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Journal Name

COMMUNICATION

Modelling flavoenzymatic charge transfer events: development of catalytic indole deuteration strategies.

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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The formation and chemistry of flavin–indole charge transfer (CT) complexes has been studied using a model cationic flavin. The ability to form a CT complex is sensitive to indole structure as gauged by spectroscopic, kinetics and crystallographic studies. Single crystals of sufficient quality of a flavin–indole CT complex, suitable for X-ray diffraction, have been grown, allowing solid-state structural analysis. When CT complex formation is conducted in *d*₄-methanol, an efficient and synthetically useful C-3 indole deuteration is observed.

Flavoenzymes represent a wide-ranging and important family of oxidoreductase enzymes which use the flavin-centred co-factors FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide).¹ It is believed that between 1% and 3% of genes encode for flavin-containing enzymes.² Organisms which contain a high level of flavoenzymes are described as leading a 'flavin-intensive lifestyle'. Accordingly, a full understanding of the mechanisms of flavoenzymes becomes especially significant in the context of important pathogens such as *Mycobacterium Tuberculosis*.³

At the molecular level, flavoenzymes act as redox enzymes with the capacity to undergo both 1- and 2-electron transfer processes. When acting as 1-electron acceptors, flavin co-factors are able to form charge-transfer (CT) complexes with electron-rich aromatic units such as purines, pyrimidines, β -carboline and notably indoles^{4–7}, due to the presence and chemistry of the proteinogenic amino acid, tryptophan.⁷ For example, the cryptochrome enzymes are blue-light sensitive flavoenzymes, common in both plants and animals and centred on a flavin–indole CT interaction. Among the functions associated with cryptochromes are phototropism in plants,⁸ the regulation of circadian rhythms in animals⁹ and avian signalling.¹⁰

A number of theoretical and experimental studies have explored model systems in the context of charge transfer from indoles to flavin moieties.^{5, 11, 12} In terms of this progressing to an electron transfer, Skibsted observed a reaction of tryptophan with photoexcited triplet state riboflavin with indole to the radical cation which was captured with spin traps, such as 2-methyl-2-nitrosopropane (MNP), to form radical **1** (Fig. 1).¹³ Additionally, Hadad and Platz used DFT methods and time resolved IR spectroscopy to suggest the radical pair formed would ultimately lead to the formation of a new covalent bond, as seen in adduct **2**.¹⁴ Notably, the use of synthetic flavins as tuneable acceptors in charge-transfer chemistry has been investigated, with a variety of donors such as diaminopyridines and porphyrins.^{15, 16} These studies form part of an important wider body of work concerning the application of model flavins to understanding flavoenzyme mechanism. For example, flavin monooxygenases, have been extensively modelled with flavinium salt **3** and hydroperoxide **4** (Fig. 1)^{17–19} and subsequently applied in bio-relevant, organocatalytic contexts.^{20–25}

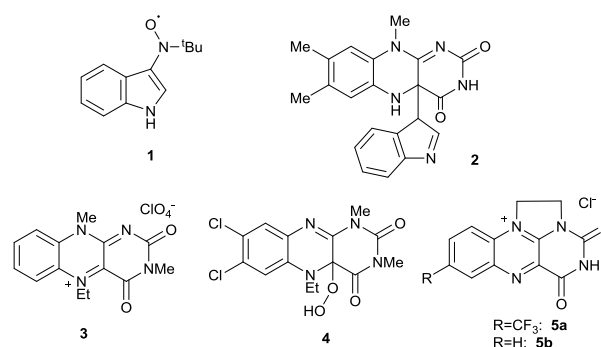


Fig. 1 Spin-trapped indolyl radical **1**, indole–flavin adduct **2** and flavoenzyme models.

Whilst spectroscopic evidence for flavin CT complexation exists, there is limited discussion relating to the ensuing chemistry that the one-electron reduction of a flavin unit by an electron-rich donor may trigger. One such example, however, is found in the biosynthesis of the nikkomycin antibiotics, which progresses through the action of the enzyme, nikD (Fig.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

system to **5a**, was gauged to be the most efficient substrate in this study, suggesting that the strength of the stacking interaction may contribute to reactivity trends, rather than the simple reactivity of an indole substrate with a D^+ source. *N*-methylindole **6k** was deuterated without concern, suggesting the *N-H* motif is not essential in mediating this pathway. An electronic dependence was also observed for method B. However, most substrates could be deuterated to varying extent, and good reactivity of indoles bearing an electron-withdrawing group (**6g-i**, entries 18, 20, 22) with lengthened reaction times. While the electron rich indole **6j** was surprisingly unreactive with methanolic HCl. This observation is possibly linked to the ability of a 5-membered intramolecular hydrogen bond to form between the indolyl C(7) methoxy oxygen centre and the indole N-H proton. This hypothesis is a key direction in ongoing work. However, limited levels of deuteration using conditions A was observed, suggesting differential effects of π donor ability vs. electrophilicity.

Reaction monitoring by *in situ* 1H NMR spectroscopy has allowed an exploratory kinetic study of the flavin-catalysed protocol, with indole **6c** as substrate. In this instance, the reaction was found to be zero-order and second-order with respect to indole and flavin concentration respectively (see ESI), suggesting two flavin molecules are involved in the rate-determining step. Whilst it is not immediately obvious what the implication of this observation is, this study will however prime a subsequent in-depth examination.

In cases where the flavin-catalysed H \rightarrow D reaction was most efficient (e.g. entries 1 and 11), the most visually impactful colour changes were also observed. Accordingly, a working hypothesis that this deuteration reaction is mediated by the formation of a flavin-indole charge transfer complex was formulated, which would be compatible with the structural pattern of indole electron-donating and electron-withdrawing groups. In addition, the less oxidising *des*-CF₃ flavin **5b**, also effected this deuteration reaction with **6a**, albeit less efficiently (entry 5).

In an effort to probe the mechanism of this deuteration reaction, we sought evidence of the hypothesised CT complex. On incubation of the Flavin with indole we observe an increase in absorption that is very broad, ranging at least from 450 nm to 700 nm (Fig 3). In flavoenzymes, such charge transfer complexes often directly precede chemical turnover, with subsequent loss of the charge transfer absorption on reduction of the flavin.⁷ The new absorption features are stable (minutes) under the conditions used, suggesting the flavin-indole complex is not turning over on the timescale of the experiment. Whilst broad, this new spectral feature has two new maxima centred at \sim 450 nm and \sim 600 nm. A broad absorbance feature centred at \sim 600 nm is typically observed in flavoenzyme charge-transfer complexes.⁷ Fitting the concentration dependence of the peak absorption to a weak binding isotherm, $A = A_{max}[indole]/(K_d + [indole])$ gives a dissociation constant (K_d) of 17.0 ± 3.3 mM and 10.5 ± 1.7 mM for the absorption features at \sim 450 nm and \sim 600 nm, respectively. That these values are significantly different within error might argue that there is more than one stable

equilibrium geometry of the CT complex, essentially with different configurations giving rise to the features at 457 nm and 600 nm. In contrast, a strong CT absorption band with **5b** was not observed, possibly due to the absence of the electron-withdrawing CF₃ group.³⁸

Previous studies in flavoenzymes have found that increasing pressure causes a significant increase in CT absorption.³⁹ This finding was taken as evidence that increasing pressure decreased the π - π orbital overlap between the flavin isoalloxazine and the cofactor nicotinamide ring and that the magnitude of the CT complex is a 'spectroscopic ruler' that accurately reflects the geometry of the flavin CT complex.^{39, 40} Figure 3B shows the difference spectrum of the CT complex absorption at 2000 bar and 1 bar for a saturating concentration of the indole. Two clear spectral features are apparent centred at \sim 450 nm and \sim 600 nm, corresponding to the approximate peak maxima from our concentration dependence studies and with a single isosbestic point at \sim 520 nm. The CT absorption increases with pressure at \sim 450 nm but decreases at \sim 600 nm. Combined with the differing K_d values for the two spectral features, these data suggest that there are at least two stable geometries of the flavin-indole CT complex. A further consideration, which may offer some insight to the observed 450/600 nm spectroscopic bifurcation, comes from a recent report discussing organic spin-crossover materials.⁴¹ In this instance, a tethered bis(viologen) dication diradical was observed to display sensitive spectroscopic switching behaviour by UV-Vis and electron paramagnetic resonance spectroscopies, which was presented as the diradical adopting either diamagnetic (singlet) or paramagnetic (triplet) electronic relationships. Accordingly, the physical perturbation applied in this pressure-temperature study may also be reporting on the spin relationship in these charge-transfer complex currently under discussion.

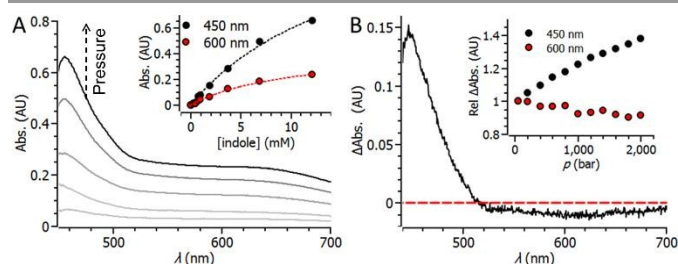


Fig. 3 A, Flavin difference spectra on incubation with indole are consistent with a flavin-indole charge-transfer complex. *Inset* Concentration dependence of indole versus absorbance changes at 457 nm and 600 nm. **B**, The flavin CT shows pressure-dependent absorption differential absorption for two distinct spectral features. *Inset* Relative absorption change for the spectral features at 450 nm and 600 nm.

While solution and solid phase forms of these enzymes can differ in stacking mode,¹² direct evidence for a CT complex has been achieved through X-ray crystallography. Complex **8**, formed from **5a** and **6a**, is amenable to crystallisation from MeOH at room temperature (Fig. 4). Co-planarity of the indole and flavin units is observed, with a flavin-indole separation of between 3.39 to 3.46 Å. The distance is comparable to that observed for the nikD enzyme, 3.17 Å.^{26, 27}

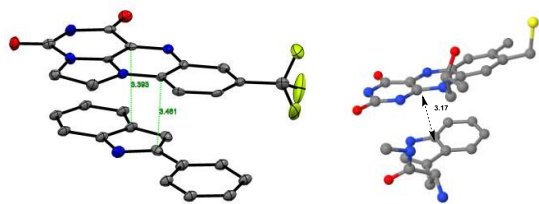


Fig 4. XRD structure of the CT complex **8**, formed from **5a** and **6a**, compared with nkd active site⁴² (PDB: 2OLO)

Significant disorder is observed in this structure with respect to the orientation of the indole unit. Presumably due to the comparable surface area of the respective π -systems of **5a** and **6a**, the indole can project the phenyl group in either a proximal or distal manner, relative to the flavin CF_3 group in this crystal. In both crystallographic “disordermers”, overlap of the flavin N(5) and indole C(3) is observed. This is consistent with overlap between the atoms carrying highest spin-density in both the indole radical cation (or neutral radical)⁴³ and the flavin semiquinone, which we have recently discussed and points to a more subtle explanation. Charge transfer complex **8** is formed from the sandwiching of two 2-D, extended planar π -systems, that of **5a** and **6a**. After formation, CT complex **8** now possess three-dimensional character which allows for enantiomeric structures to be considered (Fig. 5). It is therefore possible to describe enantiomeric and diastereomeric relationships between the four possible charge-transfer complexes using Cahn-Ingold-Prelog prioritisation rules around the flavin N(5) and indole C(3) atoms to describe which prochiral faces form the CT complex. We have as of yet been unable to correlate solution-phase π -stacking geometry of the CT complex to that observed in the solid-state data, largely because of the disordered complexity observed in the crystal structure.

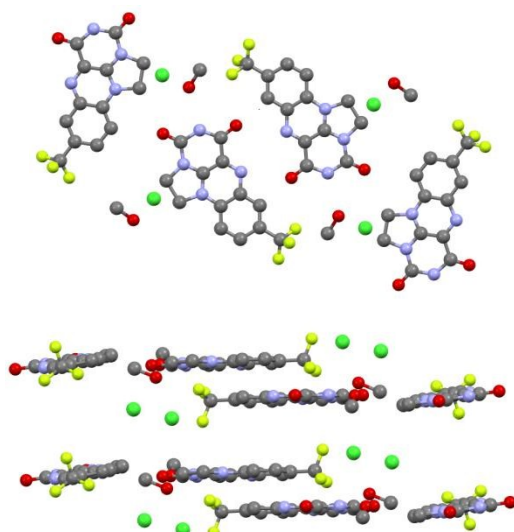


Fig 5. Flavin crystal packing. Indole units removed for clarity. Colour coding as follows: carbon - grey, nitrogen - blue, oxygen - red, fluorine - pale green, chlorine - dark green.

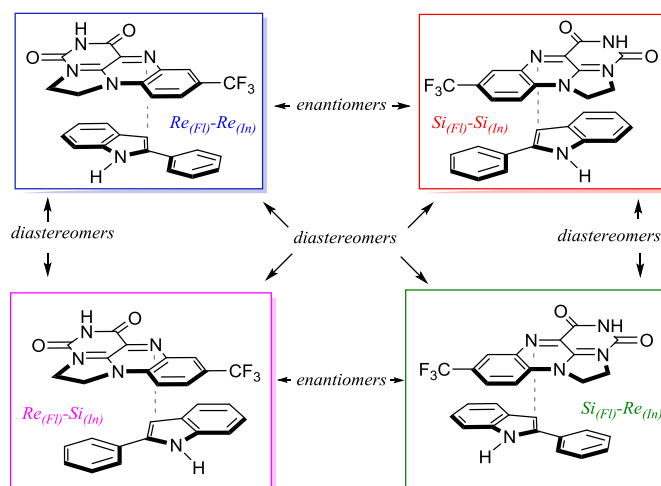
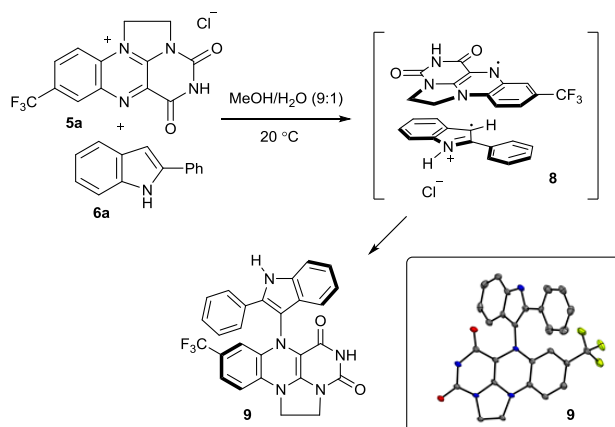


Fig 6. Stereochemical considerations in discrete flavin-indole charge-transfer complexes. For spatial graphical emphasis, flavin and indole units are displayed pre-electron transfer.

In an attempt to produce improved quality single crystals of **8**, small quantities of water was added to the crystallisation solvent, methanol, in order to improve solubility of **8** and thus offer a slower, controlled crystallisation process (Scheme 1). Instead, we observed covalent bond formation between the indole C3 and flavin, although from the flavin N(5) rather than the carbon atom as predicted by Skibsted. This can be rationalised as an electron transfer within complex **8** followed by radical recombination to form covalent adduct **9**.



Scheme 1. Formation of a flavin N(5)-indole C(3) covalent when water is added.

The sensitivity in terms of the fate of complex **8** when small proportions of water are added is notable. We offer a rationale which is centred upon the differences in the hydrogen bonding framework, in addition to an increase in solvent polarity on addition of water, which will operate around the flavin N(5). This effect may allow promotion of a second electron transfer, after formation of the charge-transfer complex, to complete the 2-electron transfer process.³¹

It is worth noting that rapid, mild and selective deuteration of organic compounds is an important synthetic operation which continually opens new opportunities in mechanistic

physical organic studies.⁴⁴ Additionally, recent metabolic stability studies of a number of medicines and other biologically active molecules has led to the possibility that drugs featuring site-specific ²H isotope incorporation may offer improved pharmacokinetic profiles.⁴⁵⁻⁴⁷ With indoles generally acting as important biological and organic substrates, current methods for indole C-3 deuteration arguably suffer from a number of disadvantages, such as high temperatures, the use of strongly acidic or basic conditions, long reaction times, and the use of expensive deuterium sources such as D₂SO₄ or ^tBuOD.^{48, 49} With a view towards a simple, efficient and reliable protocol for indole deuteration, the AcCl-initiated protocol will offer a valuable synthetic tool in indole chemistry.

In conclusion, cationic flavinium salts in CD₃OD are efficient catalysts for promoting an efficient C-3 deuteration of indoles. This process is consistent with a charge-transfer complex initiated, but acid-mediated process, which may have relevance to the mechanistic activity of enzymes with flavin-indole CT interactions in their active sites, such as nikD and some cryptochromes. Crucially, the observation of modulating one-electron and two-electron transfers from indole donor to flavin acceptor with the addition of water to the reaction medium offers an improved understanding of how flavoenzymes may modulate electron-transfer processes by controlling the H-bonding network surrounding the flavin N(5) centre.

Additionally, direct observation of the relevant charge transfer interactions by UV/visible spectroscopy, in addition to the isolation and study of a flavin-indole charge-transfer complex have assisted with this proposal. An analogous acid-mediated process is additionally observed with *in situ* generated DCI. Further work with respect to an elucidation of how the flavin CT event relates to the overall deuteration sequence is ongoing.

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We acknowledge GSK, EPSRC and University of Bath for a studentship (ATM). We thank the EPSRC for support (EP/J005118/1 and EP/K004956/1).

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