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Model systems for flavoenzyme activity: an electrochemically tuneable model of roseoflavin†

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The electrochemically tuneable hydrogen bonding interactions between roseoflavin analogue 2 and apoenzyme mimic 3 are described.

Although the biological function of roseoflavin (**1**) is largely unknown,[1] it has been suggested that the dimethylamino group attached to the 8-position of the flavin is likely to play a key role in defining its function.[2] For example, the electron donating nature of this functional group is responsible for the relatively high reduction potential of this flavin. Moreover, it has been postulated that protonation by an acidic residue of an associated apoenzyme could significantly decrease the reduction potential of the flavin, by transforming the electron donating amino group (*e.g.* Hammett σ value for a *p*-NH_2 is -0.66) to an electron withdrawing ammonium group (*e.g.* Hammett σ value for a *p*-NH_3^+ is +0.60) (Fig. 1).

Although little is known about the binding site of roseoflavin in biological systems, it has been shown that the mononucleotide analogue of **1** can be reconstituted into the apoenzyme of flavoprotein *Aerococcus viridans*, resulting in a significant positive shift of its half-wave potential ($E_{\frac{1}{2}}$) compared to that of the free flavin.[3] Furthermore, it has been shown using competition studies with labelled riboflavin, that roseoflavin has the propensity to compete for the membrane binding site of *Phycomyces blakesleeanus*.[4] Model systems are likely to provide a valuable insight into how an associated apoenzyme could modulate the properties of this flavin, by reducing the complexity of the natural system and thereby allowing specific interactions to be probed in a systematic manner. For example, roseoflavin models have been prepared in order to investigate the role solvent polarity has in modulating the reactivity of these systems.[5] Although these investigations have indicated that roseoflavin may become activated when bound to the hydrophobic pocket of an enzyme, the role more specific interactions (*e.g.* hydrogen bonding) have in controlling the redox properties of this unit has not been reported.

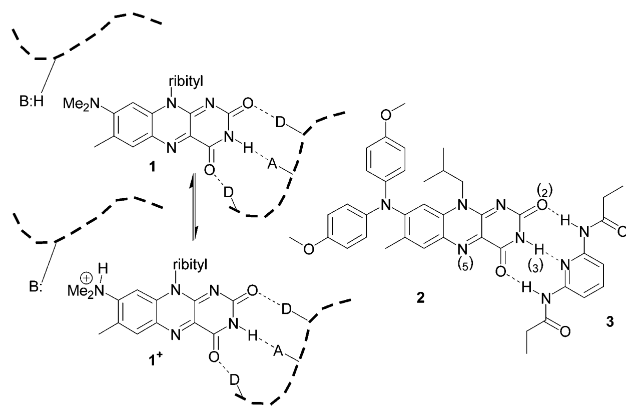

Fig. 1 Schematic representation of the possible interactions between roseoflavin **1** and an apoenzyme and structural formulae of model compounds **2** and **3**.

In systems that contain more conventional flavin cofactors (*e.g.* riboflavin), it has been shown that hydrogen bonding interactions between the apoenzyme and the cofactor play an important role in modulating the redox properties of the latter.[6] Previously, it has been shown that diamidopyridine derivatives may be used as simple yet informative mimics of the apoenzyme of flavoproteins.[7] These receptors recognise the flavin through three-point hydrogen bonding to the imide moiety of the cofactor in a manner reminiscent of most natural flavoproteins (*e.g.* lipoamide dehydrogenase). Here, we report the role molecular recognition has in modulating the redox properties of roseoflavin analogue **2**. The triarylamino moiety of **2** was selected for this study in view of its structural similarity to the dimethylamino unit of **1**, and its well-documented ability to undergo electrochemical oxidation to yield a stable radical cationic species.[8] This electrochemical transformation will allow us to convert the electron donating triarylamino species in the 8-position to an electron withdrawing radical cation species *via* electrochemical umpolung. This will allow us to conveniently mimic the changes in the electronic properties of the nitrogen in the 8-position of the flavin following protonation by an apoenzyme, thereby allowing a facile method for probing how this process could modulate hydrogen bonding to an associated apoenzyme.

Roseoflavin analogue **2** was synthesised from 8-chloro-7-methyl-10-isobutylflavin[9] and 4,4′-dimethoxydiphenylamine using a previously reported Ni-catalysed amination procedure.[10] Hydrogen bonding between **2** and **3** was confirmed using ^1H NMR spectroscopy. Addition of aliquots of receptor **3** to a solution of **2** in $CDCl_3$ resulted in a smooth downfield shift in the resonance of H(3) of the flavin. The resulting curve was fitted to a 1 : 1 binding isotherm and gave a binding constant of 877 M^{-1} for the complex (see ESI†). This value is in accordance with our recently reported NMR titration data for **3** and flavins bearing strongly electron donating substituents attached to their 8-position.[9]

Cyclic voltammetry (CV) measurements were performed on **2** in order to investigate the formation of its radical cation ($\mathbf{2_{rad}}^+$) and radical anion ($\mathbf{2_{rad}}^-$) states. In order to mimic the non-polar environment of a typical flavoenzyme binding pocket, CH_2Cl_2 was used as solvent for these measurements.[7] Firstly, the electrochemical oxidation of the triarylamino unit was investigated and revealed the irreversible formation of two oxidation waves and at least two re-reduction waves (see ESI†). Although it has been established that triphenylamine radical cations readily undergo radical coupling reactions through their *para* positions to form tetraphenylbenzidine units,[11] in our system this process will be largely prevented by the *para* methoxy groups and the N(5) nitrogen of the flavin unit.[12] Therefore, we assume that the second oxidation wave observed at a higher potential is due to further oxidation processes of the $\mathbf{2_{rad}}^+$ species. However, when the CV measurements were switched at a potential before the onset of the second oxidation wave (*e.g.* +1.3 V *vs.* Ag/AgCl), a pseudoreversible oxidation wave ($E_{\frac{1}{2}}$ = +1.03 V *vs.* Ag/AgCl) was observed, which is presumably due to the formation of $\mathbf{2_{rad}}^+$ (see ESI†).[13] We have investigated the formation of $\mathbf{2_{rad}}^+$ using UV-vis spectroelectrochemistry measurements performed on **2** in CH_2Cl_2 (see ESI†). For the neutral flavin **2**, an absorption in the visible region centred around λ = 536 nm was observed. However, upon electrochemical

† Electronic supplementary information (ESI) available: synthesis of **2**, CV and spectral data. See http://www.rsc.org/suppdata/cc/b4/b401470g/

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oxidation of the solution this absorption became largely suppressed, and a new absorption at 830 nm gradually appeared, which can be assigned to the formation of $\mathbf{2}_{\mathbf{rad}}^{+}$.[14]

Upon reduction of the flavin, a single reduction wave and two distinct reoxidation waves were observed. The reduction and first oxidation waves are due to the reversible formation of $\mathbf{2}_{\mathbf{rad}}^{-}$ ($E_{\frac{1}{2}}$ = −0.87 V *vs.* Ag/AgCl), whereas the second reoxidation wave (E = −0.53 V *vs.* Ag/AgCl), arises from an electrochemical–chemical–electrochemical (e–c–e) process where a portion of the $\mathbf{2}_{\mathbf{rad}}^{-}$ formed at the electrode surface rapidly deprotonates **2** in the bulk medium (see ESI†). The protonated flavin radical ($\mathbf{2}_{\mathbf{rad}}\mathbf{H}$) produced in this process undergoes a further one-electron reduction at the working electrode surface to form the relatively stable fully reduced flavin anion ($\mathbf{2}_{\mathbf{red}}\mathbf{H}^{-}$), which is subsequently reoxidised at a less negative potential than $\mathbf{2}_{\mathbf{rad}}^{-}$.[15]

With the electrochemistry of the isolated roseoflavin analogue studied, we next turned our attention to investigating the role hydrogen bonding interactions to apoenzyme analogue **3** have in modulating the redox properties of **2**. Square wave voltammetry (SWV) performed on **2** before and after the addition of excess **3** is shown in Fig. 2, and reveals a +25 mV shift in the oxidation potential of **2** when an excess complementary guest **3** was added. This positive shift indicates over a two-fold decrease in the efficiency of the hydrogen bonded complex upon oxidation of the triarylamine moiety (K_a = 331 M^{-1}), and corresponds to a 2.4 kJ mol^{-1} destabilisation of the $\mathbf{2}_{\mathbf{rad}}^{+}$ state.[16] The addition of **3** to a solution of flavin **2** displaced the redox wave corresponding to the formation of $\mathbf{2}_{\mathbf{rad}}^{-}$ by +100 mV, which indicates a 50-fold increase in the binding efficiency (K_a = 43067 M^{-1}), and corresponds to a 9.6 kJ mol^{-1} stabilisation of the $\mathbf{2}_{\mathbf{rad}}^{-}$ state (Fig. 3). An interesting

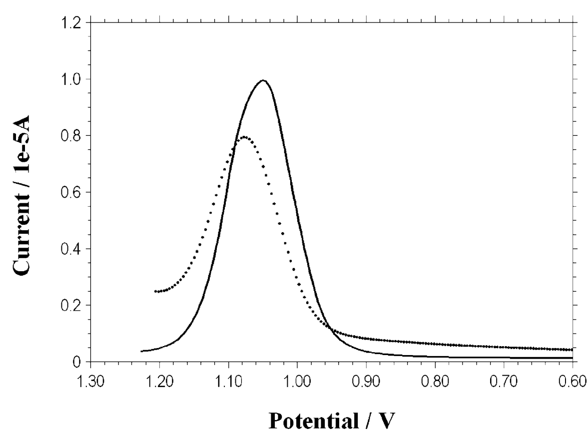

Fig. 2 SWV of **2** (—) (~7 × 10^{-4} M) and upon the addition of an excess of **3** (···) (~7 × 10^{-2} M).

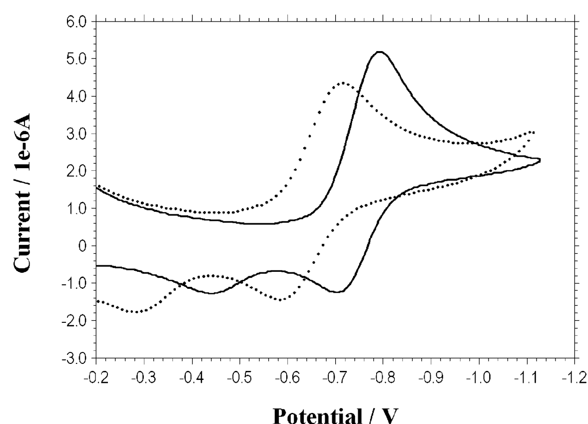

Fig. 3 CV of **2** (—) (~7 × 10^{-4} M) and upon the addition of an excess of **3** (···) (~7 × 10^{-2} M).

feature of the CV upon electrochemical reduction of **2** in the presence of excess **3**, is the non-suppression of the redox wave corresponding to the aforementioned e–c–e process. Usually this redox wave disappears when electron rich flavins are electrochemically reduced in the presence of excess **3**.[9] However, for the present system the wave is typically shifted by +160 mV and shows a marked increase in current intensity. This is likely due to the influence of the triarylamino unit increasing the pK_a of the N(5) of $\mathbf{2}_{\mathbf{rad}}^{-}$ to such an extent that it is sufficiently basic to deprotonate the weakly acidic guest **3**.

In conclusion, the electrochemical reduction of roseoflavin analogue **2** in the presence of excess **3**, results in a significant decrease in the reduction potential of the flavin, corresponding to an increase in the binding efficiency of the host–guest complex. On the other hand, when the electrochemical oxidation of the triarylamino moiety of **2** was recorded in the presence of excess **3**, a positive shift in the oxidation potential was observed, which corresponds to a decrease in the binding efficiency of the hydrogen bonded complex. As the hydrogen bonding efficiency between **2** and **3** can either be increased by reduction, or decreased by oxidation of the flavin, this study paves the way for the implementation of analogues of **2** as novel redox controllable molecular switches and devices, and our investigations towards these goals will be reported in due course.

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