{-3}) and 1.0×10^{-4} dm^{-3} of the solution was injected.

Assay for *o*-methoxyphenol oxidation

The *o*-methoxyphenol oxidation activity of haem in the presence or absence of the peptides was assayed by measuring the amount of the produced tetramer.[37] The reaction was initiated by the addition of hydrogen peroxidase (the final concentration, 0.5×10^{-3} mol dm^{-3}) to mixtures of *o*-methoxyphenol (substrate) (1.0×10^{-2} mol dm^{-3}), haem (5.0×10^{-6} mol dm^{-3}) and peptide (1.0×10^{-5} mol dm^{-3}) in 0.1 mol dm^{-3} Tris HCl buffer, pH 7.4, at 25 °C. The reaction was followed by monitoring the absorbance change at 470 nm ($\varepsilon = 2.66 \times 10^4$ cm^{-1} mol^{-1} dm^3).

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