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The possibility that the spectrum of intermediate two, seen in the course of reaction of flavoenzyme phenol hydroxylases, may be attributable to iminol isomers of a flavin-derived 6-arylamino-5-oxo(3*H*,5*H*)uracil

(flavin mixed-function oxidase/hydroxylation)

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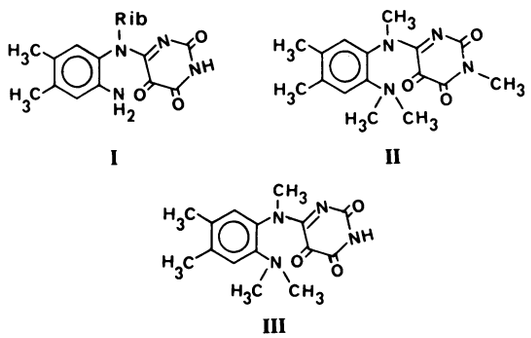
Contributed by Thomas C. Bruice, September 19, 1983

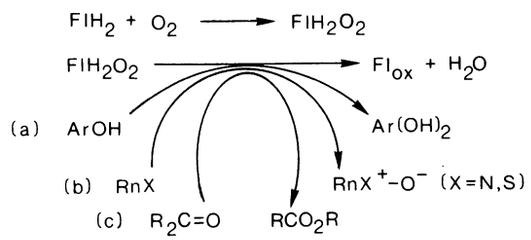
ABSTRACT **A commonly held view of the mechanism of flavin mixed-function oxidases is that enzyme-bound 4a-hydroperoxyflavin (4a-FlHOOH) undergoes ring opening to provide a carbonyl oxide (IV), which, after transferring an oxene equivalent to substrate, yields a 6-arylamino-5-oxo(3*H*,5*H*)-uracil (I). The latter is then thought to undergo ring closure to form a 4a-hydroxyflavin (4a-FlHOH), which by loss of water yields flavin (scheme I). A close structural analogue of I (i.e., III) has been synthesized. Comparison of the spectra of III (and II), taken in solvents of widely differing dielectric constants and in a strongly basic medium, with those of the intermediate(s) observed to be formed in time between 4a-FlHOOH and 4a-FlHOH has shown that the enzyme-bound intermediate(s) does not resemble spectrally I nor its iminol tautomers.**

6-Amino-5-oxo(3*H*,5*H*)uracil (I) has been of interest to chemists and enzymologists since it was first postulated as a transient intermediate in the reaction cycles of flavin-



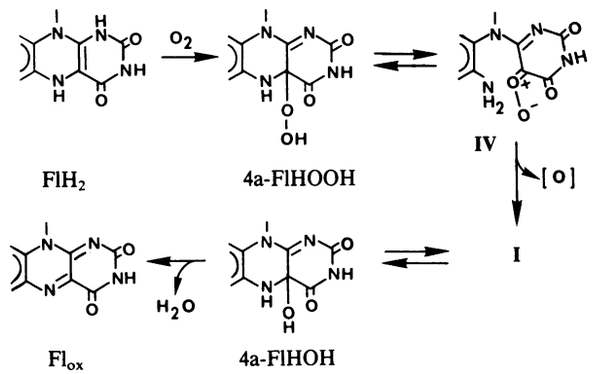
I II III

dependent monooxygenases (1, 2). These enzymes, containing no cofactor other than a flavin, catalyze the insertion of one atom of molecular oxygen into phenols, amines, thiols, sulfides, and carbonyl compounds via reductive activation of O_2 by the 1,5-reduced flavin cofactor (FlH_2, Eq. 1) (3–5). Most flavin-dependent monooxygenases play vital roles in bacterial metabolism (6) and therefore in environmental detoxification. In higher animals, microsomal flavin monooxygenase (7, 8) (catalyzing reaction 1b) is the only representative of this enzyme class. The monooxygenation of aromatic amino acids (i.e., phenylalanine, tyrosine, and tryptophan) in higher animals is catalyzed by tetrahydrobiopterin-dependent enzymes (4, 9). Tetrahydrobiopterin and dihydroflavin have in common the pteridine nucleus. However, assumption of a similarity in mechanism for these two enzyme classes deserves some caution because of the requirement of a stoichiometric amount of iron as an additional cofactor (10, 11) in the case of the tetrahydrobiopterin enzymes.



[1]

Dioxygen complexes or hydroperoxides of flavin were first invoked to explain the oxidation kinetics of free (12–17) and enzyme-bound (18, 19) flavins. It is now firmly established that the intermediates formed on reaction of O_2 with free dihydroflavins (20) and monooxygenase-bound dihydroflavin coenzymes (5) are 4a-hydroperoxides (cf. 4a-FlHOOH in Scheme I).



Scheme I

The suggested rearrangement of 4a-FlHOOH to **IV** (Scheme I) was derived from a concept in which hydroperoxides were divided into two classes, differentiated by the hybridization of their α-carbon atoms (1, 2). Hydroperoxides, bound to sp^3-hybridized carbons (e.g., 4a-FlHOOH), were classified as good nucleophiles but bad electrophiles. For electrophilic oxygen-donating ability (necessary for reactions 1a and 1b), a hydroperoxide with an sp^2-hybridized α-carbon (**IV**, Scheme I; formed from a flavin hydroperoxide by ring opening) seemed to be a logical candidate. Apart from the 4a,5-ring opening and closure (depicted in Scheme I) 1,10a- (1, 2), 10,10a- (1, 2, 4), and 9a,10- (21) ring openings (preceded by rearrangement of an initially formed 4a-hydroperoxide) have been postulated for various reasons, mostly because of lack of a reliable model for 4a,5-ring-opened flavins.

It has recently been shown (22, 23) that electrophilic oxygen-donating ability is not simply a function of the hybridiza-

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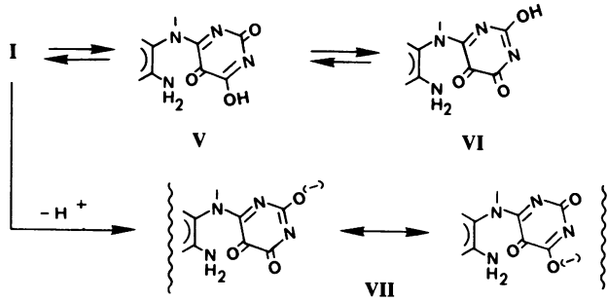
tion of the α-carbons of hydroperoxides. Gradual enhancement of reactivity toward iodide, nitrogen, and sulfur occurs with increase in the electronegativity of the carbon to which the hydroperoxide moiety is bonded. Indeed, a linear free energy relationship exists between the monooxygen donation potential of hydroperoxides and the pK_a of the leaving group (alcohol or acid), with no indication for a change in mechanism in the case of sp^2-hybridized peracids. Because of their π-electron-deficient heterocyclic nuclei, flavin 4a-hydroperoxides have a low pK_a (9.1–9.5) (22) and therefore the best oxygen-donating ability of the hydroperoxides tested, surpassed only by peracids. In accordance with this concept, the only intermediates observed during the reaction cycle of microsomal amine oxygenase are (enzyme-bound) 4a-FlHOOH and 4a-hydroxyflavins (4a-FlHOH) (24, 25), as predicted by results obtained with models (26–28).

In contrast to monooxygenation of amines and sulfur compounds, a simple S_N2-type nucleophilic displacement by phenolate on the terminal oxygen of 4a-FlHOOH does not appear likely in the flavin monooxygenase hydroxylation of phenols. That phenol monooxygenation is more complicated in the enzymatic case is evident from the observation that a strongly absorbing species [λ_{max} 390 (pH 6.6) to 420 (pH 8.7) nm; ε, 15,000 $M^{-1}\cdot cm^{-1}$] is formed from 4a-FlHOOH and is converted to 4a-FlHOH at the active site (29–31). Structure **I** was assigned to this intermediate at a time prior to the synthesis of **II**. The apparent complexity of the enzyme catalysis has its counterpart in model reactions, in which monooxygenation of phenols by flavin hydroperoxides is accomplished only via a dioxygen transfer (32–35). We have recently succeeded in preparing **II** (36, 37). The UV/visible spectrum of **II** (λ_{max} 342 nm; ε, 7,100 $M^{-1}\cdot cm^{-1}$; acetonitrile) was found to be quite different from that of the enzyme-bound intermediate postulated to be **I**. However, with **II** the tautomeric structure of the pyrimidine ring is fixed because of a methyl group at position N-3, and it can be argued that the enzyme binds preferentially to the (probably less stable) tautomers **V** or **VI** or the anion **VII** (Scheme II) (20, 36, 37).

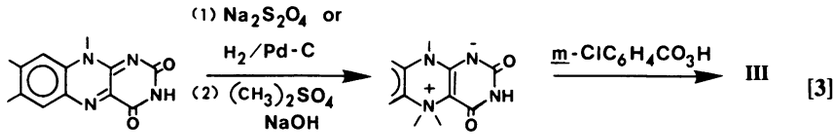


Scheme II

In this communication, we report the preparation of the 4a,5-ring-opened flavin **III** (which is not blocked at position N-3) and experimental data concerning its spectral characteristics. Since the uracil **I** has recently been postulated as the unknown cofactor of the copper-dependent amine oxidase (38) (catalyzing the reaction of Eq. 2), our results may also be relevant for this enzyme.

$$RCH_2NH_2 + O_2 \xrightarrow{H_2O} RCH{=}O + NH_3 + H_2O_2 \quad [2]$$

Synthesis of **III** in low yield was accomplished in much the same manner as that of **II** (Eq. 3). The procedures are described herein because we found it difficult to dialkylate the N-5 position of FlH_2 without also alkylating the N-3 position.



[3]

EXPERIMENTAL

Reduction of Lumiflavin. Lumiflavin was reduced to dihydrolumiflavin (or its anion) by using sodium dithionite as follows (reduction may also be effected by hydrogenation over palladium with ethanol as solvent). To an ice-cold suspension of **I** (1.00 g, 3.91 mmol) in 95% ethanol (200 ml), there was added, under a nitrogen atmosphere, a suspension of 85% sodium dithionite (2.00 g, 10 mmol) in 10 M sodium hydroxide (10 ml). Stirring in the ice bath was continued for 1 hr.

Dimethylation. The 1,5-dihydrolumiflavin so formed was dimethylated by the addition of dimethyl sulfate (20 ml, 211.4 mmol) dropwise at such a rate that the temperature did not rise above 5°C. Stirring was continued at room temperature overnight. The reaction mixture was removed from the nitrogen atmosphere, the ethanol was evaporated, and water (100 ml) was added. The aqueous solution was adjusted to pH 7–8 with sodium hydrogen carbonate solution and washed with chloroform to remove most of the starting material and 3-methyllumiflavin. The solution was filtered to remove inorganic salts, concentrated to low volume, and filtered again. This solution was absorbed onto silica (6 g) and put onto a silica column (*ca.* 50 g), which was eluted with CH_2Cl_2/methanol (7:3) to yield crude 1,5-dihydro-5,5-dimethyl-lumiflavin (**VIII**), which was further purified by stirring in sodium chloride solution (5 ml) and sodium acetate buffer (5 ml) and extracting the mixture five times with chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate and evaporated to a light yellow solid: yield, 70 mg (6%); mp 236–240°C; ^1H NMR [$(C^2H_3)_2SO$, CF_3COOH] δ 7.94 [s, 1H, C(6)H], 7.18 [s, 1H, C(9)H], 4.15 [s, 6H, N(5)$(CH_3)_2$], 3.44 [s, 3H, N(10)CH_3], 2.33 [s, 6H, C(7,8)$(CH_3)_2$]; TLC (silica) R_f = 0.67 (CH_2Cl_2/methanol, 7:3). The composition of the solvent system in the reaction mixture was critical for achieving the necessary selectivity in the alkylation.

Oxidation of VIII. **VIII** was oxidized and the 4a,5-ring of the product was opened to give 6-[(2′-dimethylamino-4′,5′-dimethylphenyl)methylamino]-5-oxo(3*H*,5*H*)uracil (**III**) by the following process. A solution of **VIII** (50 mg, 0.175 mmol) in chloroform (15 ml) was added to 85% *m*-chloroperbenzoic acid (36 mg, *ca.* 0.177 mmol) in chloroform (15 ml). The mixture was stirred at room temperature for 1 hr and then washed with sodium hydrogen carbonate solution and then with water. After drying over anhydrous magnesium sulfate, the chloroform was evaporated to low volume and the residue was applied to two 20 × 20 cm silica GF plates (1 mm thick), which were eluted with CH_2Cl_2/methyl ethyl ketone/methanol (14:2:1). The brown product was removed and stirred in chloroform. Filtration followed by evaporation yielded **III** as a brown crystalline solid, which was crushed in a little ether, and the ether solution was filtered and dried over potassium hydroxide pellets: yield, 3 mg (6%); mp 214–220°C (dec); TLC (silica) R_f = 0.60 (CH_2Cl_2/methyl ethyl ketone/methanol, 14:2:1); UV (acetonitrile) λ_{max} (ε M^{-1} × cm^{-1}) 226 (23,140), 245 (sh), 283 (10,320), 343 (7,120) nm; ^1H

Table 1. Spectrum of III in solvents of various dielectric constants

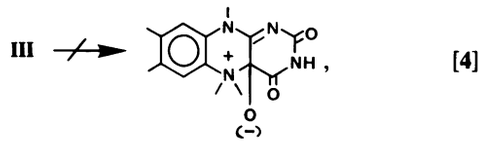
Solvent	Dielectric constant	Wavelength, nm (extinction, $M^{-1}\cdot cm^{-1}$)			
Hexane	1.9	224 (20,800),	245 (sh),	285 (9,440),	336 (5,840)
Dioxane	2.2	225 (25,360),	245 (sh),	285 (11,180),	337 (7,160)
Chloroform	4.8		245 (sh),	283 (11,300),	345 (6,660)
Acetonitrile	38	226 (23,190),	245 (sh),	283 (10,320),	343 (7,120)
Dimethyl formamide	37	Unknown*	Unknown*	285 (11,560),	343 (6,840)
Dimethyl sulfoxide	47	Unknown*	Unknown*	283 (13,120),	343 (7,520)

Hexane, chloroform, and acetonitrile were spectral grade; dioxane and dimethyl formamide were distilled under N_2 from the blue anion radical of benzophenone; dimethyl sulfoxide was filtered from CaH_2 down a previously dried neutral alumina column.
*Because of increase in the absorbance of the solvent.

NMR (C^2H_3CN) δ 5.61 (s, 1H), 5.35 [s, 1H, C(3',6')H_2], 3.46 [s, 3H, C(6)NCH_3], 2.39 [s, 6H, C(2')N$(CH_3)_2$], 2.24 (s, 3H), 2.22 [s, 3H, C(4',5')$(CH_3)_2$]; mass spectrum (70 eV) M^+ obs. 302 (M^+ calc. 302); high-resolution mass spectrum (70 eV) M^+ obs. 302.1404 fits the formula $C_{15}H_{18}N_4O_3$. A sample recrystallized from acetonitrile melted at 220–225°C (dec).

RESULTS AND DISCUSSION

That III has been synthesized in a nonhydrated form is established by low- and high-resolution mass spectrometry. The high-field position (2.39 ppm) of the ^1H NMR signal assigned to the protons on the two methyl groups attached to the amino function at C-2' excludes the addition of this amino function to the carbonyl function at C-5—Eq. 4, because in the zwitterionic product the signal would be expected at



[4]

higher ppm. Also, the UV/visible spectrum of III is similar to that of II.

Spectral studies in aprotic solvents of various dielectric constants show that the long wavelength maxima at approximately 280 and 340 nm are in no way strongly dependent on the solvating power of the solvent (Table 1). It has been observed previously with flavins that change in the solvent environment does not affect the 450-nm absorption and therefore the charge transfer from N-10 into the pyrimidine ring (39). The same conclusion holds for charge transfer from the C-6—N into the pyrimidine ring of III. The position of tautomeric equilibria is another factor influenced by the dielectric properties of the solvent (40–42). No change in the UV spectra in different solvents means either that the relative weight of the different tautomeric forms (cf. Scheme II) does not change over the range of the dielectric constants applied or that there is no difference in the energy of the electronic transitions of the amide and the iminol forms (see below).

Deprotonation of III (14 μM) in acetonitrile with the "proton sponge" 1,8-bis(dimethylamino)naphthalene (140 μM) was apparently unsuccessful because there could be observed no spectral change on addition of the proton sponge. In the case of potassium *t*-butoxide (140 μM) and III (14 μM) in dry *t*-BuOH, there was a change of the spectrum of III immediately after mixing (Thunberg cuvette under a N_2 atmosphere). The spectral change amounted to an increase

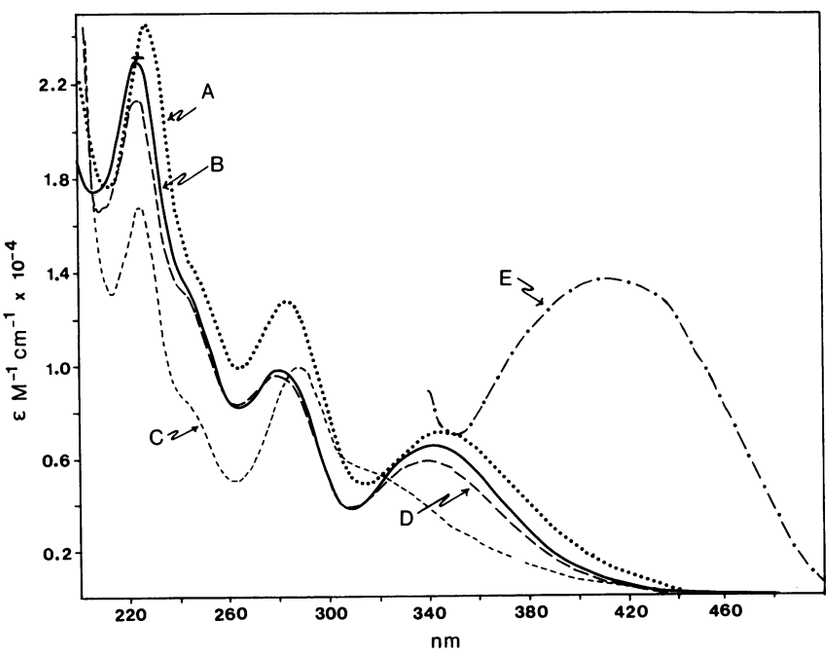
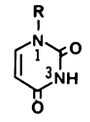


FIG. 1. Comparison of the UV/visible spectra of II [in CH_3CN (line A)] and III [in CH_3CN (line B)] in *t*-BuOH in the presence of a 10-fold excess of *t*-BuO^-K^+ (line C) and in *t*-BuOH (line D) with the spectra of the intermediate observed to arise in time between enzyme-bound 4a-FlHOOH and 4a-FlHOH in the reaction of *p*-hydroxybenzoate hydroxylase with the alternative substrate 2,4-dihydroxybenzoate (taken from ref. 5).

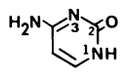
in absorbance at 287 nm and a decrease in absorbance at 343 nm (Fig. 1). This is analogous to the spectral behavior observed with 1-methyluracil (40–42) (**IX**, $R=CH_3$). If sufficient acetic acid was added to the reaction mixture to



IX

neutralize the *t*-butoxide, the original spectrum of **III** reappeared. The anion of **III** slowly decomposes in *t*-butanol/*t*-butoxide and after 22 hr there is obtained a species [290 nm (sh), 245 nm (sh), and 215 nm (sh)] that does not undergo a change in spectrum on acidification. Neither **III** nor its anion possesses a spectrum that resembles that of the intermediate observed on the reaction path between enzyme-bound 4a-FlHOOH and 4a-FlHOH with flavoenzyme phenolate monooxygenases (Fig. 1).

The properties of **III** are very much like those of its N-3 methylated analogue **II**. The spectra of both seem to be rather unaffected by solvent. This observation indicates that the iminol isomers (**V**, **VI**) represent higher energy forms, as is usually encountered in the heterocyclic chemistry of six-membered rings in polar solvents (40–42), but drawing conclusions without knowledge of the spectra of the iminol tautomers is perhaps premature. On the other hand, a well-known empirical rule ("Jones Rule") states that the spectra of enolate anions of keto tautomers resemble the enol tautomers. This correlation has been shown to hold for cytosine (**X**) (43) and for the ionization of N-3 of uracil (**IX**) (44), despite the fact that ionization at the N-3 position (N-1 in the



X

case of cytosine) leads to extensive delocalization of the electron pair involved.† The virtual identity of the 450-nm maximum of flavin, flavin anion, and a methyl blocked 2-iminol tautomer has been demonstrated (46) in aqueous solution (47).‡ We therefore think it safe to assume from the longest wavelength peaks of **III** and its ionized form that the iminol tautomers of **III** (Scheme **II**) do not show lower energy transitions in their spectra than the keto tautomer (as **III** is formulated in this paper). Simple tautomerism does not seem to provide a spectrum for **III** that approaches that of the enzyme-bound species that appears in time between 4a-hydroperoxyflavin and 4a-hydroxyflavin. This casts doubt on the proposal that the appearance of the intermediate supports the "catalysis by ring opening" approach of Scheme **I**.

It is of course possible that, when **I** is enzyme bound its spectrum is different than in solution. Certainly at the active site of an enzyme there can be presented to a bound molecule a heterogeneous solvent milieu that cannot be reproduced in the laboratory by use of pure or mixed solvents. It should be noted, however, that the spectra of enzyme-bound flavins and their 4a-hydroperoxy and hydroxy derivatives do not vary greatly from the spectra of these species in solution. Of further consequence may be steric constraints, either in the model or imposed by the apoprotein on the enzyme bound intermediate. They are difficult to assess in an experimental way. Perhaps the models exist in solution with an extended conformation but at the active site of the enzyme in a closed conformation (as drawn herein). Steric effects may be of crucial consequence if the excited state of the ring-opened intermediate were to be of a "twisted intramolecular charge transfer type" (refs. 48 and 49; citations in ref. 49). These considerations aside, the present results do not support the mechanism of Scheme **I**.

This work was supported by grants from the National Institutes of Health and the National Science Foundation.

†Conclusions concerning the electron distribution (i.e., predominance of a certain resonance structure) from this empirical correlation of UV data would be unjustified (43, 46) and are therefore not intended.

‡This is at variance with theoretical calculations that predict a red shift (47).

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