

Oxaziridines as Possible Intermediates in Flavin Monooxygenases

(oxaziranes/pteridines/molecular orbital calculations)

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ABSTRACT The enzymatic hydroxylation reactions of flavin monooxygenases are suggested to involve an oxaziridine as the active oxygenating agent. The chemistry of these monooxygenases and their nonenzymatic model systems are consistent with an oxaziridine intermediate. Several advantages of the oxaziridine model over the previously proposed "carbonyl oxide" and flavin peroxide models are discussed.

Oxygenases (1) are enzymes that catalyze the incorporation of molecular oxygen into various organic substrates. Two subclasses are recognized: dioxygenases, which catalyze the incorporation of both atoms of molecular oxygen into the substrate, and monooxygenases, which catalyze incorporation of one oxygen atom into the substrate with concomitant reduction of the other to water. All dioxygenases contain either heme or inorganic iron as cofactors, whereas a variety of cofactors (pyridine and flavin nucleotides, pteridine derivatives, ascorbic acid with copper, and heme) have been associated with monooxygenases (2).

Ternary complexes of oxygen, enzyme, and substrate are well known for several mono- and dioxygenases containing metal cofactors (1), and recently similar intermediates have been observed for monooxygenases with non-metal cofactors (3, 4). However, the means by which these non-metal-containing monooxygenases bind and activate molecular oxygen have been the subject of much speculation (5). We propose here an oxaziridine as the form of activated oxygen for the flavin-cofactored monooxygenases§ and compare it to the previously proposed flavin peroxide intermediate of Mager and Berends (6) and the "carbonyl oxide" intermediate of Hamilton (5).

Flavin as the Site of Oxygen Activation. Many of the flavin monooxygenases, such as *p*-hydroxybenzoate hydroxylase (7), salicylate decarboxylase (8), imidazoleacetate monooxygenase (9), lysine oxygenase (10), and lactic oxidative decarboxylase (11), have been crystallized and found to be metal-free. The oxygenating cycle of these enzymes is shown in Fig. 1. The enzyme with the oxidized flavin (10-*R* isoalloxazine)

Abbreviations: MO, molecular orbital; HMO, Hückel molecular orbital; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; EXTHUC, extended Hückel; SCF, self-consistent field.

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§ The structural similarity of the flavin nucleotides to pteridine derivatives allows the extension of the oxaziridine model to the pteridine-cofactored monooxygenases as well.

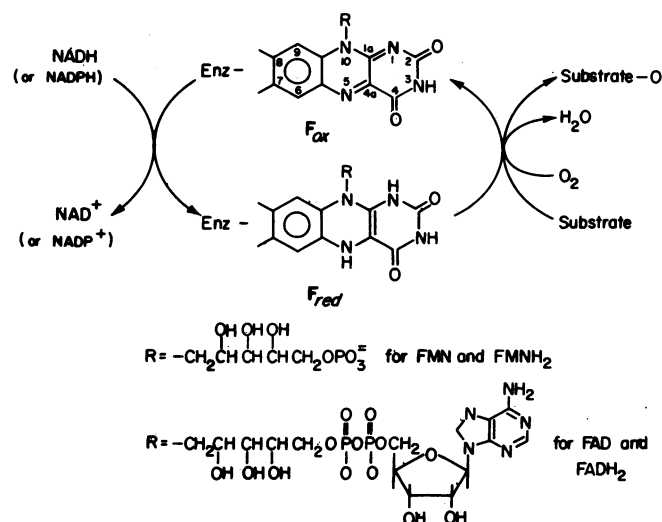


Fig. 1. Oxygenation cycle for flavin monooxygenases.

moiety (F_{ox}) is reduced to (F_{red}) either by an external reductant, such as NADH or NADPH, or by the substrate itself. The reduced flavo-enzyme with the bound substrate reacts with molecular oxygen to produce the monooxygenated substrate, water, and the intact oxidized flavo-enzyme ready for another catalytic cycle.

Although no reduced flavin-oxygen adduct has yet been fully characterized (12), experimental work, molecular orbital (MO) calculations, and model system analogies all suggest such an intermediate. Indeed Hastings *et al.* (13) have recently isolated and spectrally characterized a reduced flavin-oxygen adduct. Mager and Berends (6) have shown that reduced flavins can hydroxylate certain aromatic substrates nonenzymatically in the presence of O_2 . The work of Hemmerich *et al.* (14) has attested to the high reactivity of the 4a flavo carbon and Muller's (12) work on the reaction of flavins with alcohols has demonstrated reactivity at carbon 1a. Moreover, kinetic studies by Massey *et al.* (3, 15) on the reaction of reduced flavins with O_2 also strongly support an oxygenated flavin intermediate.

Molecular orbital (16) calculations on reduced flavin moieties also suggest their suitability as the enzymatic site of oxygen fixation. Although the EXTHUC (Extended Hückel) calculations of Malrieu and Pullman (17) and the self-consistent field (SCF) calculations of Fox *et al.* (18) indicate that the energy of the highest occupied molecular orbital (HOMO) varies slightly with the geometrical configuration about N_5

TABLE 1. Flavin HOMO energy variation as a function of geometrical configuration about N_5 and N_{10}

	Planar			10 intra 5 extra ^a		10 extra 5 intra		Observed (20)
	HMO (16)	EXTHUC (17)	SCF (18)	EXTHUC	SCF	EXTHUC	SCF	
ΔE (HOMO - LUMO) in eV	2.89	3.15	2.30	4.51	3.48 (2.92) ^b	4.17	3.81 (3.18)	3.10 ^d
HOMO Energy (β) ^c	-0.10	-0.03	-0.29	+0.39	+0.07 (-0.10)	+0.29	+0.17 (-0.03)	(-0.05)

^a For explanation of terminology see refs. 17 and 18.

^b Parenthesized numbers were obtained using trigonal parameters at N_5 and N_{10} , whereas with the nonparenthesized numbers tetrahedral parameters were used (18).

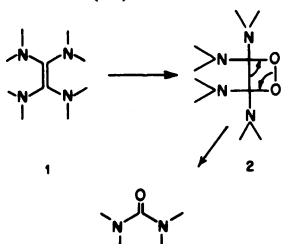
^c The SCF β values were obtained using a β -value to -3.26 eV (see ref. 18) and noting that the LUMO (lowest unoccupied molecular orbital) energy level is invariant with configurational changes, remaining at $\alpha - 0.99$ (see ref. 17).

^d Shoulder.

and N_{10} [†], all MO calculations, regardless of the level of sophistication, show the energy of the HOMO to be nonbonding or slightly antibonding (Table 1). Such an energetically high HOMO makes the reduced flavin an attractive target for electrophilic molecular oxygen.

Moreover, our calculations[‡] (Fig. 2) show that the HOMO electron density distribution, the π charge distribution, and the total charge distribution favor oxygen attack at carbons $4a$ and $1a$ (actually, these factors all give preference to initial attack at $4a$), as shown in Fig. 2.

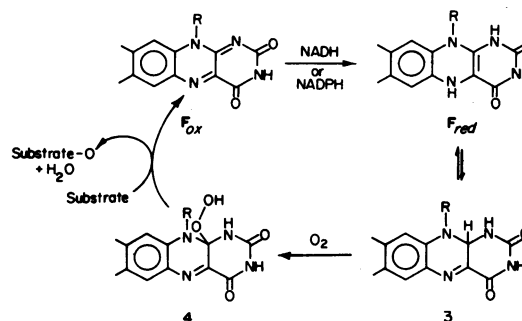
Furthermore, the structural similarity of the reduced flavins to tetraaminoethylenes should also be noted. The great majority of tetraaminoethylenes act as strong electron donors (22). The well-studied tetrakis(dimethylamino)ethylene, **1**, reacts vigorously with molecular oxygen, and in the presence of a proton source decomposes, giving tetramethylurea as the major product. This reaction is best envisioned as the reaction between the olefin **1** and oxygen to give the dioxetane **2**, which then collapses to the urea (23).



The reduced flavin might similarly react with oxygen to give

[†] The x-ray crystal structure of a reduced flavin, 5-acetyl-3,7,8,10-tetramethyl-1,5-dihydroalloxazine, has been determined. The analysis confirms the nonplanarity about N_5 and N_{10} predicted by the extended Hückel and SCF calculations. See ref. 19. Considering the HOMO's energetic fluctuation with geometry, it is conceivable that the enzyme regulates oxygen fixation by controlling the flavin geometry at N_5 and N_{10} .

[‡] Calculations were carried out using a revised version of the EXTHUC program originally described in ref. 21, with the following parameters: bond lengths; all ring bonds 1.40 Å, C—H 1.09 Å, C=O 1.23 Å, C—CH₃, 1.54 Å; all bond angles 120° except for the tetrahedral methyl groups; coulomb integrals (eV): $2s_C$, -21.34 ; $2s_N$, -27.50 ; $2s_O$, -35.30 ; $2p_C$, -11.54 ; $2p_N$, -14.32 ; $2p_O$, -17.91 ; hydrogen Slater exponent, 1.2; Wolfsberg-Helmholz k factor, 1.75.

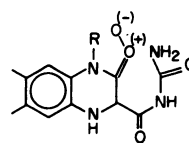


SCHEME 1. Oxygen activation mechanism proposed by Mager *et al.* (6).

the dioxethane. However, in this case another mode of decomposition (*vide infra*) is available.

Mechanism of Oxygen Activation by Flavins. The oxygen activation mechanism for flavins proposed by Berends and Mager (6) involves the flavin peroxide **4** as the hydroxylating agent, as shown in Scheme 1. The reduced flavin is postulated to be in equilibrium with its tautomer **3**, which reacts with molecular oxygen to give the flavin peroxide **4**, Berends and Mager's postulated hydroxylating agent. However, there is no evidence to date for such tautomerization of reduced flavins and, considering the accompanying interruption of cross conjugation, such tautomerization and further reaction is unlikely. Additionally, Hamilton (5) has pointed out that, in the absence of metals, alkyl hydrogen peroxides are not hydroxylating agents. He therefore suggests an alternate mechanism (Scheme 2) involving the "carbonyl oxide" intermediate **6**. Initial oxygen addition at carbon $4a$ ** gives the intermediate **5**. Rearrangement of **5** as depicted in Scheme 2 gives the carbonyl oxide intermediate **6**. Reaction between substrate and the "oxenoid-" like terminal oxygen of **6** would

** The possibility of a $1a$ carbonyl oxide has also been discussed by Hamilton (5).



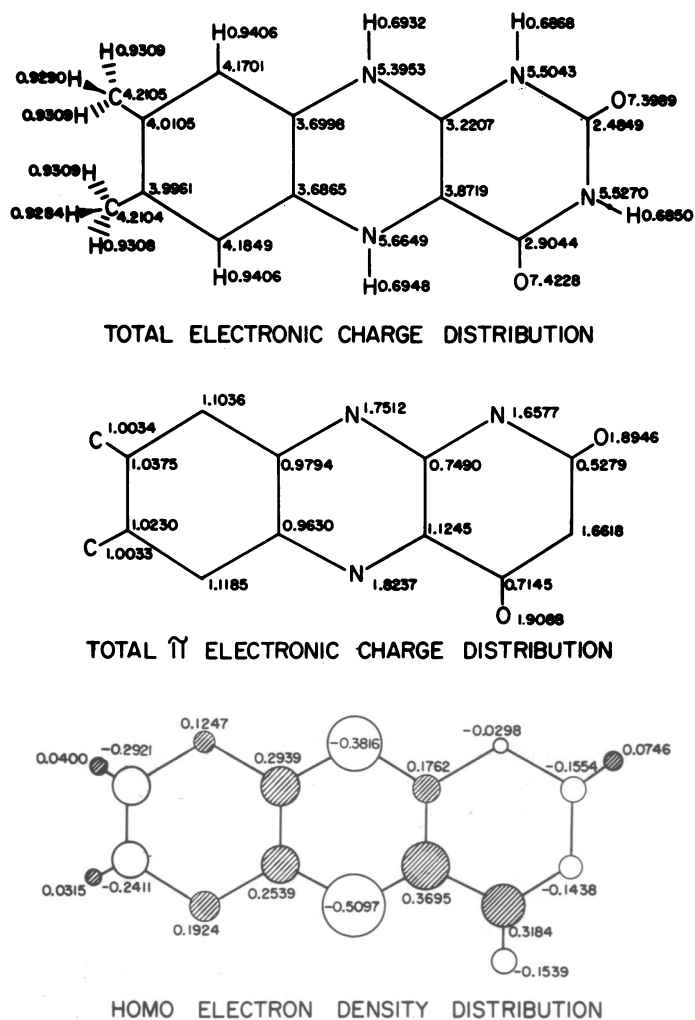
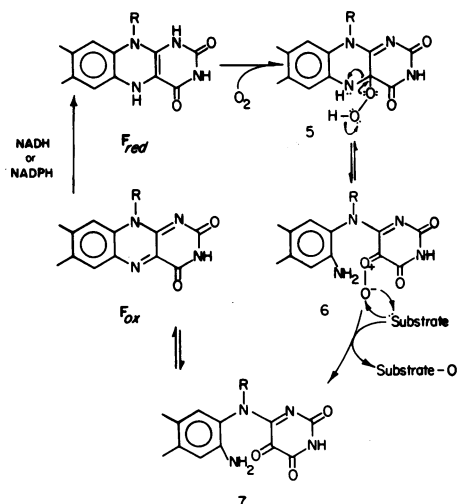


Fig. 2. Results of extended Hückel calculations on reduced isoalloxazine.

result in an oxygenated substrate and the flavin derivative 7. Finally, intramolecular Schiff's base formation would regenerate the oxidized flavin (F_{ox}).

Hamilton postulates that the terminal oxygen in θ is highly electrophilic if one considers the resonance forms $6c$ - $6e$ shown



SCHEME 2. Oxygen activation mechanism proposed by Hamilton (5).

in Fig. 3, and that enzymatic protonation of the heterocyclic ring would further enhance the electrophilicity of the terminal oxygen. These resonance forms must, however, inherently assume planarity of the oxygen moiety with the ring. Non-planarity would detract from the electrophilicity of the terminal oxygen by diminishing the electron density "drainage" by the heterocyclic ring. Furthermore, our Hückel calculations $\dagger\dagger$ on the planar system (Fig. 4) show that there is a high π electron density at the terminal oxygen and that the π bond order of the oxygen-oxygen bond is comparable to the carbonyl π bond orders. This suggests that resonance structures $6a$ and $6b$ are important contributors, and that the terminal oxygen is not likely to be electrophilic. Moreover, the opening of the C_{4a} - N_5 bond not only appears to be an uneconomical step but would result in the formation of a highly oxidizable *ortho*-phenylenediamine system, whose stability in close proximity to the site of activated oxygen is questionable. Although it could be argued that enzymatic intervention prevents self-hydroxylation of θ from occurring, no such rationalization can be offered to explain the results of Mager and Berends (6), who observed the continuous, nonenzymatic consumption of molecular oxygen by 10-methylisoalloxazine in the presence of NADH.

$\dagger\dagger$ The following parameters were used in the Hückel calculations: coulomb integrals; $C(\alpha)$, $O(\alpha + \beta)$, $N(\alpha + 1.5\beta)$ except for N_5 ($\alpha + 0.5\beta$); all resonance integrals taken as 1β .

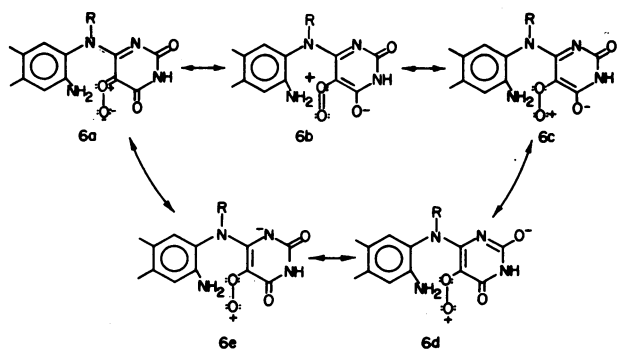
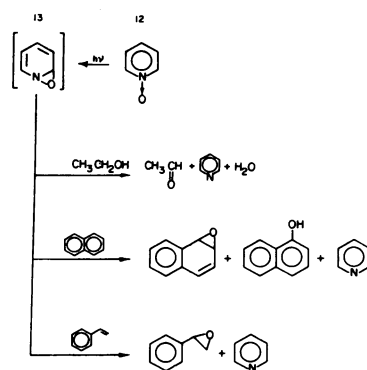
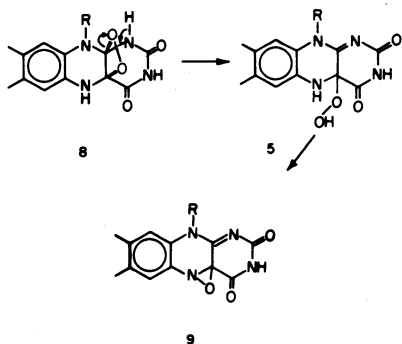
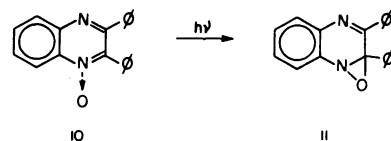


FIG. 3. Resonance forms of "carbonyl oxide" 6.

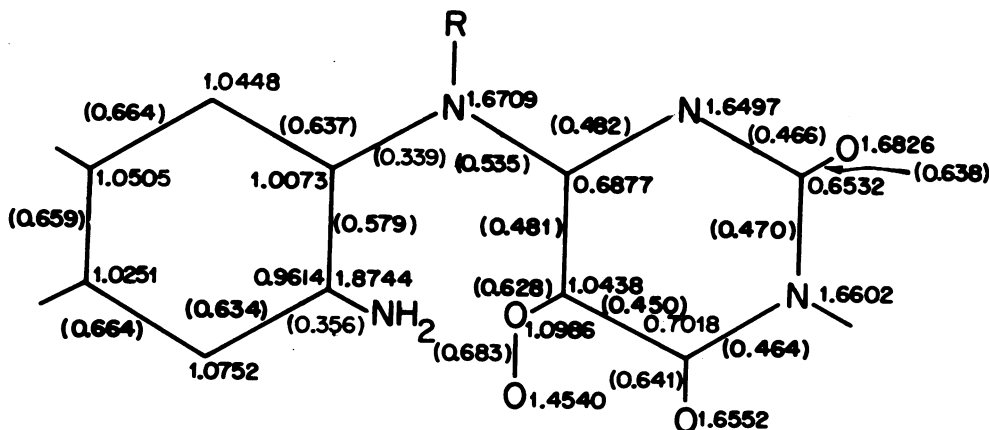
Since the likelihood of both the flavin peroxide and the carbonyl oxide as candidates for the enzymatic hydroxylating agent can be questioned, we have sought other possibilities. Although the formation of a flavin peroxide (at either 1a or 4a) via a tautomer of a reduced flavin appears unlikely, one can readily envisage its formation from the dioxetane 8 derived from the reduced flavin by a reaction analogous to that with tetrakis(dimethylamino)ethylene. For while the fully substituted dioxetane 8 has few paths available for its further reaction, 8 by virtue of the N₁ proton might react as follows to give the previously proposed flavin hydroperoxide intermediate 5. However, rather than have this hydroperoxide act as the hydroxylating agent, or have it open up to the carbonyl oxide, we suggest that a further intramolecular displacement, assisted by enzymatic protonation, occurs to give the oxaziridine 9 (24), and that it is this oxaziridine which serves as the hydroxylating agent.

FIG. 5. Photolysis of pyridine-*N*-oxide in the presence of oxidizable substrates.

Little is known concerning the chemistry of oxaziridines (25). A number of stable aliphatic oxaziridines have been prepared, most commonly from the peracid oxidation of Schiff's bases (24), and from the photolysis of imine-*N*-oxides (26). More recently, stable arene oxaziridines have been prepared by the photolysis of pyridine-*N*-oxides (27). Thus upon irradiation 2,3-diphenyl-quinoxazine-1-oxide 10 gives a high yield of the corresponding oxaziridine 11 (28). It has so far not been possible to isolate the oxaziridine from the photolysis of pyridine-*N*-oxide 12 itself.

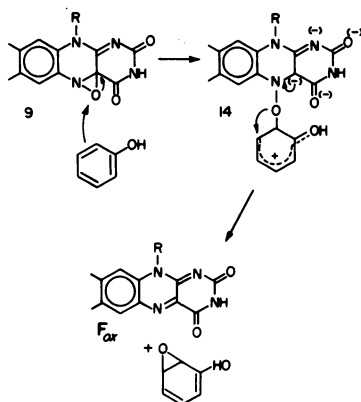


However, the course of this and related photolyses (29, 30) strongly implicates the pyridine oxaziridine 13 as an intermediate. It is of interest to note that the photolysis of pyridine-*N*-oxide in the presence of naphthalene gives both naphthalene oxide and α -naphthol (30), a reaction that in terms of both the intermediate oxide and the hydroxylated product appears to mimic exactly the biosynthetic course of aromatic hydroxylations (Fig. 5). Furthermore, Calvin and Alkaitis (31) have argued that the formation of large amounts of acetaldehyde during the photolysis of pyridine-*N*-oxide in ethanol necessitates the formation of a powerful, pyridine oxaziridine, 13, oxidant.



π ELECTRON DENSITIES (σ BOND ORDERS)

FIG. 4. Results of Hückel π -calculations on planar "carbonyl oxide" 6.



SCHEME 3. Oxidation of phenol via flavin oxaziridine.

The reactivity of the flavin oxaziridine **9** is expected to parallel that of the arene oxaziridines, since in both cases loss of oxygen is accompanied by regeneration of the conjugated system. Furthermore, we anticipate that the oxygen of the oxaziridine **9** will be electrophilic^{††}, since attack of the nucleophile at the oxygen will generate a *highly* stabilized carbanion **14** that can react further to give the oxidized flavin (F_{ox}) (Scheme 3). It should be noted that throughout the whole of this scheme the skeletal framework of the flavin remains intact.

Summary. Experimental work, molecular orbital calculations, and model system studies all indicate the ability of reduced flavins to bind molecular oxygen. Formation of a flavin hydroperoxide **5** is suggested, by analogy with tetrakis(dimethylamino)ethylene, to arise from the dioxetane adduct **8**. Further intramolecular reaction of **5** could lead to the flavin oxaziridine **9** which, by semblance to the chemistry of model oxaziridines, is proposed to be the active oxygen form of the flavin monooxygenases.

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^{††} That oxaziridines contain an electrophilic oxygen atom, and are oxidizing agents, has been shown by Emmons, who was able to titrate oxaziridines with potassium iodide (32).

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