Membrane-Peptide Mimetics

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β-Hairpin Peptides: Heme Binding, Catalysis, and Structure in Detergent Micelles**

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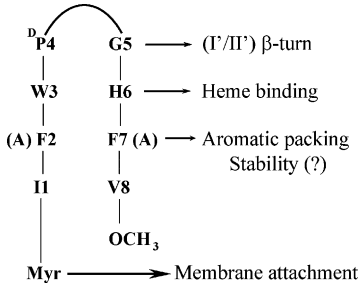
Designed peptides that demonstrate both structure and function in a lipid environment are of considerable interest for developing synthetic protein mimics for membrane-active biological processes, including enzymes, electron transfer, ion channels, and energy conversion.[1–4] Designed peptides containing heme as a co-factor would be of significant practical consideration for energy production and catalysis in membranes.[1,2] Based on the remarkable progress achieved for water-soluble heme-binding designed peptides and proteins on helical scaffolds,[5–9] membrane-soluble designed helical peptides binding to heme or porphyrin were recently described.[10–14] The β-hairpin (or two stranded antiparallel β-sheet), the fundamental unit of β-sheet proteins or β-barrel membrane proteins,[15,16] serve as an attractive system for functional and structural mimic in membranes. Designed autonomously folded water-soluble β-hairpin peptides have been extensively investigated, generating important insights for conformational preferences, energetic and functional mimetics.[17–27] However, the structure and stability of autonomously folded membrane-soluble β-hairpins or β-sheets may be complicated owing to heterogeneous aggregation.[28–30] Furthermore, heme-binding designed β-hairpin peptides have yet to be reported. Herein, we demonstrate heme binding, peroxidase activity, and structural characterization of designed β-hairpin peptides, termed IV8 and IV8FA, in dodecylphosphocholine (DPC) detergent micelles. The primary structures of the IV8 peptide (Myr-I-F-W-^{D}P-G-H-F-V-OCH_3) and its analogue IV8FA (Myr-I-A-W-^{D}P-G-H-A-V-OCH_3) contain a ^{D}P-G segment for the nucleation of a type I′ or type II′ β-turn (Scheme 1). Notably, water-soluble β-hairpin peptides have been designed using either with ^{D}Pro-Gly[31–33] or Asn-Gly[34–36] nucleating type I′ or type II′ β-turns. We have chosen ^{D}Pro residue over Asn because of its higher hydrophobicity. Further, residue W3 would anchor the peptide into the water–lipid interface, whereas residue H6 is expected to coordinate with the heme cofactor (Scheme 1). The two Phe residues, at positions 2 and 7, in IV8 peptide may stabilize the β-hairpin structure through aromatic packing.[37–40] To correlate aromatic interactions of β-hairpin stability with activity, the analogue peptide IV8FA has been synthesized. Finally, the N-termini of the peptides are myristoylated (Myr) to achieve a stable insertion into lipid membranes. It is worth mentioning that lipidation is a naturally occurring post-translational modification for membrane attachment of proteins.[41] Moreover, peptides containing long acyl chains are of therapeutic interest for targeting cellular proteins.[42,43]



Scheme 1. Primary structure and design of peptides intended to adopt a β-hairpin structure, bind heme, and catalyze oxidation of a substrate in detergent solutions.

The designed peptides were reconstituted in DPC detergent solution for functional and structural characterization. Figure 1 shows heme binding of the IV8 and IV8FA peptides by UV/Vis spectroscopy, whereby a fixed concentration of heme was titrated with increasing concentrations of the designed peptides. In the absence of peptide, heme shows a broad absorption or Soret band at 380 nm (Figure 1).

Upon addition of IV8 and IV8FA peptides, the Soret band of heme sharpens and shifts to 412 nm, at a peptide concen-

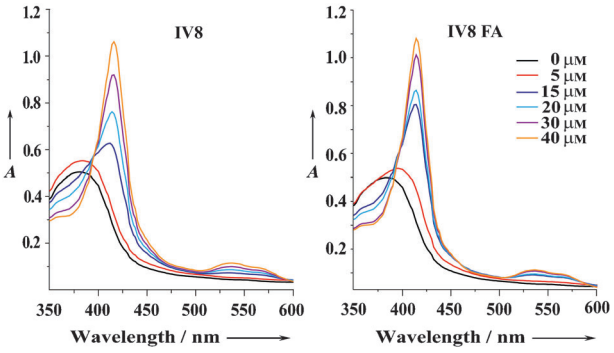


Figure 1. UV/Vis spectra of heme (10 μM) with increasing concentrations of IV8 (left panel) and IV8FA (right panel) in phosphate buffer (pH 7.5) containing 1.5 mM DPC. The critical micelle concentration (CMC) of DPC under the solution conditions was estimated to be 1.1 mM (Supporting Information, Figure S1) using dye-binding assays.[44]

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tration of more than 15 μM, showing an isobestic point at about 400 nm (Figure 1). Furthermore, less-intense α and β bands of heme can be seen between 500 and 600 nm in the presence of peptides (Figure 1). Further, reduction of ferric heme–peptide complexes to ferrous forms has caused a red shift of the Soret band to 430 nm and a resolved band at 560 nm (Supporting Information, Figure S2). These spectral changes of cofactor are diagnostic of the bishistidine–heme ligation with the heme containing six-coordinate low-spin Fe^{III}.[45,46] In other words, a single heme moiety could be sandwiched between two molecules of peptides for a bishistidine-type ligation. The apparent equilibrium dissociation constant ($K_{d,app}$) values and binding stoichiometry of heme–peptide interactions were estimated from the absorbance change at the Soret band using Hill plots (Supporting Information, Figure S3). Peptide–heme binding is cooperative, whereby two molecules of peptides are required to coordinate with the heme moiety (Supporting Information, Figure S3). Further, IV8 and IV8FA bind to heme with similar affinity: 5.02 ± 0.2 μM and 3.0 ± 0.3 μM, respectively, and with a 2:1 peptide/heme stoichiometry determined from a Job plot[47] (Supporting Information, Figures S3, S4). The heme binding affinity of the designed peptides were further examined at high detergent concentrations, micelle-free solvent, in acetonitrile, and in denaturing solution containing guanidinium hydrochloride (Supporting Information, Figure S5). Peptides were found to contain significantly reduced heme binding affinity under these conditions, as suggested by the absence of heme absorption maxima at 412 nm. Furthermore, very few NOEs were detected for IV8 in deuterated actonitrile solution, indicating a lack of folded conformations (Supporting Information, Figure S5). These observations plausibly imply important roles of the micelle environments for the function and structure of the designed peptides.

The activities of IV8 and IV8FA as peroxidases were examined following oxidation of an organic substrate 2,2′,5,5′-tetramethylbenzidine (TMB) in the presence of hydrogen peroxide.[48] The product formation is monitored following the formation of TMB-ox at 450 nm. Figure 2 shows a representative time course of TMB oxidation by IV8 and IV8FA in detergent solution. IV8 and IV8FA are both able to catalyze peroxide-dependent oxidation of TMB; however, with a slightly different rate (Figure 2). Neither the heme nor IV8 independently show any detectable peroxidase activity (Figure 2). The mutant peptide IV8FA also did not show peroxidase activity in the absence of cofactor (data not shown). Peroxidase activity was estimated, in terms of specific velocity (v_o/[heme]), to be 1620 and 1363 $Abs_{450}S^{-1}M^{-1}$ for IV8 and IV8FA, respectively. In a previous study, helical TM domain of glycophorin A had been engineered by minimal sequence modification, creating a heme binding site through bishistidine ligation.[13,49] The derived protein or ME1 interacted with heme and showed peroxidase activity in DPC micelles. Under similar conditions, ME1 exhibited a value of 2300 $Abs_{450}S^{-1}M^{-1}$ for TMB oxidation.[13] In an another study, heme-binding helical proteins from a designed library were screened using TMB as a substrate. The best catalyst in the library, protein 86, showed a value of 1600 $Abs_{450}S^{-1}M^{-1}$.[50] Remarkably, β-hairpins IV8 or IV8FA possess comparable peroxidase activity with ME1 and protein 86, despite the much shorter length.

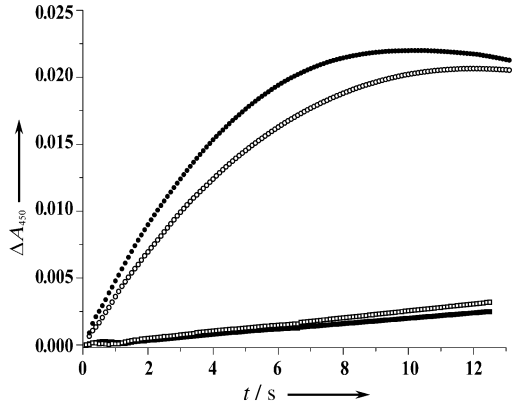

Figure 2. TMB oxidation as a function of time by peptide–heme complexes in DPC (1.5 mM) containing sodium phosphate buffer solution (pH 7.4). Enzymatic assays were carried out with complexes of 2 μM heme and 4 μM of each peptide (● IV8, ○ IV8FA) and also for only heme (■, 2 μM) or only IV8 (□, 4 μM with 10 μM TMB and hydrogen peroxide 10 mM (see the Supporting Information).

Far-UV CD and FTIR spectroscopy were used to probe secondary structures of the designed peptides in DPC micelles. Both peptides showed a negative CD band and a positive CD band at 212 nm and 225 nm, respectively (Supporting Information, Figure S6). Model β-hairpin peptides with aromatic (and especially Trp) residues have been observed to yield similar CD spectra arising from exciton coupling contribution of Trp side chain.[21,23,40] There were no significant changes in the CD spectra of IV8 and IV8FA in the presence of cofactor, indicating peptides do not undergo any major conformational rearrangement in complex with heme (Supporting Information, Figure S6). Further, binding of heme with the designed peptides produces an induced CD band for heme at the Soret region of the absorption spectra, indicating that the heme is experiencing a chiral environment (Supporting Information, Figure S7). FTIR studies indicate β-sheet conformations of the designed peptides (Supporting Information, Figures S8, S9). The strong IR band, in the amide I region, either at 1642 cm^{-1} or 1638 cm^{-1} for IV8 and IV8FA, respectively, are characteristic of a β-sheet structure.[51] IR spectra were largely invariant upon addition of heme, indicating similar structures for the apo and holopeptides. The β-hairpin structures of the designed peptides are further confirmed by NMR spectroscopy.

Addition of heme has caused severe line broadening of NMR signals of the designed peptides (Supporting Information, Figure S10). On the other hand, NMR spectra of IV8 have been found to be unperturbed in the presence of protoporphyrin IX, indicating probable lack of interactions between metal-free porphyrin with the peptide (Supporting Information, Figure S11). By contrast, well-dispersed NMR spectra can be seen for IV8 and IV8FA in DPC micelles in the absence of heme (Figure 3). Therefore, 3D structures, micelle insertion, and localization of the peptides have been characterized by NMR spectroscopy in DPC micelles in absence of

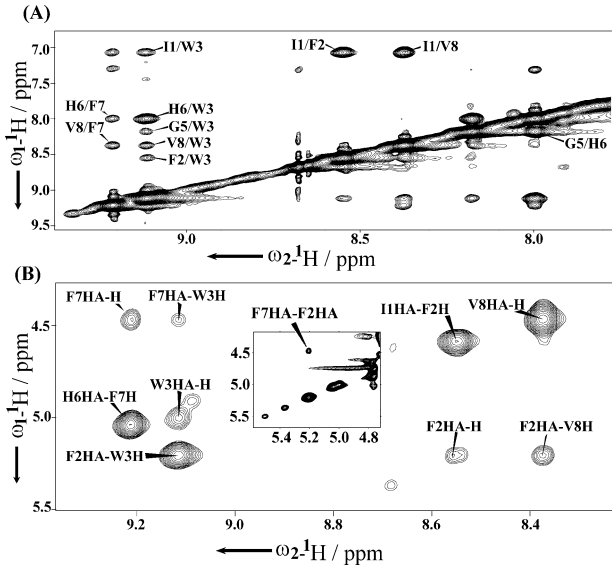

Figure 3. Selected regions of two-dimensional NOESY spectra of IV8 in solution of DPC micelles showing NOE connectivites, among the downfield shifted amide protons (panel A), among the amide protons with $C^{\alpha}H$ protons (panel B), and $C^{\alpha}H$ protons of residues F2 and F7 (panel B, inset).

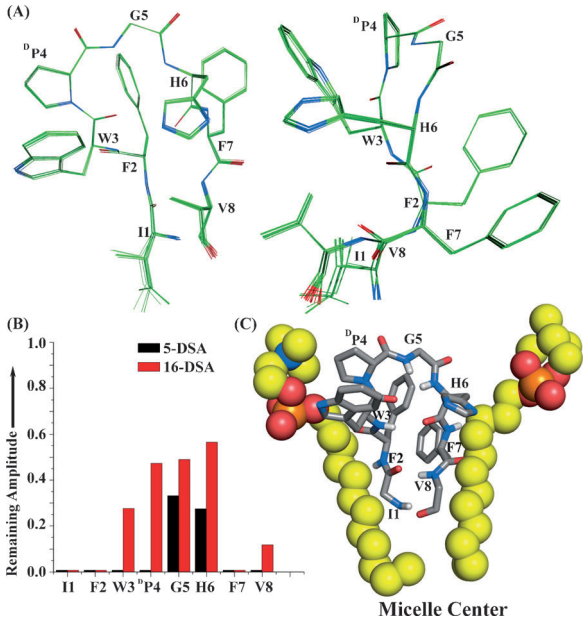

Figure 4. A) Superposition of twenty lowest-energy structures of IV8 in different orientation. B) Bar diagram showing remaining amplitude of backbone resonances of IV8 in the presence of 2 mM spin-labeled doxyl lipids. C) Plausible localization of IV8 with respect to acyl chains of DPC micelles. Only two molecules of DPC are shown to indicate an inserted orientation of the peptide in micelles.

heme. Sequence-specific resonance assignments were achieved by combined analyses of two-dimensional ^{1}H–^{1}H TOCSY and NOESY spectra (Supporting Information, Tables S1 and S2). Notably, chemical shifts of CαH resonances of residues F2, W3, and H6 are found to resonate far downfield (ca. 5.2 to 5.00 ppm), expected for β-sheet or β-hairpin structures[52] (Supporting Information, Tables S1 and S2). Further, chemical shifts of the two beta protons of residue ^{D}P4 are non-degenerate, which is presumably due to the ring current effect from the aromatic side chain of residue Trp3 (Supporting Information, Tables S1 and S2). Analyses of NOESY spectra of IV8 and IV8FA reveal diagnostic long-range NOEs involving backbone NH and $C^{\alpha}H$ resonances (Figure 3 A,B; Supporting Information, Figure S12). Specifically, backbone-mediated long-range NOEs are observed between residues I1NH/V8NH, W3NH/H6NH, W3NH/V8NH, F2 or A2 $C^{\alpha}H$/V8NH, F7 or A7$C^{\alpha}H$/W3NH and F2 or A2$C^{\alpha}H$/H6NH (Figure 3 A,B; Supporting Information, Figure S12). Further, an NOE is observed between F2$C^{\alpha}H$/F7$C^{\alpha}H$ (Figure 3 B, inset). Owing to overlapping resonances, A2$C^{\alpha}H$/A7$C^{\alpha}H$ NOE could not be detected for IV8FA peptide. Furthermore, in case of IV8 peptide, a number of long-range NOE contacts are detected for residues F2/V8, F2/F7 ,and W3/V8 involving side chain–backbone and/or side chain/side chain resonances. Residues ^{D}P4 and G5 delineate only sequential and medium-range NOEs. The N-terminal myristoyl chain showed fewer NOE contacts, only with residue I1, and was not included in the structure calculation. Ensembles of structures of IV8 and IV8FA have been determined based on NOE driven distance and dihedral angle restraints using Cyana (see the Supporting Information). A summary of the structural statistics is given in the Supporting Information, Tables S4 and S5. Both peptides assume β-hairpin structure in DPC micelles (Figure 4; Supporting Information, Figure S13). The β-hairpin structures of IV8 or IV8FA are determined by a type II′ β-turn centering at ^{D}P4-G5 residues. The backbone dihedral angles (ϕ, ψ) of residues I1-W4 and residues H6 V8 remain in extended or β-sheet conformations (Supporting Information, Tables S5 and S6). The side chains of residues W3, ^{D}P4, and H6 are in close proximity at the one face of β-hairpin (Figure 4 A). The phenyl rings of residues F2 and F7 of IV8 are involved in cross-strand packing interactions, showing an edge-face orientation, at the opposite face of the hairpin conformation (Figure 4 A). On the other hand, CH_3 groups of A2 and A7 are in proximity in the β-hairpin structure of IV8FA (Supporting Information, Figure S13).

Localization and micelle interactions of IV8 and IV8FA peptides have been probed by detergent–peptide NOEs, amide proton exchange, or H/D exchange, with solvent D_2O and resonance perturbations by the spin-labeled doxyl lipids, 5-doxyl stearic acid (5-DSA), and 16-doxyl stearic acid (16-DSA). NOE contacts can be identified for DPC protons, C_6H_2–$C_{14}H_2$, at 1.1 ppm, with the aromatic ring protons of residues F7/F2, H6, and W3 of IV8 (Supporting Information, Figure S14). Intermolecular NOE contacts are also observed for IV8FA involving W3 and H6 with the acyl chains of DPC micelles (Supporting Information, Figure S14). These data indicate a close proximity of the aromatic side chains of peptides with the acyl chains of detergent micelles. In H–D exchange NMR, amide protons of residues I1, F2, W3, and V8 demonstrate a slow exchange with solvent (Supporting Information, Figure S15). Backbone amide resonances of residues I1, A2, and V8 are found to be retained for IV8FA peptide (Supporting Information, Figure S15). For spin-

labeled studies, two-dimensional TOCSY spectra were obtained either in the absence or presence of 2 mM doxyl lipids, and intensity changes of backbone $C^{\alpha}H/NH$ correlations or remaining amplitude (RA) are estimated for IV8 (Figure 4B) and IV8FA (Supporting Information, Figure S13). Note that changes in $C^{\alpha}H/C^{\beta}H_2$ cross-peak intensity have been considered for ^{D}Pro residue. Residues I1, F2, W3, F7, and V8 contain a lower RA value in 5-DSA and 16-DSA in comparison to the other residues (Figure 4B). These observations would imply that the residues I1, F2, W3, F7, and V8 are inserted into the hydrophobic region of micelles and are plausibly be located above the micelle center.[53,54] Residue W3 might have a somewhat lower depth of insertion as it has experienced a lesser perturbed by 16-DSA (Figure 4B). Further, D_2O exchange protection, as discussed above, of these amide protons, except for F7, would support a largely nonpolar micelle environment with decreased solvent accessibility.[55] A rather rapid exchange of the amide proton of F7 may be occurring owing to a local dynamical fluctuation of the micelle–peptide complex at that region.[55] Residues ^{D}P4, G5, and H6 are perturbed largely by 5-DSA (Figure 4B) and also showed a fast exchange of amides, indicating their localization at the micelle surface. Taken together, the aforementioned results demonstrate that residues of the β-strands of the hairpin are inserted into nonpolar core of DPC micelles, whereas residues at the tip of the hairpin or at the β-turn seems to be localized in the proximity to the micelle–water interface or polar environment of the micelles (Figure 4C). For IV8FA, 5-DSA demonstrated a higher perturbation of resonances in comparison to 16-DSA (Supporting Information, Figure S13). 16-DSA induced perturbation can be seen only for residues I1, A2, A7, and V8 of IV8FA. Also, residues I1, A2, and V8 are protected against exchange with D_2O. In other words, the β-hairpin structure of IV8FA appears to be maintaining a relatively shallower penetration into DPC micelles in comparison to IV8.

In conclusion, we have characterized, functionally and structurally, designed β-hairpin peptides in detergent solution. This is the first report of designed β-hairpin structures engaged in heme binding and performing peroxidase activity. Our studies show that the designed acylated octapeptides are inserted into DPC micelles with the β-turn of the hairpin is located in proximity to the micelle head groups, whereas the residues at the β-strands are into the hydrophobic milieu of the micelles. The observed organization of the β-hairpins seems to be typically mimicking the β-hairpins or β-sheets of membrane proteins.[16,17] Further, interfacial localization of the β-turn residues is likely to be responsible for a facile His-heme coordination and peroxidase activity. Our results also demonstrate that the β-hairpin folding and activity in micellar environment can be sustained in the absence of potential stabilizing interactions, through aromatic packing, between the β-strands. This observation is in marked contrast with the structures of autonomously folded β-hairpins in water, whereby replacement of interstrand aromatic interactions found to be highly destabilizing.[37,38] Thus, these results suggest that a larger variation in side chain topology can be plausibly utilized for designing of β-hairpins in membrane mimicking environment. Furthermore, novel multi β-strand TM proteins of high-affinity cofactor binding and catalytic activity could be developed using the current model system as a starting template.

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