BIOORGANIC CHEMISTRY 6, 421–429 (1977)

On the Mechanism of Oxygen Activation by Tetrahydropterin and Dihydroflavin-Dependent Monooxygenases

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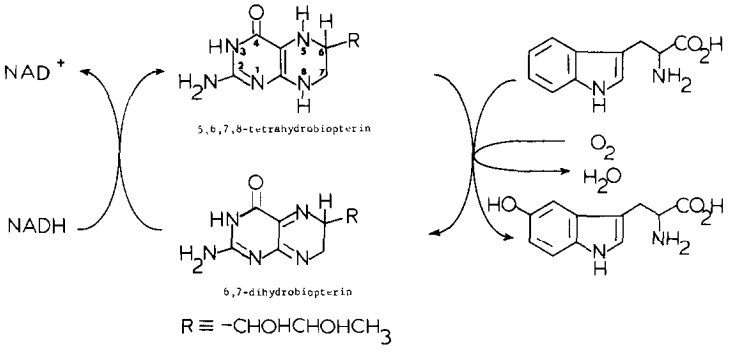
Received February 10, 1977

A brief critical survey of the current theories of molecular oxygen activation in the hydroxylation of aromatic compounds by dihydroflavin- and tetrahydropterin-dependent monooxygenases is presented. A reinterpretation of the available data has resulted in the proposal of a new mechanistic hypothesis. It is suggested that the dihydroflavin (or tetrahydropterin) cofactor interacts with ground-state molecular oxygen to give a C_{4a}–C_{10a} singlet perepoxy dihydroflavin (or a C_{4a}–C_{8a} perepoxy tetrahydropterin) intermediate which functions as the oxenoid reagent in this class of biological hydroxylations.

The hydroxylation of the benzenoid aromatic ring of each of the aromatic amino acids phenylalanine, tyrosine, and tryptophan is a key step in the metabolism of these substances in animals and some bacteria (*1*). The monooxygenases which catalyze these reactions utilize molecular oxygen and a tetrahydropterin cofactor as illustrated in Scheme 1. In mammalian brain tissues these enzymes catalyze essential steps in the biosynthesis of the neurotransmitter serotonin and the catecholamines and as a result play an important role in normal brain function.



SCHEME 1. Tetrahydropterin-dependent monooxygenases (tryptophan-5-monooxygenase).

In bacterial systems the hydroxylation of aromatic substrates is catalyzed by a related group of monooxygenases which utilize a dihydroflavin as cofactor, as illustrated in Scheme 2 (*2*). Because of the obvious structural similarity of tetrahydropterins and dihydroflavins, it is generally assumed that the mechanisms of hydroxylations catalyzed by these two classes of enzymes are very similar (*3*).

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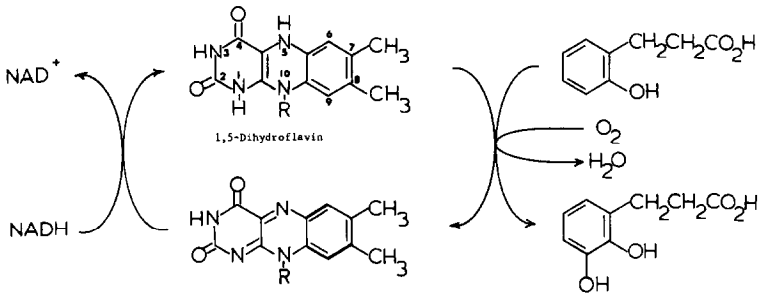
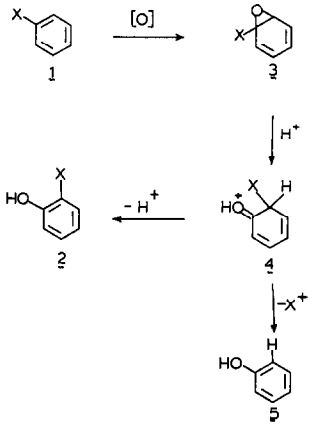
ISSN 0045–2068



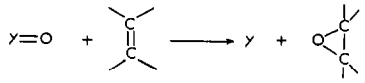
SCHEME 2. Dihydroflavin-dependent monooxygenases (melilotate hydroxylase).



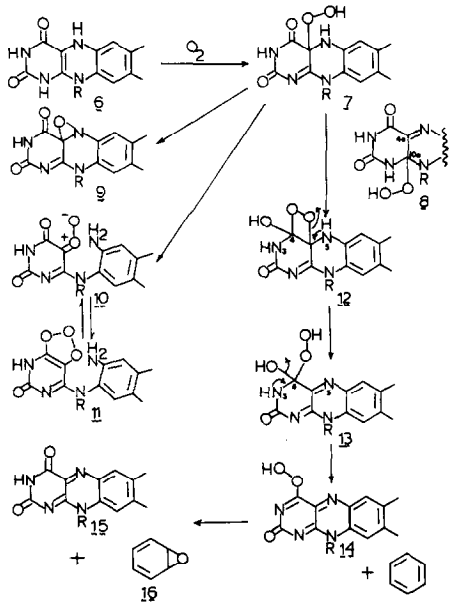
SCHEME 3. The NIH shift.

Enzymatic hydroxylations of the aromatic amino acids have been shown to be accompanied by a phenomenon called the NIH shift, illustrated in Scheme 3 (*1*). An aromatic substrate bearing a substituent (often deuterium or tritium) at the normal site of hydroxylation gives a product which retains the substituent (to varying degrees depending upon the nature of X) bound to a carbon atom adjacent to the site of hydroxylation. There is now a large body of chemical evidence which suggests that the NIH shift phenomenon is a consequence of the intermediacy of an arene oxide **3** which rearranges as shown in Scheme 3. The extent of retention of X in **2** is dependent upon the relative ease of loss of X^+ or H^+ from the intermediate **4** (*4*). The formation of arene oxides such as **3** strongly suggests that the actual oxidizing agent in these monooxygenase catalyzed reaction is an oxenoid reagent (Y=O); that is, one which transfers a single oxygen atom in a single step as shown in Scheme 4.

Since no NIH shifts accompany aromatic hydroxylations mediated by dihydroflavin at oxidation, they are poor models for enzymatic hydroxylations (*5*).

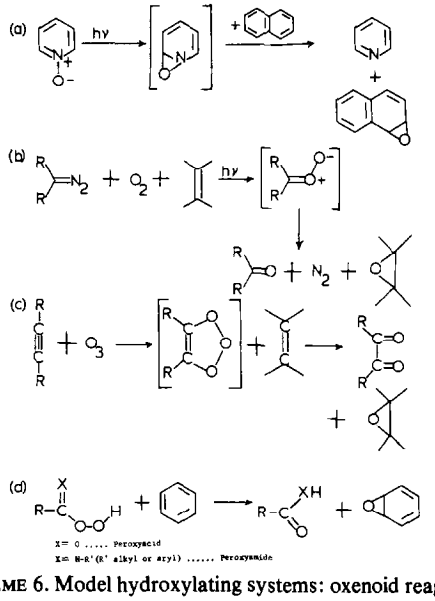


SCHEME 4. Oxenoid reaction.



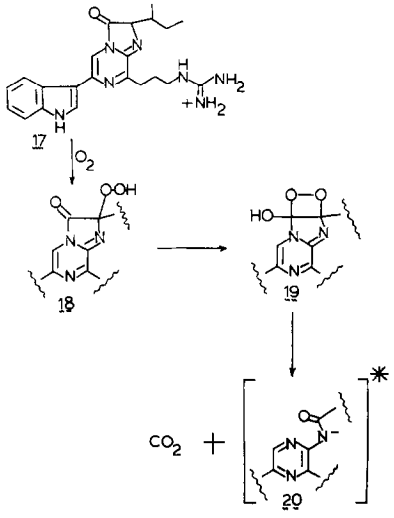
SCHEME 5. Hypothetical oxenoid intermediates for flavin-dependent monooxygenases.

Since neither ground-state molecular oxygen nor any of its known reduced forms (HOO·, H_2O_2, HO·) are capable of functioning as oxenoid reagents, it has been quite naturally assumed that the oxenoid reagent in these systems is some form of oxygen–dihydroflavin (or oxygen–tetrahydropterin) adduct. Rapid reaction studies with *p*-hydroxybenzoate hydroxylase have resulted in the detection of a modified flavin intermediate, very likely a flavin–oxygen adduct (*2*). Some of the current hypotheses regarding the structure of the oxenoid species involved in these systems are outlined in Scheme 5.



SCHEME 6. Model hydroxylating systems: oxenoid reagents.

Mager and co-workers have suggested that hydroperoxide **7** or **8** is the hydroxylating species (*6*). Their hypothesis has been criticized by others who have pointed out that there is no chemical analogy for the hydroxylation of aromatic compounds by alkyl hydroperoxides in the absence of metal ions (*7, 8*). As an alternative, Orf and Dolphin have suggested that an oxaziridine such as **9** is the oxenoid species in this class of biological hydroxylation (*8*). In support of this mechanistic proposal they cite the reported formation of arene oxides in the photolysis of pyridine-*N*-oxide in the presence of aromatic substrates. They suggest that a transient pyridine–oxaziridine is the oxenoid species in this model system and that this is a reasonable chemical analogy for their hypothetical mechanistic scheme for the flavin-dependent monooxygenases (see Scheme 6). Hamilton has pointed out that no well-characterized oxaziridine has been shown to possess oxenoid properties (*9*). Furthermore, the photolysis of pyridine-*N*-oxide is a complex, poorly understood reaction and, consequently, the oxenoid properties of the putative pyridine–oxaziridine are not firmly established. Hamilton has suggested that a chemically more reasonable oxenoid species is a carbonyl oxide such as **10** (Scheme 5) or its ring-closed tautomer **11** (*9, 10*). Evidence which suggests that carbonyl oxides can function as oxenoid reagents comes from the studies of photosensitized interaction of diazo compounds with singlet oxygen in the presence of aromatic substrates such as naphthalene as illustrated in Scheme 6 (*11*). The presence of carbonyl oxides can be detected chemically in this system and the isolation of arene oxides as products suggests that carbonyl oxides have oxenoid character. Recently it has been demonstrated that a product formed in the low-temperature ozonolysis of certain acetylenes is a potent oxenoid reagent (*10*). There is good reason to believe that the intermediate formed in this ozonolysis is the ozonide shown in Scheme 6c and this is suggestive evidence for the oxenoid character of a species such as **11** of Scheme 5.

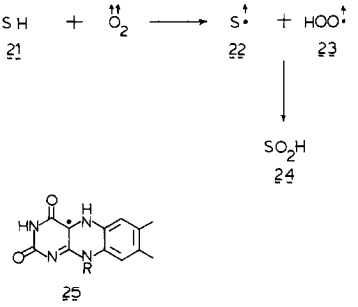


SCHEME 7. Cyprinda luciferin.

Recent studies in our own laboratory have been concerned with yet another possibility. We have been considering the possibility that the hydroperoxide **7** might react as do other α-hydroperoxy amides to give the dioxetane intermediate **12.** Cyprinda luciferin **17** is known to form an α-hydroperoxy amide **18** which undergoes subsequent

reaction via a dioxetane intermediate **19** to give an excited-state amide **20** which reverts to its ground state by light emission, as illustrated in Scheme 7 (*12*). For the dioxetane **12** there is another possible mode of ring-opening. Ring-opening with the assistance of the lone pair electrons of the adjacent N_5 could give the hydroperoxide **13** which, upon dehydration, would yield the peroxyimidic acid **14**. Peroxy acids are the only well-characterized organic compounds which have been shown to hydroxylate aromatic compounds with a substantial NIH shift as shown in Scheme 6d (*13*). The closely related peroxyimidic acids also shown in Scheme 6 are known to be somewhat more powerful oxenoid reagents than peroxy acids (*14*). An interesting consequence of this mechanism, as illustrated in Scheme 5, is that one oxygen atom of molecular oxygen is incorporated into the hydroxylated substrate and the other is incorporated into the C_4 carbonyl group of the cofactor. This observation forms the basis of experiments utilizing $^{18}O_2$, which are now in progress.

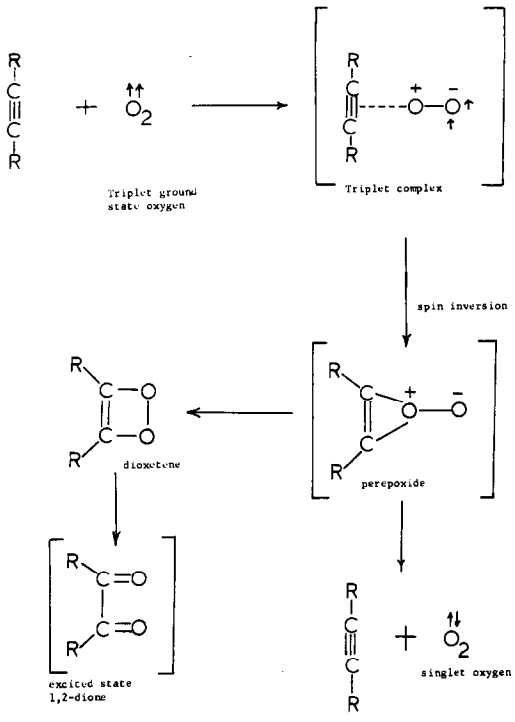
At present there is insufficient experimental evidence with which to confirm or reject the intermediacy of any of the hypothetical oxenoid reagents shown in Scheme 5 and no doubt still other potential oxenoid intermediates can be suggested for these systems.



SCHEME 8

Comparatively few mechanistic possibilities have been considered for the first step in the formation of these putative oxidizing agents, namely, the direct reaction of molecular oxygen and the reduced cofactor. The limitations on the mechanistic possibilities for the interaction of ground-state triplet oxygen with organic substrates have been outlined succinctly by Hamilton (*3*). Since the spin-inversion process required for the direct interaction of a triplet species such as ground-state oxygen and a singlet species such as ground-state aromatic substrates to give a ground-state hydroxylated product is normally a slow process (1 to 10^{-9} sec) on the time scale of normal organic reactions (10^{-13} sec), it has generally been assumed that the initial step of "oxygen activation" involves instead the formation of two radical or doublet species (Scheme 8). For example, in the case of flavin-dependent enzymes, recombination of a flavin radical such as **25** with the superoxide **23** would give the hypothetical hydroperoxide intermediate **7** which could yield one of the oxenoid species shown in Scheme 5 or some other oxidizing agent.

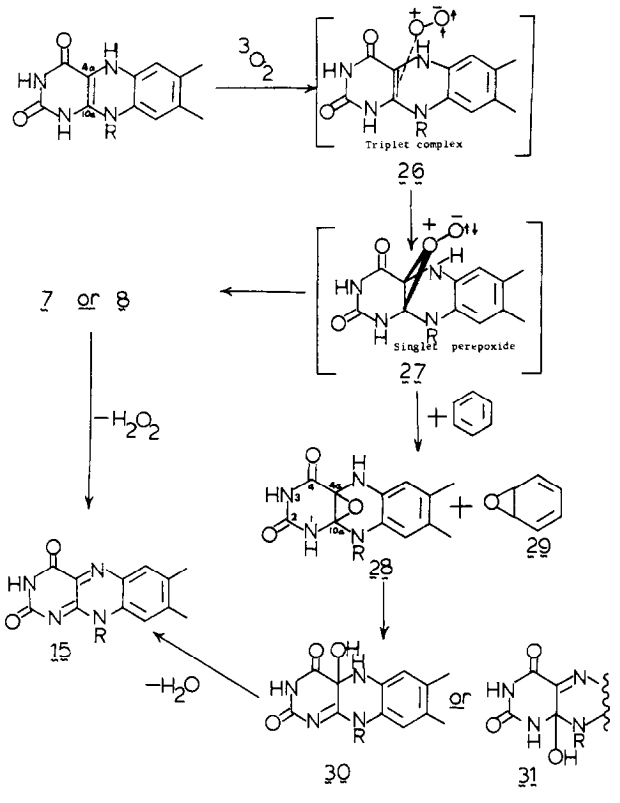
Very recently, there has been an important development in the organic chemistry of ground-state molecular oxygen which has caused us to reconsider the hypotheses upon which the mechanistic schemes for pterin- and flavin-dependent hydroxylations have been based. Turro and co-workers have demonstrated that ground-state triplet oxygen can interact with organic molecules, strained cyclic acetylenes in particular, to generate



SCHEME 9

singlet oxygen as illustrated in Scheme 9 (*15*). It has been suggested that one of the Π-bonds of the acetylene molecules studied interacts with triplet oxygen in such a way as to generate a triplet complex in which both unpaired electrons of molecular oxygen are localized on a single oxygen atom of the complex, as indicated in Scheme 9. Theoretical considerations suggest that this situation would favor spin inversion and such a complexation should lower the activation free energy and the time required for such a spin inversion (*16*). The singlet complex formed is a perepoxide which in the cases studied by Turro collapses to a dioxetene which can ring open to an excited-state dione or lose singlet oxygen and regenerate the ground-state alkyne.

We suggest that just this type of spin-inversion process may occur with ground-state molecular oxygen and enzyme-bound dihydroflavin or tetrahydropterin cofactors in monooxygenase-catalyzed hydroxylations of aromatic compounds. If the C_{4a}–C_{10a} double bond of a dihydroflavin (see Scheme 10) were to interact with a triplet oxygen molecule in a fashion analogous to the strained cyclic alkynes studied by Turro, then the singlet perepoxide **27** (Scheme 10) would be formed via a triplet complex such as **26**. Clearly a species such as **27** could be envisaged to undergo tautomerism to one of the hydroperoxides, **7** or **8**, which could then be converted to an oxenoid species as indicated previously (see Scheme 5). Loss of hydrogen peroxide from **7** or **8** to give the flavin **15** would explain the observed formation of hydrogen peroxide in the absence of an appropriate oxidizable substrate (*1*, *2*). However, there is reason to believe that such perepoxides have chemical properties of much more profound significance to the mechanism of flavin- or pterin-dependent monooxygenases.



On the basis of recent molecular orbital (MINDO-3) calculations Dewar *et al.* have predicted that perepoxides, which can be generated by the interaction of olefins with singlet oxygen, should be rather powerful oxenoid reagents (*17*). If this is indeed the case, then the perepoxide **27** formed by direct interaction of a dihydroflavin with triplet oxygen could be the oxenoid species involved in this class of biological oxidation. An oxenoid-type reaction of **27** with an aromatic compound would give an arene oxide **29** and a C_{4a}–C_{10a} epoxy flavin **28**. Ring-opening of the epoxide **28** with assistance of the lone pair of electrons on N_1 or N_5 to give **30** or **31**, respectively, followed by dehydration of **30** or **31** would give the observed flavin product.

This mechanistic proposal is an attractive one for several reasons:

(1) It is entirely consistent with the observed stoichiometry.

(2) Since it involves an oxenoid type of reaction, it is consistent with the observed NIH shift phenomenon.

(3) The oxenoid species is formed in a very simple and direct fashion. A one-step orbital interaction between the reduced cofactor and ground-state molecular oxygen yields the oxenoid reagent.

(4) Oxygen activation involves minimal structural alteration of the cofactor (i.e., no ring-openings or skeletal rearrangements are required).

(5) The observed oxidized cofactor structure **15** arises in a direct and natural manner from the epoxy flavin **28** formed immediately upon singlet oxygen atom transfer to the substrate.

(6) The formation of hydrogen peroxide by such enzyme systems in the absence of a suitable substrate is readily explained by the loss of H_2O_2 from the hydroperoxide **7** or **8** formed by simple tautomerism of the perepoxide **27**.

(7) The failure of superoxide dismutases to inhibit monooxygenases of this class is readily understandable since superoxide is not an intermediate. To interpret these observations in terms of other mechanisms of oxygen activation which invoke the intermediacy of superoxide, it must be argued that the superoxide which is formed remains in the active site, inaccessible to superoxide dismutases (*1*).

Clearly, much experimental effort must be expended before any of the hypothetical mechanistic schemes proposed previously or those proposed herein can be considered to be realistic representations of biological hydroxylations of aromatic substrates. We have presented this discussion because we feel that it is of the utmost importance to consider all of the implications of the known organic chemistry of molecular oxygen in devising experiments aimed at the elucidation of the mechanisms of biological oxidations.

Subsequent to the submission of this manuscript we became aware of the very recent report by McCapra and Leeson (*18*). They have proposed that a hydroperoxide such as **8** actually exists as a quasi-perepoxide in which there is a substantial bonding interaction between the C_{4a}- and the C_{10a}-bound oxygen and that this is the oxenoid species involved in flavin-dependent monooxygenase reactions. It is important to note that although McCapra and Leeson have not suggested a mechanism for formation of their putative oxenoid reagent, their mechanistic proposal is similar to ours to the extent that the structural differences between the perepoxide **27** and their quasi-perepoxide are very subtle.

ACKNOWLEDGMENTS

The authors wish to thank the National Research Council of Canada and the Ontario Mental Health Foundation for financial support of this work, and M. Parniak and F. Widmer for helpful discussions.

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