Published in final edited form as:

Tetrahedron Lett. 2007 July 30; 48(31): 5517–5520. doi:10.1016/j.tetlet.2007.05.165.

A Flavin Analogue with Improved Solubility in Organic Solvents

Ronald L. Koder^{a,b,*}, Bruce R. Lichtenstein^a, Jose F. Cerda^a, Anne-Frances Miller^c, and P. Leslie Dutton^a

^aThe Johnson Research Foundation and Department of Biochemistry and Biophysics, the University of Pennsylvania, Philadelphia, PA, 19104

^bDepartment of Physics, The City College of New York, New York, NY 10031

^cThe University of Kentucky Department of Chemistry, Lexington, KY 40506-0055

Abstract

We report the synthesis and initial electrochemical characterization of a benzene-soluble flavin analogue: N(10)-2,2-dibenzylethyl-7,8-dimethylisoalloxazine (DBF, **1**). This analogue, which has an unmodified flavin headgroup, is intended for use in the spectroscopic examination of the electronic effects of flavin hydrogen bonding in simple model systems in aprotic, non-hydrogen bonding solvents. With future spectroscopic studies in mind, we have developed a synthetic route which allows the incorporation of isotopic labels using inexpensive starting materials.

Keywords

flavin model; isotopic labeling; electrochemistry

Flavins are versatile enzyme prosthetic groups or cofactors essential to enzymes found in all five kingdoms of life, where they catalyze a remarkably diverse chemistry that draws on its rich oxidation-reduction chemistry and strong coupling to proton release and uptake. This includes their role in light signaling and transcription activation, in light activated DNA repair, in respiratory electron chains, and in dehydrogenation, dehalogenation, hydroxylation and oxygenation reactions.¹ It is clear that for each enzyme the specific interactions between the cofactor and the protein control both the type and driving force behind specific types of flavin chemistries. Thus, flavoenzymes vary widely with respect to their one- and two-electron midpoint potentials and associated pK_a values in the multiplicity of states that describe the redox-coupled acid-base chemistry of the flavin cofactor.¹⁻⁴

Despite many years of research, this area remains poorly understood. Powerful spectroscopic methods such as infrared spectroscopy, resonance Raman (RR) spectroscopy, Stark spectroscopy and solution nuclear magnetic resonance (NMR) spectroscopy have all been utilized in the examination of both free flavins (i.e. FMN and FAD in aqueous buffers) and a number of flavoproteins,⁵⁻⁹ but global analysis of these data supports only qualitative conclusions due to the complexity of the protein environment and the attendant difficulty in deconstructing the simultaneous effects of the large number of flavin-protein interactions.

*Corresponding author: E-mail E-mail: koder@sci.ccny.cuny.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The Rotello group and others¹⁰⁻¹⁴ have examined flavin oxidation-reduction in simplifying aprotic solvents. This work demonstrated that, in model hydrogen bonding complexes between a chloroform-soluble flavin analogue and mono- and diamidopyridines, the 1-electron reduction potentials (from neutral oxidized to semiquinone radical anion) of the flavin are modulated by over 4 kcal/mol. These hydrogen bonded complexes offer the opportunity to examine, in the atomic detail we are currently lacking, the changes wrought in the electronic structure of the flavin by individual flavin-cosolute interactions previously connoted only indirectly by the aforementioned changes in the flavin reduction potentials. Examination of these model systems promises significant new insights into the manner in which flavoproteins direct flavin reactivity.

The flavin analogue used in Rotello's pioneering experiments bear an isobutyl side chain at the N(10) position of the isoalloxazine moiety to increase solubility in organic solvents (see Figure 1). The oxidized form was examined by ¹H and ¹³C solution NMR, and its reduction to the semiquinone was studied by cyclic voltammetry (CV), in conjunction with electron paramagnetic resonance (EPR).⁴ The oxidized form of this molecule was found to be soluble to <1 mM in chloroform, less so in methylene chloride and almost insoluble in benzene (J.D. Walsh and A.-F. Miller, unpublished observations). Moreover, the reduced anionic forms of the flavin are considerably less soluble than the oxidized in any of these solvents. Sub-millimolar concentrations are insufficient for many forms of spectroscopic analysis. Additionally, chloroform, the solvent in which the magnetic resonance studies have by necessity been performed, is a solvent which possesses considerable hydrogen bond-donating ability.¹⁵ Hydrogen bonding to solvent has been shown to significantly affect ¹⁵N chemical shifts in other cases.¹⁶ Pyridine, for example, has been demonstrated to exhibit a difference in chemical shift between vacuum and chloroform of over 30 ppm.¹⁷ As an ideal model system should examine the effects of single or multiple hydrogen bonds in isolation, nonpolar solvents such as benzene, toluene, carbon tetrachloride or cyclohexane must be used. Therefore the non-redox-active N(10) side chain must be highly soluble in these solvents.

It will prove informative to expand the examination of these complexes to include ¹⁵N NMR (both in solution and the solid state¹⁸), IR, RR and Stark spectroscopies. In order to circumvent solubility problems, we have undertaken the synthesis of novel flavin analogues with improved solubility in aprotic, non-hydrogen bonding solvents. We have chosen benzene as a model solvent because its relatively high melting temperature is ideal for solid state NMR spectroscopy on frozen flavin solutions, although there remains a possibility that flavin energetics in this solvent will be perturbed by π stacking effects.¹⁹ As each of NMR, infrared and raman spectroscopy necessitates the synthesis of multiple flavins, each isotopically labeled at different positions, our intent was to find a synthetic approach based on the most inexpensive isotopically labeled starting materials.

Some high-yield synthetic approaches to flavin analogues have been reported,^{20,21} but each has the disadvantage of expensive and/or synthetically challenging isotopically labeled precursors. In 1947, Tishler *et al.* reported the condensation of 1-(D-ribitylamino)-2-arylo-4,5-dimethylbenzene and barbituric acid to form riboflavin.²² While this synthesis was performed in aqueous acid, which would not be amenable to the synthesis of more hydrophobic flavin analogues, the method does have the advantage that the N(5) nitrogen can be readily and inexpensively labeled using ¹⁵N-sodium nitrite, and the uracil ring can be inexpensively labeled at many positions by synthesizing barbituric acid from urea and diethyl malonate.²³ Thus, every atom predicted to exhibit large changes in chemical shifts or vibrational frequencies²⁴ can be readily labeled using our strategy, with the exception of the N(10) nitrogen.

Our approach is outlined in Scheme 1 (synthetic details, including product characterization, are include in the Supplementary material.) 2,2-dibenzyl-diethylmalonate (**2**), synthesized using the method of Maslak,²⁵ was saponified with KOH and decarboxylated by heating to form 2,2-dibenzylacetic acid **3**. Coupling of **3** with dimethylaniline using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and dimethylaminopyridine followed by LiAlH₄ reduction yielded the secondary amine **5**, which was diazotinated with ¹⁵N-sodium nitrite and p-toluidine using the method of Tishler,²² substituting THF for water and performing the reaction at 0°C to maximize the formation of the *meta*-substituted kinetic product. The resultant compound, **6**, was condensed with barbituric acid **7** in refluxing glacial acetic acid for 16 hrs forming N(10)-(2,2-dibenzylpropyl) isoalloxazine (DBF) **1** in 69% yield. The moderate yield of this final step is compensated for by the fact that this is the step in which the positions most likely to bear isotopic labels, those on the uracil ring of the dimethylisoalloxazine, are attached. This greatly reduces label loss in comparison to other possible approaches in which there are several synthetic steps subsequent to uracil ring formation, each of which would entail a geometrically growing loss of expensive labeled material.

DBF is soluble to 1.5 mM in benzene and 20 mM in methylene chloride as determined optically. This is a 15-fold improvement over the benzene solubility of tetraacetylriboflavin (TARF), the molecule most often used in spectroscopic investigations of free flavins.⁶ Thus, DBF is sufficiently soluble for solution NMR, IR, RR and Stark spectroscopies. Importantly, DBF lacks the multiple ester functionalities of TARF, so it is less likely to form intermolecular hydrogen bonds to the alloxazine N(3) proton.²⁶ DBF's solubility in nonpolar solvents has already proven useful in the generation of dry, powdered, reduced flavin samples for solid state NMR spectroscopy.¹⁸

As expected, DBF is redox-active as demonstrated by CV, displaying a reversible single-electron oxidation/reduction transition between neutral oxidized and the anionic semiquinone radical at -1287 mV vs ferrocene (see Figure 2). We observe a reduced peak area in the reduction half-wave, presumably due to some degree of protonation of reduced flavin anions by oxidized flavins in the bulk solution.⁴ To our knowledge, this is the first report of flavin electrochemistry in benzene. Spectroscopic examination of DBF and its complexes is currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Colin Wraight of the Department of Biochemistry, the University of Illinois at Urbana-Champaign for the kind donation of a sample of 2,2-dibenzylacetic acid to be used as a synthetic standard. This work was supported by DOE Grant DE-FG02-05ER46223, NIH grants GM41048 (P.L.D.), GM063921 (A.F.M.), NSF-MRSEC Grant DMR05-20020 (P.L.D.) and the Petroleum Research Fund via PRF #44321-AC4 (A.F.M.). R.L.K. was a NIH Postdoctoral Fellow (GM64090).

Bibliography

1. Hemmerich, P.; Massey, V. Oxidations and Related Redox Systems. Pergamon Press; Oxford: 1982. p. 379-403.
2. Schopfer, LM.; Ludwig, ML.; Massey, V. Flavins and Flavoproteins. Curti, B.; Ronchi, S.; Zanetti, G., editors. Walter de Gruyter; New York: 1990.
3. Koder RL, Haynes CA, Rodgers ME, Rodgers DW, Miller AF. Biochemistry 2002;41:14197–14205. [PubMed: 12450383]

4. Niemz A, Imbriglio J, Rotello VM. *J Amer Chem Soc* 1997;119:887–892.
5. Stanley RJ. *Antioxidants & Redox Signaling* 2001;3:847–866. [PubMed: 11761332]
6. Muller, F. *Chemistry and Biochemistry of Flavoenzymes*. Muller, F., editor. CRC Press; Boca Raton, FL: 1991. p. 2-72.
7. Muller, F. *Chemistry and Biochemistry of Flavoenzymes*. Muller, F., editor. CRC Press; Boca Raton, FL: 1991. p. 558-95.
8. Wille G, Ritter M, Friedemann R, Mantele W, Hubner G. *Biochemistry* 2003;42:14814–14821. [PubMed: 14674755]
9. Altose MD, Zheng YG, Dong J, Palfey BA, Carey PR. *Proc Nat Acad Sci USA* 2001;89:3006–3011. [PubMed: 11248022]
10. Breinlinger E, Niemz A, Rotello VM. *J Amer Chem Soc* 1995;117:5379–5380.
11. Deans R, Cooke G, Rotello VM. *J Org Chem* 1997;62:836–839.
12. Cuello AO, McIntosh CM, Rotello VM. *J Amer Chem Soc* 2000;122:3517–3521.
13. Goodman AJ, Breinlinger EC, McIntosh CM, Grimaldi LN, Rotello VM. *Organic Letters* 2001;3:1531–1534. [PubMed: 11388859]
14. Hasford JJ, Kemnitzer W, Rizzo CJ. *J Org Chem* 1997;62:5244–5245.
15. Kamlet MJ, Taft RW. *J Chem Soc-Perkin Trans* 1979;2:349–356.
16. Kamlet MJ, Dickinson C, Taft RW. *J Chem Soc-Perkin Trans* 1981;2:353–355.
17. Witanowski M, Sicinska W, Biernat S, Webb GA. *J Mag Res* 1991;91:289–300.
18. Koder RL, Walsh JD, Pometum MS, Dutton PL, Wittebort RJ, Miller AF. *J Amer Chem Soc* 2006;128:15200–15208. [PubMed: 17117871]
19. Breinlinger EC, Rotello VM. *J Amer Chem Soc* 1997;119:1165–1166.
20. Yoneda F, Sakuma Y, Ichiba M, Shinomura K. *J Amer Chem Soc* 1976;98:830–835. [PubMed: 1245698]
21. Epple R, Wallenborn EU, Carell T. *J Amer Chem Soc* 1997;119:7440–7451.
22. Tishler M, Pfister K, Babson RD, Ladenburg K, Fleming AJ. *J Amer Chem Soc* 1947;69:1487–1492.
23. Murray, JI. *Organic Synthesis, Collected Volume 4*. John Wiley & Sons, Inc; New York, N.Y.: 1963. p. 744-746.
24. Walsh JD, Miller AF. *J Phys Chem B* 2003;107:854–863.
25. Maslak P, Varadarajan S, Burkey JD. *J Org Chem* 1999;64:8201–8209. [PubMed: 11674737]
26. Stanley RJ, Siddiqui MS. *J Phys Chem A* 2001;105:11001–11008.
27. Euler HV, Karrer P, Malmsberg M, Schöpp F, Benz F, Becker B, Frei P. *Helv Chim Acta* 1935;18:522–35.
28. Bojic U, Elmazar MMA, Hauck RS, Nau H. *Chem Res Tox* 1996;9:866–870.
29. Nemoto T, Matsumoto T, Masuda T, Hitomi T, Hatano K, Hamada Y. *J Amer Chem Soc* 2004;126:3690–3691. [PubMed: 15038703]

Riboflavin

FMN

FAD

TARF

Rotello¹¹

Figure 1.

The flavin moiety and some common natural and synthetic derivatives.

Figure 2. Cyclic voltammogram of 37 μM DBF in benzene with 0.2 M tetrahexylammonium perchlorate as a supporting electrolyte. A 1.6-mm diameter platinum electrode was used for the measurements. The voltammogram was recorded at a scan rate of 100 mV/s. The potential is internally referenced vs ferrocene and the positive peak denotes reduction of the flavin analogue.

Scheme 1.