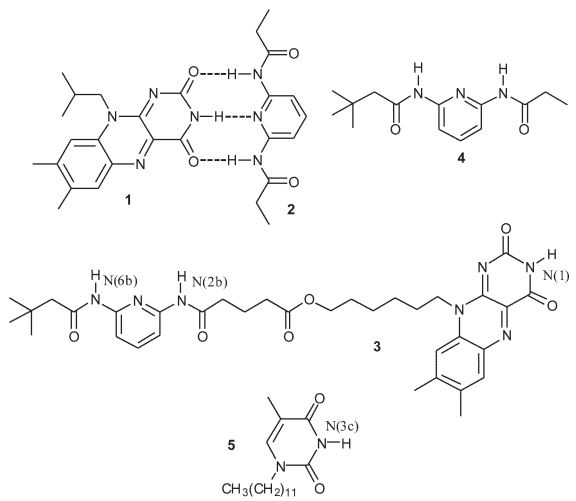
Model systems for flavoenzyme activity: a tuneable intramolecularly hydrogen bonded flavin–diamidopyridine complex†

Alan S. F. Boyd,[a] Joseph B. Carroll,[b] Graeme Cooke,*[a] James F. Garety,[a] Brian J. Jordan,[b] Suhil Mabruk,[a] Georgina Rosair[a] and Vincent M. Rotello[b]

Received (in Cambridge, UK) 8th February 2005, Accepted 14th March 2005
First published as an Advance Article on the web 29th March 2005
DOI: 10.1039/b501887k

We report the electrochemically tuneable intramolecular hydrogen bonding interactions between a covalently linked flavin–diamidopyridine unit.

Flavoenzymes are a ubiquitous series of proteins that utilise the $FADH_2$–FAD redox cycle to catalyse a variety of biological processes such as redox transformations, signal transduction and electron-transfer.[1] In the majority of systems, the flavin cofactor is non-covalently bonded to the apoenzyme, however, there are examples of systems where the flavin unit is covalently bound to the polypeptide backbone. The covalent attachment is usually through the C8 position of the flavin unit, *via* an ether, thioether or imidazolo link to tyrosine, cysteine or histidine moieties of the peptide backbone, respectively.[2] In another example, the flavin cofactor of triethylamine dehydrogenase is covalently attached to the apoenzyme through a C6 thioether link.[3]



Supramolecular interactions (*e.g.* hydrogen bonding and π-stacking) between the flavin and apoenzyme have been implicated in the modulation of the redox properties of the flavin unit by over 500 mV.[4] To reduce the complexity inherent in the biological systems, model systems have been developed so that the effect individual interactions have on tuning the redox potential of the flavin moiety can be probed more directly.[5] For example, Rotello and co-workers have shown that intermolecular hydrogen bonding interactions between isobutylflavin **1** and complementary 2,6-diethylamidopyridine **2**, results in a +150 mV shift in the half-wave potential of the redox wave due to the formation of the flavin radical anion (in CH_2Cl_2), indicating that a significant stabilisation of this state occurs.[5a] Moreover, the shift in redox potential corresponds to a 500-fold increase in the binding efficiency of the host–guest complex upon electrochemical reduction of the flavin unit. Here, we report that compound **3** self-assembles into an intramolecularly hydrogen bonded complex, both in solution and the solid-state. The highly ordered and compact complex is reminiscent of the binding pocket of flavoenzymes where the flavin unit is covalently attached to the apoenzyme, and therefore, provides an attractive model system in order to probe the role intramolecular hydrogen bonding interactions have in controlling the redox properties of the flavin cofactor.

The flavin derivative **3** was synthesised from *N*-(10)-hydroxy-hexyl flavin[6] and 4-[6-(3,3-dimethyl-butyrylamino)-pyridin-2-ylcarbamoyl]-butyric acid using an EDCI–DMAP catalysed esterification procedure (see ESI†). Slow crystallisation of this compound from CH_2Cl_2–ethyl acetate gave rise to crystals suitable for X-ray analysis (see ESI†).‡ The crystal structure is shown in Fig. 1, and clearly shows the 12-atom spacer unit folds to accommodate intramolecular hydrogen bonding interactions between the flavin and the diamidopyridine moieties. Three hydrogen bonds formed between the imido group of the flavin

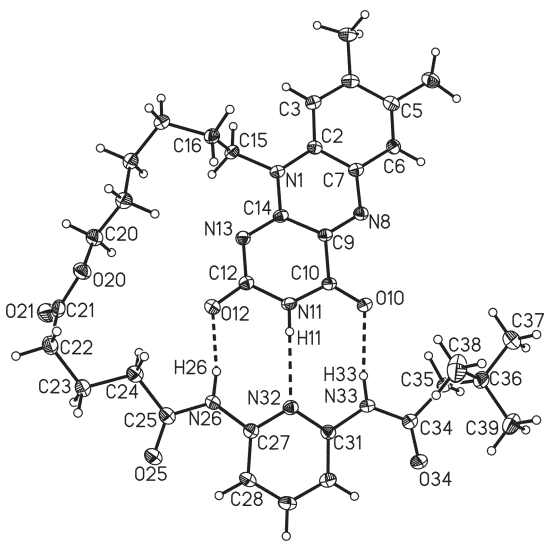


Fig. 1 X-Ray crystal structure of **3** showing intramolecular hydrogen bonds.

† Electronic supplementary information (ESI) available: synthesis and characterization details for **3**. See http://www.rsc.org/suppdata/cc/b5/b501887k/

*g.cooke@hw.ac.uk

and the diamidopyridine moiety hold the structure intact, with N–H···O lengths of approximately 1.9 Å, typical of the optimum bond lengths found in intramolecularly hydrogen bonded structures.[7] Furthermore, the N–H···O angles (167°) are similar to the theoretical optimum of 160° for an intramolecular hydrogen bonded complex.[8]

The ^{1}H NMR spectrum of compound **3** in $CDCl_3$ clearly indicates hydrogen bonding occurs in this solvent, *via* the downfield position of the imido NH [N(1) δ = 12.65 ppm] of the flavin unit and the two amido NH groups [N(2b) δ = 10.50 ppm, N(6b) δ = 9.77 ppm] of the pyridine unit, compared to the resonances of these protons of **1** (δ = 8.42 ppm) and **4** (δ = 7.40 and 7.60 ppm, respectively) when recorded at the same concentration. It is noteworthy that the signal for the proton attached to N(2b) of **3** appears significantly more downfield compared to the analogous proton attached to N(6b).

There is clear evidence from a ^{1}H ROESY[9] spectrum of **3** (8×10^{-3} M), that the three hydrogen bonds formed between the heterocyclic moieties are linked; ROE contacts between the hydrogen attached to N(1) and the hydrogens attached to N(2b) and N(6b) are clearly visible. In addition, a chain of ROE contacts was established from the flavin end of the molecule through to the ester link, and then from the other side of the ester link to the pyridyl moiety. Connection across the ester link was established with a long-range C–H correlation (HMBC) experiment.[10] However, there were no ROE contacts between non-adjacent protons in the molecule (except at the hydrogen bonding site), so it is not possible to differentiate between a cyclic (intramolecularly) hydrogen bonded structure as opposed to an intermolecular hydrogen bonded structure. However, all the NMR lines were sharp, suggesting that the molecule was tumbling rapidly, which may be taken as indirect evidence for a monomeric cyclic structure. Any intermolecular binding would be expected to extend over several molecules giving rise to polymeric aggregates or clusters which would tumble slowly and give broad NMR signals. Moreover, ^{1}H NMR spectra recorded in $CDCl_3$ (298 K) showed negligible concentration dependence from 10^{-2} down to 10^{-4} M (see ESI†). Therefore, it is unlikely that supramolecular oligomers are formed to any great extent, and intramolecular hydrogen bonding interactions are likely to predominate under the conditions examined.

The solution electrochemistry of **1** and of **3** (0.1 M Bu_4NPF_6 in CH_2Cl_2) has been studied using cyclic voltammetry (CV) (Fig. 2).[11] Upon reduction of **1**, a single reduction wave and two distinct reoxidation waves were observed.[12] The former process is due to the reversible formation of $\mathbf{1}_{rad}^-$ ($E_{½}$ = −1.36 V), whereas the second reoxidation wave (E = −1.07 V), arises from an electrochemical-chemical-electrochemical (e-c-e) process where a portion of the $\mathbf{1}_{rad}^-$ formed at the electrode surface rapidly deprotonates **1** in the bulk medium (see ESI†). The protonated flavin radical ($\mathbf{1}_{rad}\mathbf{H}$) produced in this process undergoes a further one-electron reduction at the working electrode surface to form the relatively stable, fully reduced flavin anion ($\mathbf{1}_{red}\mathbf{H}^-$), which is subsequently reoxidised at a less negative potential than $\mathbf{1}_{rad}^-$. Upon the addition of an excess of compound **4** to the electrolyte solution, the redox wave corresponding to the $\mathbf{1}/\mathbf{1}_{rad}^-$ redox couple is immediately shifted by +80 mV (see ESI†), corresponding to a substantial stabilisation of the flavin radical anion of 7.7 kJ mol^{-1}, which is consistent with an over 22-fold increase in the binding

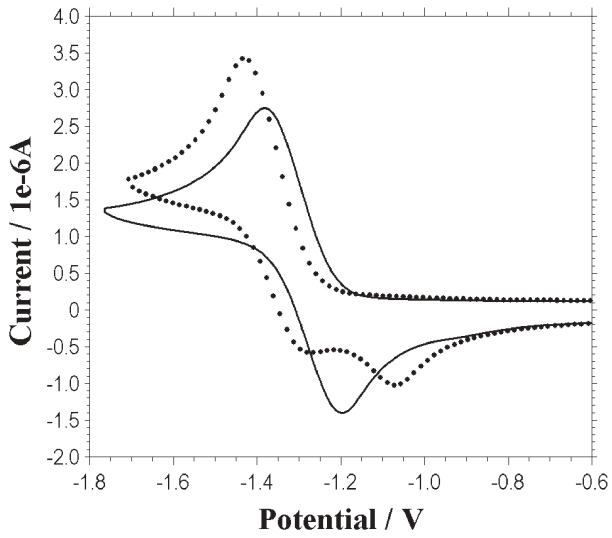


Fig. 2 CVs of separate equimolar solutions of **3** (—) and **1** (•••) ($\sim 3 \times 10^{-4}$ M) in CH_2Cl_2. Scan rate = 0.1 V s^{-1}.

strength of the complex ($\mathbf{4}\cdot\mathbf{1}_{rad}^-$, K_a = 3140 ± 160 M^{-1}).[13] The CV data for **3** are in accordance with the CV data for flavin derivatives recorded in the presence of a complementary diamidopyridine, as the wave for the reoxidation of the $\mathbf{3}_{red}\mathbf{H}^-$ to **3** is virtually fully suppressed due to the prevention of the e-c-e process due to complexation.[14] Furthermore, the $\mathbf{3}/\mathbf{3}_{rad}^-$ redox couple is shifted by +80 mV, compared to the CV data recorded for compound **1** (see ESI†). Therefore, the data are consistent with a significant stabilisation of the $\mathbf{3}_{rad}^-$ state and a concomitant increase in the hydrogen bonding efficiency upon the electrochemical reduction of the flavin moiety of the complex.

With electrochemically tuneable intramolecular hydrogen bonding verified, we then turned our attention to investigating whether we could create a molecular switch from **3**, whereby intramolecular bonding can be switched off under the influence of an external stimulus.[5a] Firstly, we investigated the thermal control of intramolecular hydrogen bonding using variable temperature (VT) NMR and square wave voltammetry (SWV) techniques. VT-^{1}H NMR measurements recorded for a 2×10^{-3} M solution of **3** in $C_2D_2Cl_4$ (from −20 °C to 90 °C) revealed, through a complicated series of events, that at higher temperature the imido and amido proton resonances undergo a substantial upfield shift, indicating a disruption of the hydrogen bonded structure (see ESI†). VT-SWV further confirms the thermally controlled disruption of the intramolecular hydrogen bonding interactions, as illustrated by the −150 mV shift in the reduction wave due to the $\mathbf{3}/\mathbf{3}_{rad}^-$ redox couple upon heating a solution of **3** in 1,1,2,2-tetrachloroethane from ambient temperature to 80 °C (Fig. 3).[15] This large negative shift in reduction potential is consistent with the flavin moiety of **3** being no longer hydrogen bonded to its complementary pyridine unit.

As an alternative strategy for disrupting the intramolecular hydrogen bonding of **3** in solution, we have investigated the addition of a host unit capable of competing for its pyridine binding moiety. In particular, we have used ^{1}H NMR spectroscopy to determine whether thymine derivative **5** has the ability to disrupt intramolecular hydrogen bonding in **3**. The addition

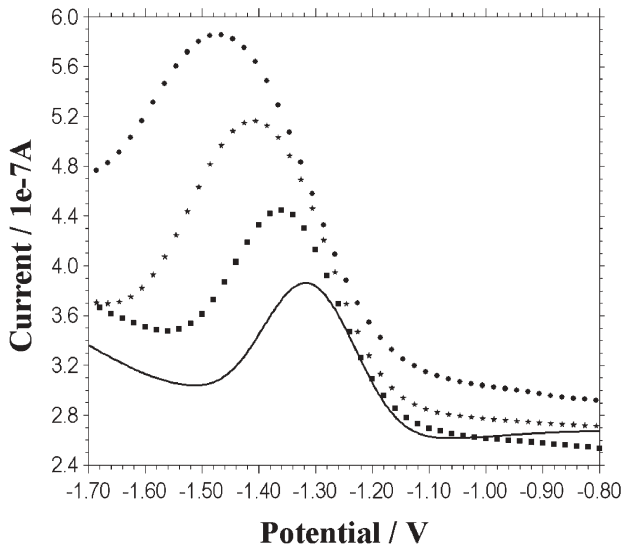


Fig. 3 Variable temperature SWV for **3** (initially $\sim 3 \times 10^{-4}$ M) recorded in a 0.1 M solution of Bu_4NPF_6 in 1,1,2,2-tetrachloroethane. (—) = 20 °C; (■■) = 40 °C; (★★★) = 60 °C; (•••) = 80 °C.

of aliquots of **5** to an NMR tube containing **3** (7×10^{-4} M in $CDCl_3$) resulted in the gradual downfield shift in the resonance for the proton attached to N(3c) of **5**, and the concomitant upfield migration of the signal for the proton attached to N(1) of the flavin moiety of **3** (see ESI†). Thus, these data are in accordance with **5** having the propensity to disrupt the intramolecular hydrogen bonded architecture of **3**, by forming intermolecular hydrogen bonds to the pyridine moiety of **3**.

In conclusion, we have shown that **3** has the propensity to form intramolecular hydrogen bonds in solution and solid-state. Cyclic voltammetry experiments have shown that the hydrogen bonding interactions between the diamidopyridine and flavin moieties have the propensity to significantly stabilise the flavin's radical anion state. Furthermore, these studies have also shown that the intramolecular binding efficiency can be significantly increased upon the electrochemical reduction of the flavin moiety. We have also shown that intramolecular hydrogen bonding can be switched off by either heating the species to above ambient temperature or by the addition of a thymine derivative which competes for the pyridine binding site of **3**. We are currently exploiting the molecular electronic properties of **3**, and preparing analogues which possess shorter linker units between the flavin and pyridine moieties, in the expectation of forming electrochemically tuneable supramolecular polymers. The results of these investigations will be published in due course.

GC gratefully acknowledges the EPSRC for supporting this work. VMR acknowledges the NIH for grant GM59249.

Alan S. F. Boyd,[a] Joseph B. Carroll,[b] Graeme Cooke,*[a] James F. Garety,[a] Brian J. Jordan,[b] Suhil Mabruk,[a] Georgina Rosair[a] and Vincent M. Rotello[b]

[a]Centre for Biomimetic Design & Synthesis, Chemistry, William H. Perkin Building, School of Engineering & Physical Sciences, Heriot-Watt University, Riccarton, Edinburgh, UK EH14 4AS. E-mail: g.cooke@hw.ac.uk
[b]Department of Chemistry, University of Massachusetts at Amherst, Amherst, MA 01002, USA

Notes and references

‡ $C_{35}H_{45}Cl_2N_7O_6$, M = 730.68, triclinic, a = 9.1226(12), b = 13.640(2), c = 15.435(2) Å, α = 71.272(4), β = 76.201(6), γ = 81.735(4)°, U = 1761.7(5) Å^3, T = 100(2) K, space group $P\bar{1}$ (no. 2), Z = 2, μ(Mo Kα) = 0.241 mm^{-1}, 51896 reflections collected, 8351 unique [R(int) = 0.0475] which were used in all calculations. Final $R_1$was 0.0857 and wR_2 was 0.1754 for all data. CCDC 259130. See http://www.rsc.org/suppdata/cc/b5/b501887k/ for crystallographic data in CIF or other electronic format.

1 (*a*) *Flavins and Flavoproteins 1996*, ed. K. J. Stevenson, V. Massey and C. H Willams, Jr., University of Calgary Press, Calgary, 1997; (*b*) *Chemistry and Biochemistry of Flavoenzymes*, ed. F. Muller, CRC Press, Boca Raton, FL, 1991, vol. 1–3.
2 D. E. Edmondson and R. De Francesco, in *Chemistry and Biochemistry of Flavoenzymes*, ed. F. Muller, CRC Press, Boca Raton, FL, 1991, vol. 1, pp. 73–103.
3 D. J. Steenkamp and F. S. Mathews, in *Chemistry and Biochemistry of Flavoenzymes*, ed. F. Muller, CRC Press, Boca Raton, FL, 1991, vol. 2, pp. 395–424.
4 For examples see: (*a*) V. Massey and P. Hemmerich, in *The Enzymes*, ed. P. D. Boyer, Academic Press, New York, 1975, vol. 12, pp. 191–240; (*b*) R. Swenson, G. Krey and M. Eren, in *Flavins and Flavoproteins*, ed. D. E. Edmondson, D. B. McKormick, Walter de Gruyter, Berlin, 1987, pp. 98–107.
5 For recent reviews see: (*a*) A. Niemz and V. M. Rotello, *Acc. Chem. Res.*, 1999, **32**, 44; (*b*) V. M. Rotello, *Curr. Opin. Chem. Biol.*, 1999, **3**, 747; (*c*) Y. Yano, *Rev. Heteroat. Chem.*, 2000, **22**, 151.
6 C. Frier, J.-L. Décout and M. Fontecave, *J. Org. Chem.*, 1997, **62**, 3520.
7 E. N. Baker and R. E. Hubbard, *Prog. Biophys. Mol. Biol.*, 1984, **44**, 97.
8 D. Peters and J. Peters, *J. Mol. Struct.*, 1980, **68**, 255.
9 T.-L. Hwang and A. J. Shaka, *J. Am. Chem. Soc.*, 1992, **114**, 3157.
10 W. Williker, D. Leibfritz, R. Kerssebaum and W. Bermel, *Magn. Reson. Chem.*, 1993, **31**, 287.
11 All electrochemical experiments were performed using a CH Instruments 620A electrochemical workstation. The electrolyte solution (0.1 M) was prepared from recrystallised Bu_4NPF_6 and dry CH_2Cl_2 or 1,1,2,2-tetrachloroethane. A three electrode configuration was used with a platinum disc working electrode, a platinum wire counter electrode and a silver wire *pseudo* reference electrode. Ferrocene was used as an internal reference with the ferrocene–ferrocenium couple adjusted to 0 V. The solution was purged with nitrogen prior to recording the electrochemical data, and all measurements were recorded under a nitrogen atmosphere.
12 A. Niemz and V. M. Rotello, *J. Am. Chem. Soc.*, 1997, **119**, 6833.
13 The thermodynamic data for these systems were calculated from binding constants determined by NMR titration experiments ($CDCl_3$) for complex **1·4** (K_a = 140 ± 20 M^{-1}). The redox-based enhancement in recognition can be calculated using a thermodynamic cycle which can be expressed mathematically using: $K_a(\text{red})/K_a(\text{ox}) = e^{(nF/RT)[E_{1/2}(\text{bound}) - E_{1/2}(\text{unbound})]}$. K_a(red) and K_a(ox) are the association constants in the reduced and oxidized forms, and $E_{1/2}$(bound) and $E_{1/2}$(unbound) are the half-wave redox potentials in the receptor bound and unbound states.
14 E. Breinlinger, A. Niemz and V. M. Rotello, *J. Am. Chem. Soc.*, 1995, **117**, 5379.
15 Increase in peak currents is probably due to gradual evaporation of solvent during heating.

