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# Regulation of α/β-folding of a designed peptide by haem binding

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**A designed peptide H2α17-I bound $Fe^{III}$–mesoporphyrin (haem); and the haem-binding prevented the peptide forming β-sheet aggregates by facilitating the formation of an α-helix tetramer, indicating that the folding state of artificially designed peptides could be regulated by cofactor binding.**

Iron porphyrins occur widely in nature as cofactors of haemproteins and display diverse functions. In addition to the wide range of iron porphyrin reactions, iron porphyrins are important factors in defining the three-dimensional (3D) structure of haemproteins, because the removal of a porphyrin cofactor from natural haemproteins caused perturbation and/or destabilization of protein 3D structures.[1–4] So far, in the field of *de novo* protein design, considerable effort has been devoted to construction of polypeptide 3D structure conjugated with porphyrin molecules *via* chelation[5–7] or covalent linkage with peptides.[8–11] However, to establish an artificial haemprotein that meets the minimal requirements for its function, it will be necessary to understand the role of porphyrin cofactors as structural elements of haemproteins. In a previous paper, we described the design of a two-α-helix peptide H2α17-L, whose binding to $Fe^{III}$–mesoporphyrin (haem) stabilized an α-helical structure and induced a change in the molecular-assembly of the peptide from a monomeric to a tetrameric form.[7b] These results demonstrated that the haem had a significant effect on the 3D-structure and molecular association of an artificially designed polypeptide. Here we examine the effects of amino acid disposition around the axial ligand on the haem-binding property, using novel designed peptides H2α17-I and H2α17-V. Furthermore, we found that the haem binding affected the secondary structure and molecular-association state of the peptide H2α17-I.

The design of H2α17-I and H2α17-V was based on that of the template peptide H2α17-L,[7b] which took an α-helix structure in the buffer both with and without bound haem. The amino acid sequences of H2α17-I and H2α17-V were identical to that of H2α17-L except for their hydrophobic residues (Fig. 1). In the cases of H2α17-I and H2α17-V, hydrophobic Ile and Val residues, respectively, were introduced at the 5th, 6th, 12th and 13th positions instead of the Leu residues of H2α17-L. In the designed α-helix structure, four hydrophobic residues per helix could be arranged around the His[9] to make a hydrophobic haem binding site and a haem would be deployed between the two helices. In the cases of H2α17-I and H2α17-V, however, it was predicted that the folded state of the amphiphilic α-helical sequences would be significantly destabilized by the introduction of four Ile or Val residues, which prefer a β-strand rather than an α-helix structure.[12] Additionally, when the peptide sequences were drawn as a β-sheet model, the peptide could take a kind of amphiphilic β-sheet structure. However, the haem will bind to the folded-state of an α-helical structure with higher affinity than to that of a β-structure, because the hydrophobic residues, which form the hydrophobic haem-binding site, can be arranged around the His,[9] only when the peptides take an α-helix structure. The peptides were synthesized by solid-phase method using Fmoc chemistry and purified with semi-preparative HPLC. The peptides H2α17-I and H2α17-V gave molecular ion peaks at *m/z* 4011.9 [$(M + H)^+$] (calc. 4011.7) and 3898.8 [$(M + H)^+$] (calc. 3899.0), respectively, on matrix assisted laser desorption ionization of time-of-flight mass spectrometry.

Circular dichroism (CD) studies revealed that the peptides H2α17-I and H2α17-V showed typical α-helical patterns with double negative maxima at 207 and 222 nm in trifluoroethanol (TFE), which was known to be an α-helix stabilizing solvent.[13] Upon dilution in $2.0 \times 10^{-2}$ mol $dm^{-3}$ Tris HCl buffer (pH 7.4) from TFE stock solution of H2α17-I and H2α17-V (final TFE concentration was 1.0%), the peptides showed CD spectra typical of a β-structure with a single negative maximum at 218 nm and a positive maximum at 198 nm (Fig. 2). Additionally, Fourier transform infrared (FT-IR) spectra of the peptides in cast films exhibited narrow peaks for the amide I and II bands: H2α17-I, 1630 and 1555 $cm^{-1}$; H2α17-V, 1627 and 1553 $cm^{-1}$, respectively. These wavenumbers were characteristic of a β-structure rather than an α-helix or a random structure.[14] In the absence of haem, H2α17-I and H2α17-V gave no peak on the size exclusion column, suggesting that the peptides formed β-sheet aggregates in multiple states. The formation of β-sheet aggregates was also confirmed by the binding of Congo Red dye, which stains β-sheet proteins, such as amyloid and prion proteins.[15a] Because the template peptide H2α17-L took an α-helix structure and was in a monomeric form under the same conditions, the β-conformation of the peptides in the buffer is due to the high β-propensity of the Ile and Val residues.

On the contrary to the above results, when the TFE stock solution of H2α17-I was diluted with buffer containing haem (1.0 equiv.), the peptide showed a typical α-helical pattern with

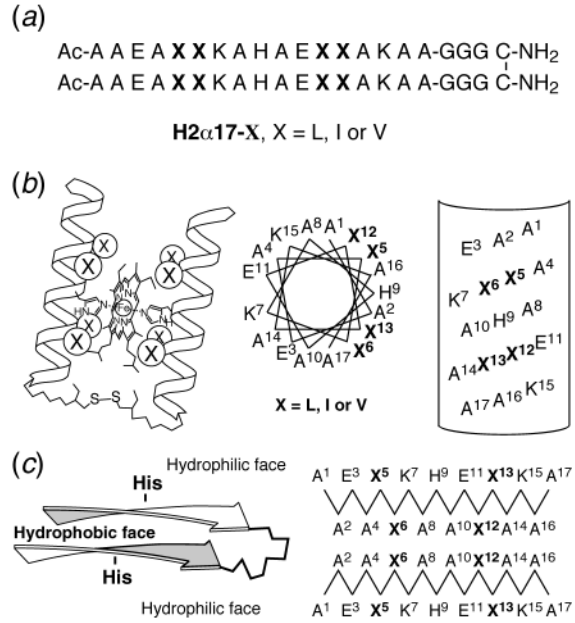


**Fig. 1** Structure of the designed peptides. (*a*) Amino acid sequence of H2α17-I and H2α17-V. (*b*) Illustration of the two-α-helix peptide structure bound to the haem, and helix wheel and net drawings of the 17-peptide. (*c*) Schematic illustration of the β-structure of the 17-peptide.

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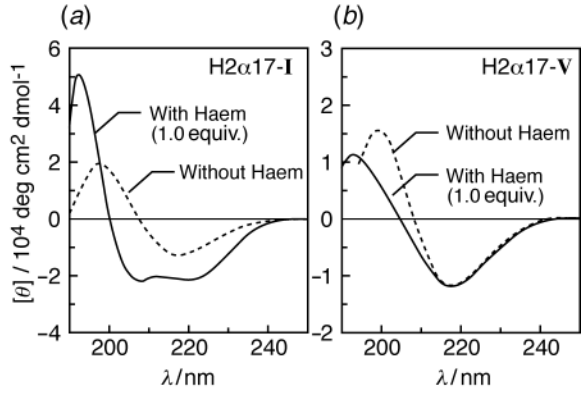


**Fig. 2** CD spectra of (*a*) H2α17-I and (*b*) H2α17-V in the absence and presence of haem (1.0 equiv.) in the buffer (pH 7.4) containing 1% TFE at 25 °C. [peptide] = $1.0 \times 10^{-5}$ mol dm$^{-3}$.

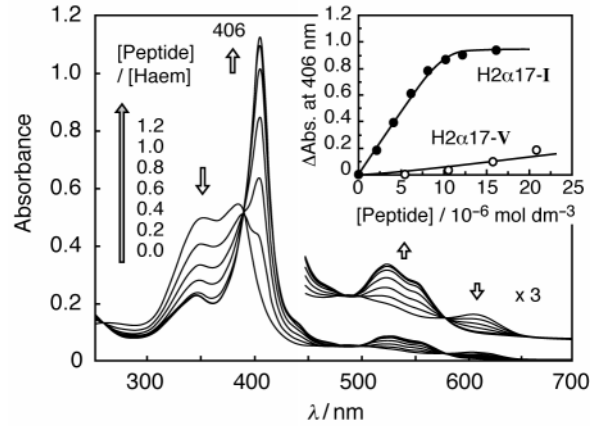


**Fig. 3** UV-VIS spectra of haem with increasing H2α17-I concentration in the buffer, pH 7.4 at 25 °C. The TFE solution of the peptide was added to the buffer solution of haem (final TFE content; 1.5%). [haem] = $1.0 \times 10^{-5}$ mol dm$^{-3}$. Inset: Plots of absorbance at the Soret band of haem as a function of concentration, peptide (●) H2α17-I and (○) H2α17-V.

double negative maxima at 208 and 222 nm in the buffer (containing 1.0% TFE) (Fig. 2). From the ellipticity at 222 nm ($[\theta]_{222}$ = −22 500 deg cm$^2$ dmol$^{-1}$), the α-helicity was estimated as 71%.[16] Size-exclusion chromatography and sedimentation equilibrium studies revealed that the peptide H2α17-I was in a tetrameric form ($MW_{obs}$ = 18 000, 3.9-mer) after haem binding, as was observed in H2α17-L.[7b] That is, the haem binding induced a drastic alteration of the secondary structure and molecular association state from β-sheet aggregates to an α-helical tetrameric assembly. The peptide H2α17-I folded into a β-structure at acidic pH (4.0–6.0) in the presence and absence of haem. Because the p$K_a$ of His is reported as *ca*. 6.4,[17] the pH effect is attributed to the protonation of the His side chains such that they cannot act as ligands. Therefore, we concluded that H2α17-I folded into an α-helix structure *via* haem binding through a ligation with His residues. In contrast, the peptide H2α17-V took a β-structure even in the presence of haem at pH 7.4, suggesting that H2α17-V could not bind the haem effectively. CD studies demonstrated that the secondary structure and molecular assembly of the designed peptide was regulated by the haem binding. Although the α-form (with haem) was completely denatured with 6.0 mol dm$^{-3}$ urea (urea denaturation midpoint; $[\text{urea}]_{1/2}$ = 2.7 mol dm$^{-3}$, free energy of unfolding; $\Delta G_{H_2O}$ = −7.6 kJ mol$^{-1}$), the CD spectrum of the β-form (without haem) was not changed even with 7.0 mol dm$^{-3}$ urea, indicating that the β-form is much more stable than the α-form. Thus, the alternation of peptide α/β folding appeared to be regulated by a kinetic mechanism rather than a thermodynamic one. Even in the absence of haem, immediately after dilution from the TFE solution, it is predicted that the peptide takes an α-helix structure,[15] although the α-helix structure is unstable and the rapid transition from the α- to the β-form follows. In the presence of haem, however, the haem binds the metastable α-form and prevents the peptide progressing to a β-form. Indeed, after formation of the β-sheet structure, the peptide could not bind the haem and the α-helix structure was not recovered by the addition of haem.

To further characterize the haem binding with the peptides, UV-VIS titration of the haem was carried out in buffer. With increasing concentration of H2α17-I an increase of the Soret band at 406 nm and a decrease of the band at 355 nm of haem were observed (Fig. 3). The UV-VIS spectrum of haem in the presence of peptide resembles those of natural cytochromes with low-spin six-coordinate iron.[7] The binding constant ($K_a$), determined from the absorbance change at the Soret band using a single site binding equation, was $1.0 \times 10^7$ dm$^3$ mol$^{-1}$ (Fig. 3, inset), comparable to that of H2α17-L ($K_a$ = $1.1 \times 10^7$ dm$^3$ mol$^{-1}$).[7b] On the other hand, addition of H2α17-V caused little increase of the Soret band, indicating that the peptide could not bind the haem effectively in this concentration range. The computer modeling study suggested that the side chain of the Val residue was too small to make effective van der Waals contact with the porphyrin plane, when the haem was positioned between the helices with energetically favorable side-chain torsional angles of the His ligands.[10,11] Additionally, the hydrophobicity of Val is lower than that of Ile and Leu,[18] These results revealed that the nature of amino acids at the haem binding site was important for the effective haem-binding by the two-α-helix peptides. There is enough hydrophobicity and van der Waals volume in Leu and Ile for effective haem binding in the two-α-helix form.

In conclusion, we successfully designed and synthesized the peptide H2α17-I with the unique property that the folding state was controlled by the haem-binding. The haem binding prevented the peptide from forming β-sheet aggregates by facilitating the formation of an α-helix tetramer. These findings indicate the importance of haem cofactor as a structural element of artificial haem-conjugated proteins and polypeptides. This kind of work will lead to studies to construct mini-haemproteins and elucidate their folding and application.

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