Peptidomimetics

A Peptide Flavoprotein Mimic: Flavin Recognition and Redox Potential Modulation in Water by a Designed β Hairpin**

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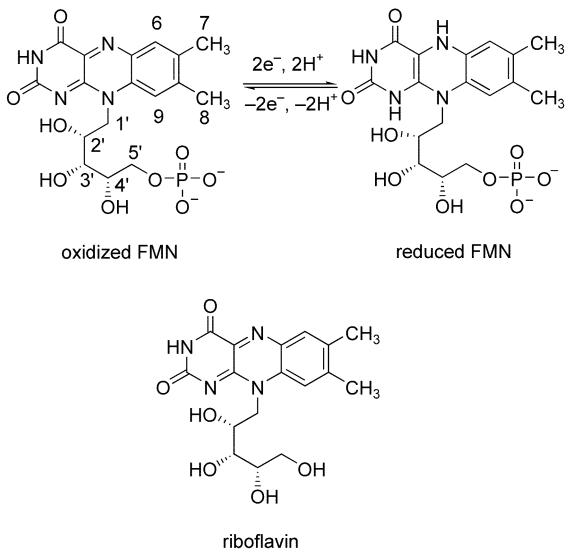
The design of miniature proteins that demonstrate both structure and function is an important goal with chemical and biomedical implications.[1] Redox systems are particularly attractive targets for these studies, because of the diversity and importance of redox-mediated processes. In the field of de novo protein design, much effort has gone into the development of designed proteins that bind metals and hemes as metalloenzyme mimics for catalytic applications.[1a] Designed proteins and peptides that incorporate cofactors including pyridoxal, thiamine, nicotinamide, and flavin functionalities have also been developed, but all of these systems feature covalent attachment to the polypeptide.[1b] Although covalent incorporation of cofactors has led to novel functional properties in designed systems, recent studies suggest that protein binding of a cofactor plays a more significant role than simply bringing it in proximity to the substrate. For example, studies of flavoenzymes have shown that noncovalent interactions are essential not only for regulating cofactor recognition, but also for tuning the redox properties of the flavin cofactor and thus controlling the overall catalytic activity of the enzyme.[2] The shift in reduction potential of flavin mononucleotide (FMN) upon binding to flavodoxin is believed to result in part from stacking interactions between the flavin ring and aromatic side chains in the protein; these interactions disfavor the two-electron reduction to the hydroquinone form of the cofactor (Scheme 1).[2a–c] This shift to a more negative potential has been described in synthetic model systems[2e,f] and counteracts the effects of hydrogen bonding, which has been shown to increase flavin reduction potentials.[2d] In addition, the presence of charged residues has been shown to influence the redox potential of FMN. For example, basic residues in the FMN binding pocket of flavodoxin have been shown to stabilize the anionic hydroquinone.[2a]



Scheme 1. Structures of the flavin cofactors and the two-electron reduction of FMN.

Given the interplay between noncovalent interactions and flavoprotein redox activity, an ideal designed flavoenzyme would incorporate a binding site for a flavin cofactor, thereby allowing modulation of the flavin redox properties through specific interactions with preorganized side chains. To this end, we have designed a 12-residue β-hairpin peptide that binds FMN strongly in water and demonstrates many of the same features as are seen in flavoproteins, including modulation of the flavin redox potential.

The recently reported peptide receptor **1**[3] has been shown to be a well-folded β hairpin.[4] The peptide sequence incorporates two Trp residues in a diagonal orientation on one face of the β hairpin; this forms a binding cleft for aromatic guests. In addition, two Lys residues were incorporated on the same face of the hairpin to allow favorable electrostatic interactions with FMN.

Ac-Arg-**Trp**-Val-**Lys**-Val-Asn-Gly-Orn-**Trp**-Ile-**Lys**-Gln-NH_2
Peptide **1**
(Bold lettering indicates the residues on the binding face of the *β*-hairpin)

Titration of peptide **1** with FMN, monitored by using NMR, UV/Vis, and fluorescence spectroscopy, demonstrates that the flavin ring intercalates between the two Trp residues. The addition of approximately ten equivalents of FMN to peptide **1** produced a 0.3 ppm upfield shift of the signals for the H-5 protons of both Trp residues of the hairpin in the NMR spectra; this result indicates a stacking interaction involving both Trp side chains.[5] Furthermore, the signals for the nonexchangeable flavin aromatic ring protons were upfield shifted substantially in the presence of the hairpin, thus supporting the participation of stacking interaction in

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binding (Figure 1). Upfield shifting of the FMN sugar proton signals was also observed and could be indicative of CH–π interactions with the Trp rings. However, the magnitude of the shift of the sugar proton signal decreases with increasing

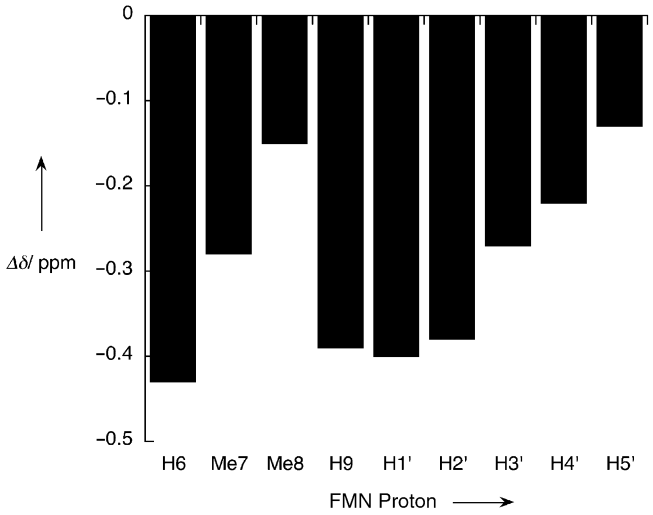

Figure 1. Change in the chemical shift values of the signals for nonexchangeable FMN protons in the presence of one equivalent of peptide **1**.

distance from the flavin ring; this suggests that the shift is a consequence of its proximity to the flavin intercalation site.

In the UV/Vis spectrum, the FMN band at 373 nm is blue-shifted by approximately 4 nm at binding saturation, which indicates that the flavin ring is in a more hydrophobic environment when it is bound by the peptide.[6] The flavin band at 446 nm also demonstrates hypochromicity in the presence of peptide **1**, as has been observed for other flavins participating in π–π stacking interactions.[7] In addition, quenching of the FMN fluorescence upon titration with peptide **1** also denotes an interaction between the Trp side chains and the flavin ring (Figure 2).[8]

NMR studies indicate that FMN is bound to the peptide in its folded state. Cross-strand NOE interactions are apparent in peptide **1** in the presence and absence of FMN, thus indicating that the peptide maintains a β-hairpin structure when FMN is bound (Figure 3a). In addition, the chemical-shift difference between the glycine diastereotopic protons in the turn is unchanged in the presence of FMN ($\Delta\delta$Gly = 0.74 ppm for both free and complexed peptide **1**); this suggests that no significant structural change occurs upon binding.[9] Upfield shifts of the signals for the α protons of peptide **1** were observed upon FMN binding, presumably because of the proximity of the flavin ring as opposed to destabilization of the β hairpin,[10] since cross-strand NOE interactions and $\Delta\delta$Gly values indicate that the peptide is well-folded in the presence of FMN. Moreover, the greater shifting of the signals for the Trp and Lys side-chain protons in the presence of excess FMN as compared to those for other residues suggests that FMN binds on this face of the β hairpin (Figure 3b). Taken together, these results indicate that FMN is binding to the peptide in the folded conformation, and the

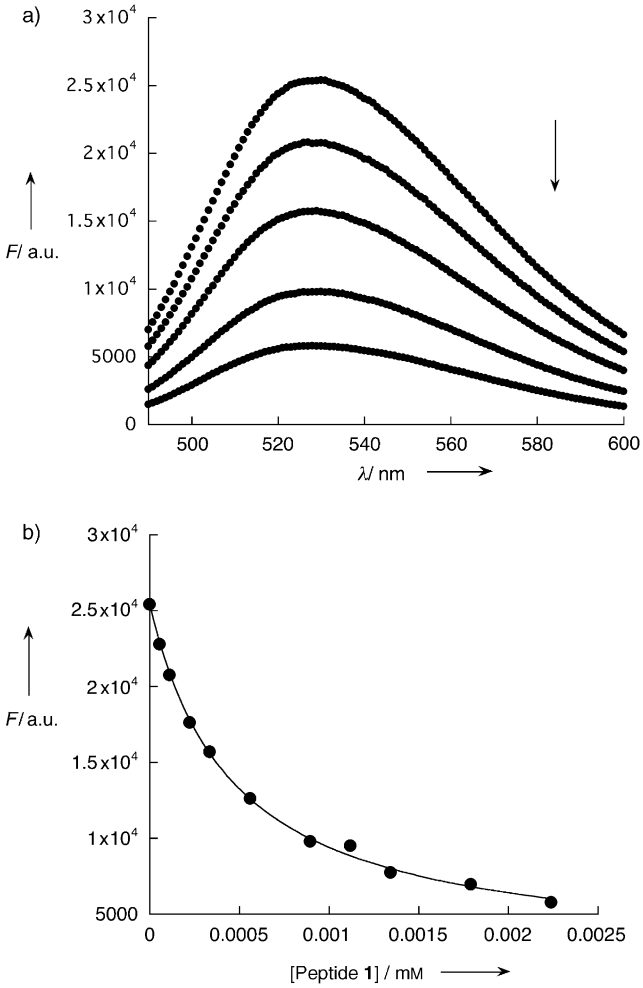

Figure 2. a) FMN fluorescence quenching in the presence of increasing concentrations of peptide **1** in 10 mM acetate buffer (pH 5.0) at 25 °C ([FMN] = 37 μM, [peptide] = 0–2.25 mM). The arrow indicates the direction of change in fluorescence intensity with increasing peptide concentration. b) FMN fluorescence at 530 nm versus the concentration of peptide **1** ([FMN] = 37 μM). The line through the data represents the best fit to a 1:1 binding equation. a.u. = arbitrary units.

cofactor interacts directly with both Trp residues on one face of the hairpin.

The binding affinity of FMN was quantified by using fluorescence quenching of the flavin. Fitting of the fluorescence quenching data as a function of peptide concentration to a 1:1 binding equation gave an association constant (K_{assoc}) of 2200 M^{-1} (Table 1), with a 1:1 binding ratio demonstrated by a Job plot by NMR spectroscopy. Variation of the FMN concentration by a factor of ten does not significantly influence the observed association constant, which suggests that higher order aggregates are unlikely. The designed hairpin and FMN therefore exhibit a strong interaction in water through noncovalent interactions of FMN with the side-chains of the peptide in the folded state.[11]

The difference in binding affinities between FMN and riboflavin (K_{assoc} = 315 M^{-1}; Table 1) indicates that electrostatic interactions involving the FMN phosphate group contribute approximately −1 kcal mol^{-1} to FMN binding,

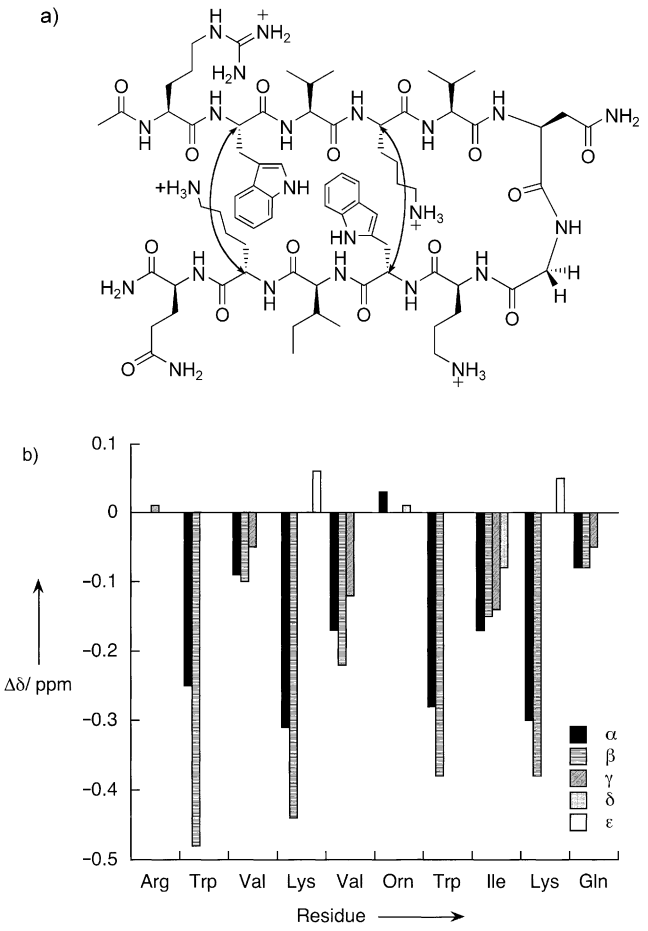

Figure 3. a) Cross-strand NOE interactions observed in the presence of ten equivalents of FMN. b) Change in side-chain proton chemical shifts for peptide **1** in the presence of ten equivalents of FMN, with turn residues (Asn–Gly) omitted ($\Delta\delta H_\alpha = \delta H_{\alpha,10FMN} - \delta H_{\alpha,0FMN}$). Measurements were made in 10 mM [D_3]acetate buffer (pH 5.0, uncorrected) at 25 °C. α–ε indicate the position on the sidechains. The γ and δ protons on Lys4 and Lys9 could not be unambiguously.

Table 1: Association and dissociation constants (K_{assoc} and K_d) for flavin recognition by peptide **1**.[a]

Flavin substrate	[KCl] [mM]	K_{assoc} [M^{-1}]	K_d [mM]	$\Delta G_{binding}$ (error) [kcal mol^{-1}][b]
FMN	0	2200	0.45	−4.6 (0.1)
riboflavin	0	310	3.2	−3.4 (0.1)
FMN[c]	100	720	1.4	−3.9 (0.1)
$FMNH_2$[c]	100	40	25	−2.2

[a] In 10 mM acetate buffer (pH 5.0) at 25 °C, determined by fluorescence titrations unless otherwise stated. [b] Errors determined by the average deviation between two to three separate titration experiments. [c] In 10 mM phosphate buffer with 100 mM KCl (pH 5.0) at 25 °C. The association constant for $FMNH_2$ was determined from electrochemical measurements (see Supporting Information).

which is in agreement with electrostatic contributions to adenosine triphosphate (ATP) recognition by peptide **1**.[3] The electrostatic contribution probably arises from interactions between the FMN phosphate group and the Lys side chains. If the recognition of riboflavin by peptide **1** is driven by aromatic interactions alone (that is, in the absence of hydrogen bonds or CH–π interactions), then the flavin–tryptophan interaction contributes approximately −3.4 kcal mol^{-1} to FMN recognition, which is nearly twice the strength of the adenine–tryptophan interaction ($\Delta G = -1.8$ kcal mol^{-1}) estimated previously for ATP recognition by peptide **1**.[3] The strength of the flavin–tryptophan interaction in our system is similar in magnitude to previously measured π–π stacking interactions involving flavin rings in water.[7a] The strong aromatic interaction between flavin and the Trp side chains is not surprising given the large π surface area of flavin, but can also be attributed to favorable electrostatic overlap between the electron-rich Trp side chains with the electron-poor flavin ring.

Electrochemical studies were performed to determine the influence of peptide binding on the FMN reduction potential. Square wave voltammetry measurements show a shift of FMN reduction potentials to more negative values with increasing peptide concentration (Figure 4). At binding

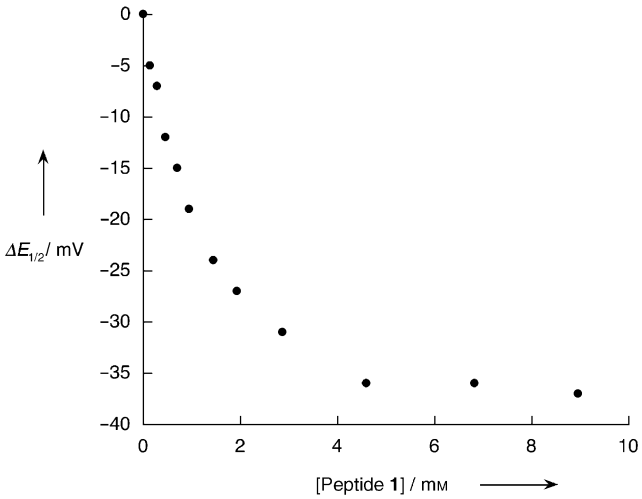

Figure 4. Change in reduction potential (*E*) with added peptide ($\Delta E_{1/2} = E_{FMN,bound} - E_{FMN,free}$).

saturation, $\Delta E_{1/2}$ is −38 mV, which indicates that the bound flavin ring is more difficult to reduce to the $FMNH_2$ hydroquinone by 1.7 kcal mol^{-1}, relative to unbound FMN. This result is consistent with previous investigations of the influence of aromatic interactions on the reduction potential of flavin. McCormick and co-workers have shown that peptides with intramolecular stacking interactions between flavin and Trp show slowed rates of FMN reduction compared to uncomplexed flavin,[12] while Breinlinger and Rotello have shown that flavin stacking with anthracene shifts the potential by −63 mV in chloroform.[2e] The contribution of aromatic stacking is substantial in peptide **1**, as a cationic environment has been shown to *increase* the redox potential of FMN, thereby counteracting the modulation provided by the stacking interactions.[1b] Although the perturbation of the FMN redox potential can be of the order of hundreds of millivolts in naturally occurring flavoproteins, stacking interactions typically only contribute a fraction of the shift, with the rest

resulting from solvent exclusion as well as proximity to several negatively charged side chains in the binding site.[2] The magnitude of the negative potential shift in our system is substantial given that it results solely from aromatic interactions at a solvent-exposed location in a cationic peptide.

From the change in reduction potential of FMN in the presence of peptide **1**, the binding affinity of the reduced FMN is calculated[13] to be $40\,\text{M}^{-1}$ in 100 mM KCl, a approximately 18-fold drop from the $720\,\text{M}^{-1}$ value observed for the oxidized flavin under the same conditions (Table 1). This finding further emphasizes the role of the electrostatic component of aromatic–aromatic interactions in regulating flavin redox processes. Favorable interactions exist between the electron-poor oxidized flavin and the electron-rich Trp side chains. These interactions are attenuated upon reduction of the flavin to the electron-rich hydroquinone form, thereby reducing the strength of the peptide–flavin interaction. The observation of an electrostatic effect on FMN binding indicates that electrostatics are a major contributor to aromatic stacking interactions, even in water.[14]

This is the first report of a designed, structured peptide with a noncovalent flavin binding site. Our results indicate that diagonal Trp residues in the non-hydrogen-bonding sites of a model β hairpin create a pre-organized recognition cleft for flavin cofactors. The electrostatic nature of the stacking interactions in the peptide–FMN complex perturbs the flavin reduction potential such that two-electron reduction to the reduced hydroquinone is disfavored relative to free FMN, as observed in many flavoenzymes. While synthetic receptors have been shown to both complex flavin and shift its redox potential, to our knowledge there have been no reports of designed systems that accomplish both of these feats in water. Catalysis by this redox-active miniature protein with a bound FMN cofactor will be explored in the future.

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