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# ON THE CHEMISTRY OF FLAVIN-DEPENDENT OXYGEN ACTIVATION III.[1] SYNTHESIS OF 1.10a-DIHYDROFLAVIN AND ITS 10.10a-RING OPENED DERIVATIVE AS MODEL CHROMOPHORES FOR ENZYME-BOUND INTERMEDIATES

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Abstract: Model chromophores of 1.10-dihydroflavin and its 10.10a-ring opened derivative have been isolated. By comparison with enzymatic intermediates, reaction mechanisms of flavin-dependent oxygenase and luciferase are discussed in terms of chemical structure.

In this journal, Mager[3] has recently given evidence for the proposal that nucleophilic addition at 1.3.10-trimethyl-alloxazinium-perchlorate (IV; $R^1=R^3=R^{10}=CH_3$, $R^7=R^8=H$; for formulae see scheme) yields a "C(10a)-adduct" of structure II ($R^1=R^3=R^{10}=CH_3$, $R^7=R^8=H$, $X=OCH_3$). The data of Mager prompt us to report on crystalline flavin derivatives obtained recently in our laboratory in the context of a more general view concerning enzyme-bound intermediates.

As summarized recently by Hemmerich and Massey[4], flavin (bio)chemistry exhibits a dichotomy, owing to the fact that the flavin chromophore has two nucleophilic sites, defined by the lone pairs of either N(5) or the N(1)/-O(2α)-region. If a given site is blocked either chemically (e.g. by alkylation) or biochemically (e.g. by regiospecific strong hydrogen bridges from the apoprotein) we obtain two kinds of flav(oprote)in reactivity:

1) in N(1)/O(2α)-blocked flavins radical formation is thermodynamically suppressed and $2e^-$-oxidoreduction, i.e. polar (de)hydrogenation is favored, while,

2) in N(3)-blocked flavin the radical is thermodynamically stabilized and hence, $1e^-$-oxidoreduction, i.e. electron transfer, is favored.

For interconversion of $1e^-$- and $2e^-$-equivalents, catalytic turnover must be accompanied by a regular shuttle between N(1)/O(2α)- and N(5)-blocking protein conformations. The third main activity, namely $O_2$-activation, requires both conformations and thus two "flavin-dioxygen intermediates" have been postulated:

1) In the "N(5)-blocked" intermediate dioxygen is linked to the flavin via C(4a). The resulting 5-RFl-4a-OOH (I; X=OOH) first postulated by Hemmerich[5] is well established chemically and biochemically[6].

2) The N(1)/O(2α)-blocked intermediate, first postulated as 1-RFl-10a-

OOH (II; X=OOH) by Mager[7] has long been disputed by Hemmerich[8] and Müller[9]. In the meantime Mager[3], as well as Müller[10], have provided convincing evidence, that no other vinylogous position than C(10a) need to be considered, though no C(10a)-monoadducts were hitherto isolated.

But this alone does not settle the bioorganic problem. The following two questions remain, concerning e.g. the turnover of p-hydroxybenzoate oxygenase [11], and bacterial luciferase[12]:

1) What is the structure of intermediate B($\lambda_{max}$=400-420 nm, $\varepsilon \cong$15.000 $M^{-1}cm^{-1}$) observed by Massey[11,13] in the reaction of p-hydroxybenzoate oxygenase (cf. eq.1):

(1) $Fl_{red}H_2 \xrightarrow{+O_2}$ 5-HFl-4a-OOH (A: I,X=OOH) $\xrightarrow{+ArOH}$ B $\xrightarrow{-Ar(OH)_2}$ 5-HFl-4a-OH (C: I,X=OH) $\xrightarrow{-H_2O}$ $Fl_{ox}$

2) What is the structure of the blue intermediate B' ($\lambda_{abs}^{max} \cong$600 nm)[14,15] which occurs in the course of the bacterial luciferase reaction? B' is a precursor to the emitter C' ($\lambda_{lum}^{max}$=490 nm), or in rapid equilibrium with it, whose properties are in good agreement with the cationic structure IV ($\lambda_{abs}^{max}$=400 nm, $\lambda_{fluor}^{max}$=490 nm, $\lambda_{excit}^{max}$=390 nm)[16,17] as visualized in eq.2:

(2) $Fl_{red}H_2 \xrightarrow{+O_2}$ 5-HFl-4a-OOH (A: I,X=OOH) $\xrightarrow{+RHCO}$ B' $\xrightarrow[-OH^-]{-H_2O}$ $1\text{-RCO-Fl}^+_{ox}$ (C': IV,$R^1$=RCO[17]) $\xrightarrow{-h\nu,\ -RCO_2H}$ $Fl_{ox}$

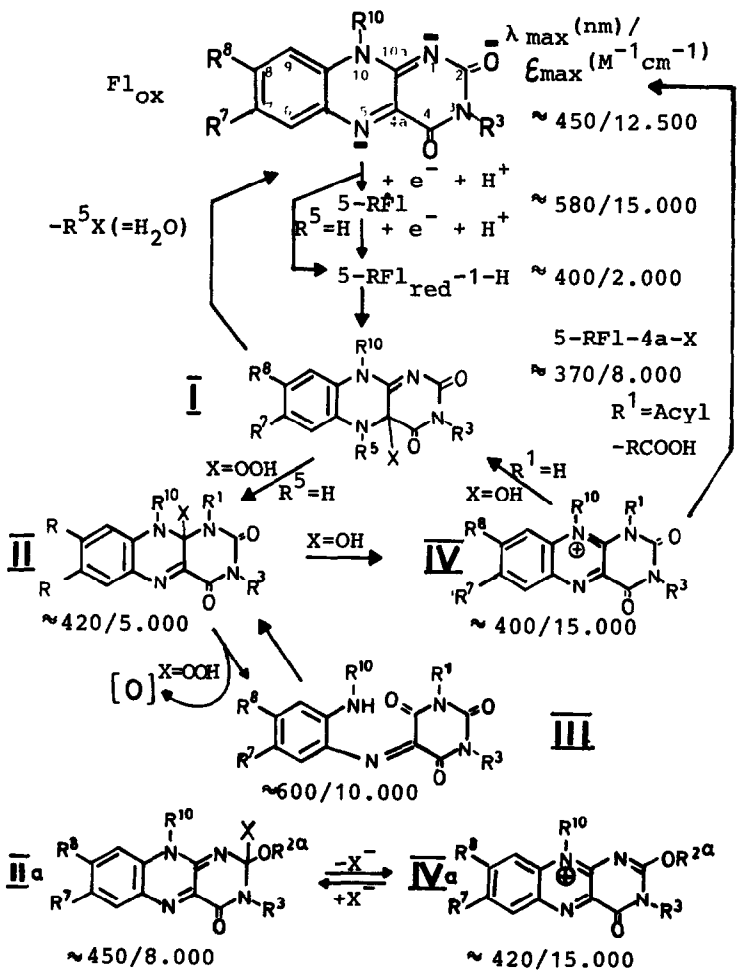
With respect to these questions we want to report the following new findings, which favor N(1) and exclude O(2α) as natural blocking position:

- By adding the 2α-blocked cation IVa[18] ($R^{2\alpha}$=$R^3$=$R^{10}$=$CH_3$, $R^7$=$R^8$=H or $CH_3$) to sodium methylate in methanol, we were able to isolate the C(2)-methoxy adduct IIa ($R^{2\alpha}$=$R^3$=$R^{10}$=$CH_3$, $R^7$=$R^8$=H or $CH_3$, X=$OCH_3$), identified by the $^1$H-NMR-signal at δ=3.24 (s; 6H, 2 C(2)-$OCH_3$). Additional structural proof was obtained by treatment of the latter with glycol which gave the spiro-compounds IIa (X-$R^2$=O-$CH_2$-$CH_2$): IR (KBr) 1667 $cm^{-1}$ (C(4)=O); UV (Methanol) $\lambda_{max}$=446 nm (8.400 $M^{-1}cm^{-1}$); $^1$H-NMR ($CDCl_3$) 7.78 (s; 1H, C(6)-H), 7.00 (s; 1H, C(9)-H), 4.50-4.10 (m; 4H, $CH_2$-$CH_2$), 3.36 (s; 3H, N(10)-$CH_3$), 3.20 (s; 3H, N(3)-$CH_3$), 2.40 (s; 3H, C(8)-$CH_3$), 2.30 (s; 3H, C(7)-$CH_3$) (for $R^7$=$R^8$=$CH_3$). Our findings confirm independently the results of Müller[10] obtained with $^{13}$C-enriched compounds.

- By treatment of the 1.10-bridged cation IV[18] ($R^1$-$R^{10}$=$CH_2$-$CH_2$), $R^3$=$CH_3$, $R^7$=$R^8$=H) with sodium methoxide in methanol, we have been able to obtain the first crystalline "1.10a-dihydroflavin", namely II ($R^1$-$R^{10}$=$CH_2$-$CH_2$, $R^3$=$CH_3$, $R^7$=$R^8$=H, X=$OCH_3$): IR (KBr) 1715 (c(4)=O), 1675 (C(2)=O); UV (Acetonitril): $\lambda_{max}$=404 nm (4.300 $M^{-1}cm^{-1}$); $^1$H-NMR ($CD_3CN$) 7.80-7.40 and 7.20-6.97 (2 m; 2H, Ar-H) 4.43-3.67 (m; 4H, $CH_2$-CH), 3.24 (s; 3H, $OCH_3$), 3.04 (s; 3H, N(3)-$CH_3$), confirming the nature of the chromophore obtained by Mager[3] and Müller[10]

only in solution.

- If the 1.10a-dihydroflavin chromophore is not linked together by a 1.10-ethylene bridge, it undergoes with smallest amounts of water slow ring opening. In polar solvents the 1.10a-bond is cleaved irreversibly[19]. We assume that, biochemically, cleavage of the 10.10a-bond is preferred, which is, in principle[20],[21], reversible. We have now synthesized (from N,N-dimethyl-alloxan and N-(t-butyl-)4.5-dimethyl-2-amino-aniline) a crystalline derivative of this 10.10a-opened chromophore as shown in formula III ($R^1$=$R^3$=$R^7$=$R^8$=$CH_3$, $R^{10}$=t-butyl) which indeed is blue: UV (Acetonitril) $\lambda_{max}$=620 nm (10.800 $M^{-1}cm^{-1}$); IR (KBr) 1700 and 1640 $cm^{-1}$ (C=O); $^1$H-NMR ($CDCl_3$) 8.03 (s; 1H, Ar-H) 6.70 (s; 1H, Ar-H) 3.43 (s; 6H, N-$CH_3$), 2.25 (s; 3H, Ar-$CH_3$), 2.12 (s; 3H, Ar-$CH_3$), 1.49 (s; 9H, t-butyl); MS (70 eV) 344 (100%, $M^+$).




The striking color difference in the open and closed isomers (if $R^1$=H) II and III is indeed surprising to us. It is, however, certain, that the 400-420 nm chromophore of Massey[13] (intermediate B of eq.1) can not be represented by the much more weakly absorbing II, while the blue diamagnetic intermediate B' of Presswood & Hastings[14],[15] is fitting with the 10.10a-opened system III. Based on these comparisons with model chromophores, we show in the formula scheme a pathway of flavin-dependent oxygenation, which implies the following main steps:

Reaction of dihydroflavin with $O_2$ yields a "5-blocked" peroxide intermediate I ($R^3$=H, $R^7$=$R^8$=$CH_3$, $R^{10}$=Rib., X=OOH),

which requires further activation by 5.1-prototropy to the unstable II. This in turn (when X=OOH) undergoes a 10.10a-ring opening, followed by "oxene" cleavage[1,8,11] to yield blue III, which then undergoes 10.10a-reclosure by formation of the cation IV. The properties of IV are fitting with those of the luciferase emitter C'[16], as well as with the Massey intermediate B'[13,22]. IV returns to the starting $Fl_{ox}$ either directly (in the case of bacterial luciferase) by 1-deacylation[17] or (in the case of p-hydroxybenzoate oxygenase) indirectly by 1,5-prototropy and loss of water from the now formed I, X=OH (Massey's intermediate C)[13].

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22. We would like to acknowledge the fact, that the idea of the cationic nature of intermediate B was advanced by Dr. B. Entsch (private communication).


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