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Aromatic Hydroxylations by Flavins: Evidence on Direct Attack of Phenylalanine by Flavin Radical Species.

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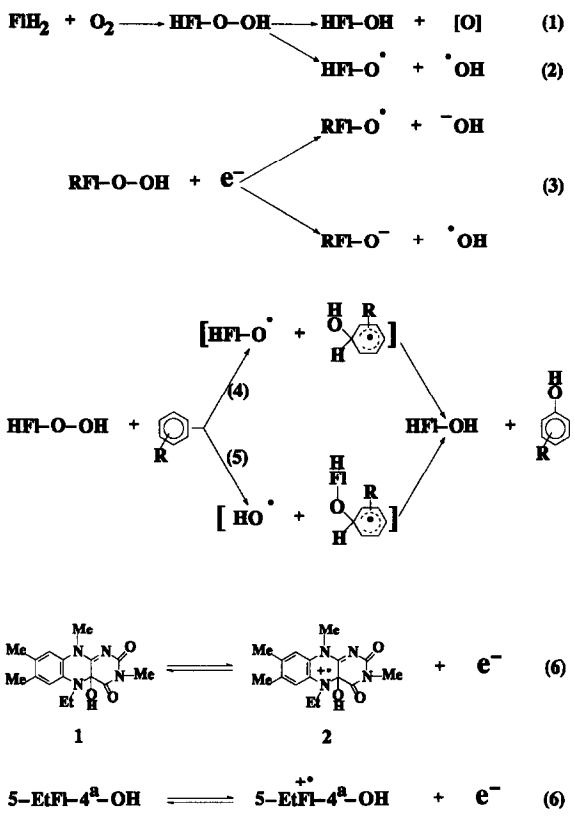
Abstract: In 0.05 - 12.0 N acidic solutions, the 5-ethyl-3-methyllumiflavosemiquinone **5** (and/or $\mathbf{5H^+}$) spontaneously arose from the corresponding flavinium cation **4**. Raising the temperature from 20 to 50 °C, considerably increased the reaction rates with no significant changes in the yields of **5** ($\mathbf{5H^+}$). The spontaneous one-electron reduction of **4** requires a coupling with a one-electron oxidation of another flavin such as 5-ethyl-4[a]-hydroxy-3-methyl-4[a],5-dihydroflavin pseudobase **1**. The latter, being in equilibrium with **4**, can be oxidized to give the transient 5-ethyl-4[a]-hydroxy-3-methyllumiflavin radical **2**. This is the protonated form of a flavinoxy radical **3**[a,b], a product of a homolysis of the O-O bond in a dihydroflavin hydroperoxide. As an alternative to the homolysis mentioned, the one-electron oxidation of **1** provides the principle to develop a new hydroxylating model system that does not require a dihydroflavin hydroperoxide as a starting compound. Using phenylalanine as a test substrate, the *anaerobic* formation of tyrosine and its *o*- and *m*-hydroxyphenylalanine isomers was established. This achievement is a strong experimental support for the hypothesis that flavin radical species like **2** may directly attack an aromatic. Evidence was obtained on some accumulation of an intermediate that is not a hydroxycyclohexadienyl radical. It was shown to react in a secondary, oxidative chain reaction, remarkably increasing the yields of aromatic hydroxylation without any further supply of flavin.

Introduction

In 1962, some monooxygenases were first reported to require a dihydroflavin[1] or a tetrahydropteridine[2] as a cofactor. The mechanistic aspects did not receive any particular attention until Mager et al.[3a-3e] published a hypothesis on the activation and transfer of oxygen. The activation of O_2 was proposed to occur through a covalent bonding at the cofactors (e.g., FlH_2= dihydroflavin; Eq.1, Scheme 1) to give cofactor-hydroperoxides (HFl-OOH). In recycling the cofactor, the intermediacy of a corresponding pseudobase (HFl-OH) was established. The hydroperoxides were formulated to react as the precursors of H_2O_2 or as key-intermediates in "coupled oxidations," e.g., in the hydroxylation of an aromatic.[4a-4i]

Systems, consisting of a dihydroflavin in the presence of O_2 and/or H_2O_2, could convert the test substrate phenylalanine to a mixture of tyrosine and its *o*- and *m*-hydroxyphenylalanine isomers. In particular cases, the yields remarkably exceeded two moles of hydroxyphenylalanines per mole of starting dihydroflavin.[4d-4f] A homolysis of the O-O bond (Eq. 2) or, more readily an assisted homolysis (Eq. 3) in the case of an N^1- or N^5-substituted flavin (generally abbreviated as RFl-OOH), would result in two types of hydroxylating radicals.[4f] In acidic solutions, the N^1- and N^5-alkylated dihydroflavinoxy radicals (RFl-$O^\bullet$) were

already concluded to be the more reactive, hydroxylating species. For comparison, in the absence of flavins, HO$^{\bullet}$ radicals were generated through Fenton's reagent or the homolysis of H_2O_2. In contrast to the flavin systems, lower yields of hydroxyphenylalanines were obtained in acidic solution and relatively higher yields in the pH range of 5 - 7. In ESR studies[5] on the hydroxylation of pyromellitic acid by HO$^{\bullet}$ radicals, using Fenton's reagent, the hydroxycyclohexadienyl radical could clearly be trapped while no such transient was observed using the RFlOOH models.



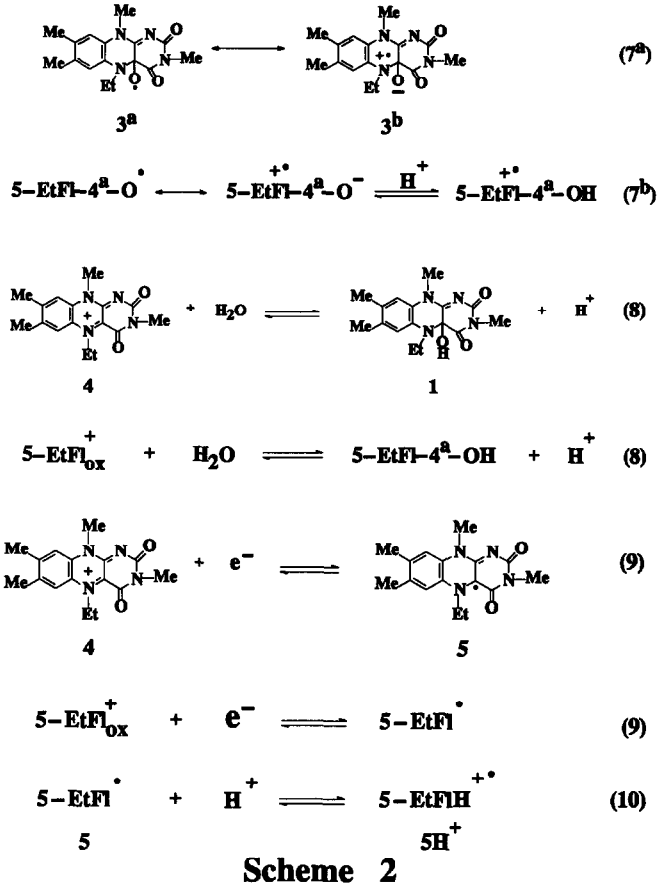
Scheme 1

The first proposal on a homolysis or an assisted homolysis of the flavin peroxide bond (Eqs. 2, 3) was not readily considered to be relevant to the mechanism of enzymatic monooxygenation. About two decades after the first publication, however, this reluctance has apparently disappeared. A homolysis (Eq. 2) is now argued to be thermodynamically very favorable.[6] Spectral findings were ascribed to the occurrence of a hydroxycyclohexadienyl radical (Eq. 4),[7] but this interpretation was questioned.[8] Instead of the HO$^{\bullet}$ radical, a flavinoxy radical was formulated to primarily attack the substrate to give a flavin-substrate adduct tentatively formulated as a flavinoxycyclohexadienyl radical (Eq. 5).

The existence of N-centered flavin radical species was substantiated by oxidizing N^1- and N^5- alkylated flavin adducts carrying a hydroxy or alkoxy substituent at the 10^a- and 4^a- position, respectively.[9] One-electron oxidation of 5-ethyl-4^a-hydroxy-3-methyl-4^a,5-dihydrolumiflavin **1** (Eq. 6) provides a radical **2**. It has become a major compound for further studies as a mechanistic model for the 4^a-hydroxydihydroflavin radical, proposed to be the key-intermediate in bacterial bioluminescence.[10] The radical **2** can be prepared chemically,[9d,e; 10a,b] electrochemically[11] and, as recently reported, in a reaction with $N_3^{\bullet}$ radicals generated by pulse radiolysis.[12] It is well defined by its redox potential, spectrum, the reversibility in an electrochemical process and by a rapid

conversion to the corresponding flavinium cation or flavosemiquinone.

Merényi *et al.*[12] have pointed out that the O- and N-centered flavin radical formulations actually represent resonance structures ($3^a \leftrightarrow 3^b$; Eq.7a) of the same radical. It implies that **2** is the protonated form of the radical generally formulated in Eq. 3 as the O-centered flavin radical RFl-O$^\bullet$ (R= 5-Et). A more complete representation is given in Eq. 7b (Scheme 2). Consequently, it should be possible to construct a new hydroxylating system that does not require a dihydroflavin or a dihydroflavin hydroperoxide (Eqs. 1-3) as the starting compound. In principle, the flavin-4^a-pseudobase **1**, subjected to a one-electron oxidation (Eq. 6), should fulfil the requirement. Basically, it would open the way to prove the postulate that a 4^a-hydroxydihydroflavin radical like **2** could directly attack an aromatic substrate. The first attempt to realize such a new model system will be dealt with in the present paper. The radical **2** was chosen to be generated chemically since the application of the electrochemical and pulse radiolytic techniques would also involve the formation of HO$^\bullet$ radicals.



Scheme 2

Results and Discussion

The pseudobase **1** is in equilibrium[9e;13] with the 5-ethyl-3-methyllumiflavinium cation **4** (Eq. 8; pK'_a= 4.15 in 0.1 M citrate buffer).[9e] Therefore, both **1** and **4** are directly applicable as starting compounds to achieve a spontaneous formation of the radicals **2** and **5**, formulated as the combination of the two redox reactions

represented by the Eqs. 6 + 9 ⇒11a. Their redox potentials were determined in acetonitrile and water on the basis of which the overall process (Eq. 11a) does not seem to be thermodynamically favorable.[11,12,14] In spite of this expectation, however, radical formation (Eq. 11a) does occur spontaneously both in apolar and polar

$$\underset{1}{\text{5-EtFl-4}^{a}\text{-OH}} + \underset{4}{\text{5-EtFl}_{ox}^{+}} \xrightarrow{(11a)} \underset{2}{\text{5-EtFl-4}^{a}\text{-OH}^{+\bullet}} + \underset{5}{\text{5-EtFl}^{\bullet}}$$

environments, apparenly due to faster, consecutive conversions of **2** (**2** disappears in water at room temperature within milliseconds[12] and in acetonitrile in less than two minutes[11]). In aqueous solution, in the pH 2-7 range at 23 °C, radical formation takes place with a slow to moderate rate. A relative initial rate / pH profile was found to be in agreement with the mechanism as given by Eqs. 8 and 11a.[9e] In the present study, excess amounts of phenylalanine were added to react with the radical **2**. This helps further to drive the radical formation (Eq. 11a) to the right.

In the pH 4-5 range, under anaerobic conditions, the flavosemiquinone **5** arose with a yield of 50 ± 3% of the total flavin consistent with the maximal theoretical value as expected from Eq. 11a. The pseudobase radical cation **2** decomposed to ring-contracted (non-flavin) products and to relatively small amounts of 3-methyllumiflavin.[9e, 15] Upon lowering the pH, the ring rearrangements of **2** decreased in favor of a rapid cleavage of the C^{4a}-OH bond ultimately resulting in a maximal recycling of **4**. Consequently, the final yields of **5**(**5H**$^+$) have now been found to approach the theoretical yield of 100% (Eq. 11b). Based on this principle, a formation of **5**(**5H**$^+$) was accomplished in a variety of solvents[9; 10a,b] but the identity of the oxidized components of the reaction mixture, as required by the stoichiometry of the process (Eq. 11b), has remained unresolved.

$$\underset{4}{2\ \text{5-EtFl}_{ox}^{+}} + H_2O \xrightarrow{(11b)} \underset{5H^{+}}{2\ \text{5-EtFlH}^{+\bullet}} + [O]$$

Anaerobically, as illustrated in Figs. 1 - 3, the process was followed by monitoring the appearance of the specific spectrum of **5** or **5H**$^+$, or a mixture of the two species, dependent on the acidity of the reaction medium (Eq. 10, the apparent pK'_a= 2.55, in 0.1 M citric acid).[9e] The calculations were performed using the molecular absorbances published earlier.[9d,e; 16]

The spontaneous spectral changes of an anaerobic solution of the flavinium cation **4** in 0.05 N H_2SO_4, containing an excess of phenylalanine (8 x 10^{-2} M), are demonstrated in Fig. 1. The starting concentration of **4** (curve a) was in the range of 1 - 4 x 10^{-4} M. Due to the excess of phenylalanine, the solution had a rather high pH of 2.74. This explains the formation of a mixture of **5** and **5H**$^+$ with a final,

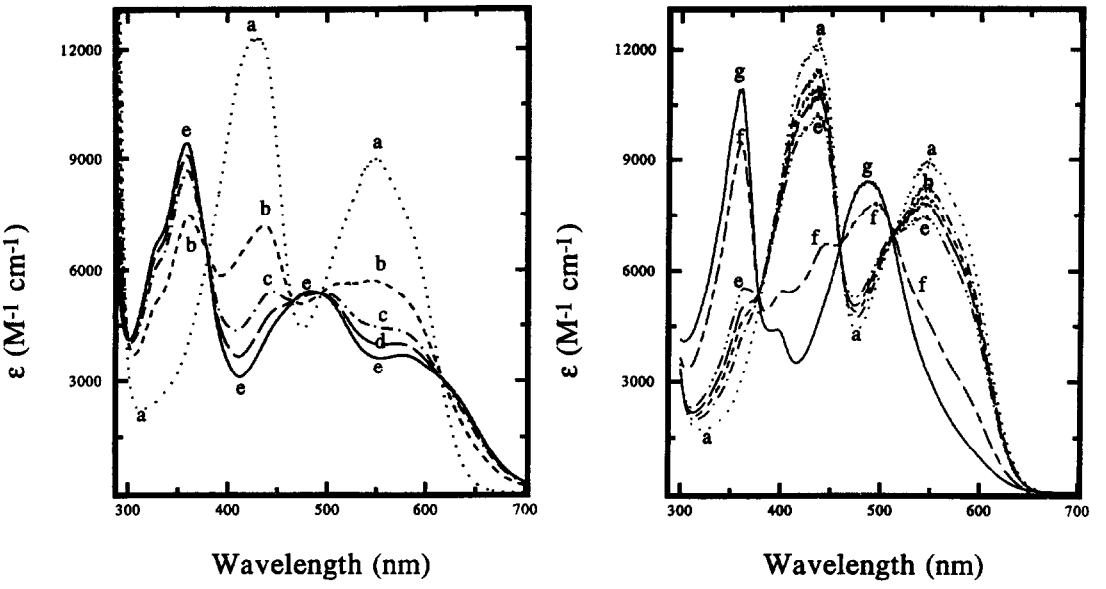


Fig. 1. Spontaneous, anaerobic formation of a mixture of **5** and **5H**$^+$ at 50 °C in 0.05 N H_2SO_4 containing **4**,ClO_4^- (1 - 4 x 10^{-4} M) and phenylalanine (0.08 M); pH= 2.74. The spectra were taken at reaction times of (**a**) 0 (**b**) 60 (**c**) 120 (**d**) 150 and (**e**) 210 min. Isosbestic points at: 300, 379, 466, 494 and 617 nm.

Fig. 2. Spontaneous, anaerobic formation of **5H**$^+$ at 23 °C in 6.0 N H_2SO_4 containing **4**,ClO_4^- (1 - 4 x 10^{-4} M) and phenylalanine (0.08 M). The spectra were taken at reaction times of (**a**) 0 (**b**) 70 (**c**) 130 (**d**) 190 and (**e**) 240 min; (**f**) 24 and (**g**) 48 h. Isosbestic points at: 300, 378, 458 and 514 nm.

combined yield of 90 ± 5% (curve e). Such findings were verified by neutralizing or acidifying the mixture, to give exclusively either **5** or **5H**$^+$ and by determining the recovery of **4** following the oxidation of **5H**$^+$ with HNO_2.

The transient spectra in Fig. 1 show five isosbestic points which are not all shared by the starting spectrum ***a*** or the final curve ***e***. This is connected with the build-up and, eventually, the disappearance of a transient complex. Particularly at low reaction rates (e.g. at 4 - 20 °C) in an aqueous environment, it was observed that some complex first arose in a low steady state concentration before the isosbestic points were detected (cf. Fig. 2). A transient complex could be accumulated in acetonitrile.[10e]

A process as in Fig. 1 took about 3 days at room temperature (20 - 23 °C) to be completed. The reaction rate was considerably enhanced (to about 3 hours for completion) by raising the temperature to 50 °C, with no significant changes in the yields of the **5**(**5H**$^+$).

Mixtures of **5** and **5H**$^+$ arose in the 0.05 - 0.2 N acid range, but in the 0.5 - 12 N acid range only **5H**$^+$

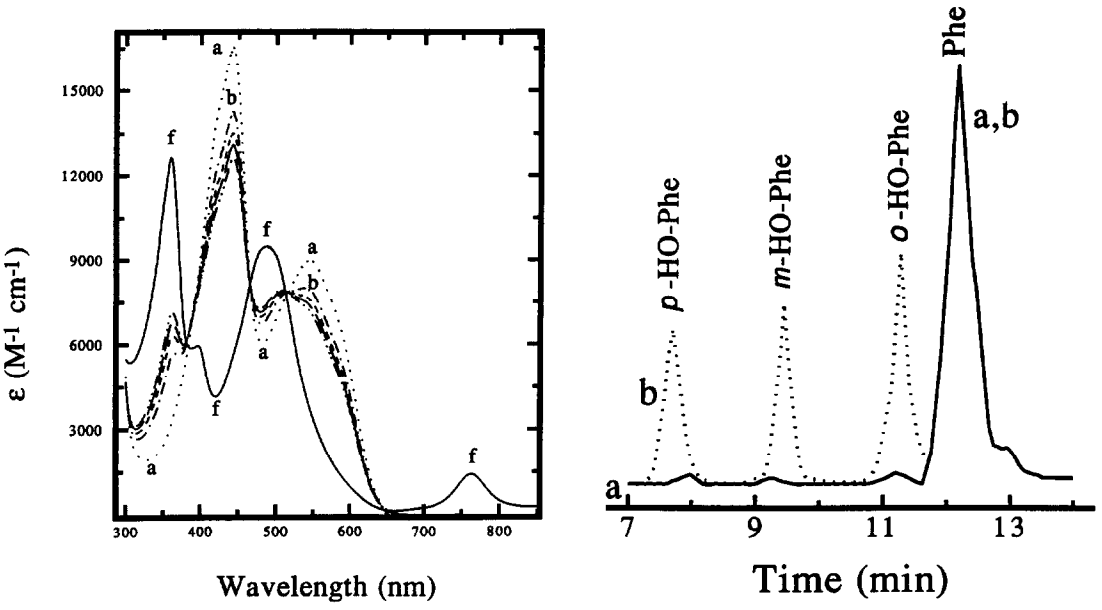


Fig. 3. Spontaneous, anaerobic formation of $\mathbf{5H^+}$ at 23 °C in 12.0 N H_2SO_4 containing **4**,ClO_4^- (1 - 4 x 10^{-4} M) and phenylalanine (0.08 M). The spectra were taken at reaction times of (**a**) 0 (**b**) 30 (**c**) 60 (**d**) 75 and (**e**) 90 min; (**f**) 24 h. Isosbestic points at: 300, 384, 465 and 515 nm.

Fig. 4. HPLC analyses (25 µl sample; flow rate= 0.8 ml/min; absorbance mode, detection at 275 nm) of: (a) a control solution of D-phenylalanine (8 x 10^{-2} M) showing contaminations at or near the retention times of *p*-, *m*- and *o*-hydroxyphenylalanine in the order of 1.5 x 10^{-6} M, 1.0 x 10^{-6} M and 2.0 x 10^{-6} M, respectively; (b) a solution of D-phenylalanine (8 x 10^{-2} M) after the anaerobic conversion of **4** (3.0 x 10^{-4} M) to $\mathbf{5H^+}$, the subsequent extraction of **5** and, eventually, the oxidative chain reaction increasing the total production of hydroxyphenylalanines to a value of HOPhe/Fl= 0.31 (cf. example 1).

appeared with a yield of 95 ± 5% (Figs. 2-3). Consistently, in the 0.5 - 12.0 N acid range, the transient spectra went through four isosbestic points. With reference to Fig 2 it is noted that in a similar experiment, but in 6 N HCl, the spontaneous formation of flavin radicals was finished about twice as rapidly as in 6 N H_2SO_4. This suggests that there is an anion effect; an interaction of anions with flavin intermediates was already observed in earlier studies.[4b, 9]

Unexpectedly, considering the equilibrium between **4** and **1** (Eq. 8), the spontaneous formation of $\mathbf{5H^+}$ (Eq. 11b) proved to proceed even in very strong acidic environment as in 12 N H_2SO_4 (Fig. 3). Actually, the rate of the accumulation of $\mathbf{5H^+}$ increased upon increasing the acidity of the solution from 0.05 N to 12.0 N H_2SO_4. While the spontaneous formation of $\mathbf{5}(\mathbf{5H^+})$ in the presence of phenylalanine,

comparable to Fig. 1, took three days in 0.05 N H_2SO_4 at 23 °C, the process in 12.0 N H_2SO_4 was completed in about six hours (Fig. 3). This further indicates an anion effect as well as the possibility of a different pathway in the conversion of a key transient in a more acidic environment. The more rapid formation of $\mathbf{5H^+}$ in very strong acidic aqueous solutions exhibits some similarity with the conversion obtained upon adding a strong acid to the pseudobase radical **2** (or derived product) accumulated in low polar, aromatic environment.[10a,b] The latter immediately produced $\mathbf{5H^+}$ implying that this conversion must have been coupled with the oxidation of another compound e.g. the hydroxylation or dimerization of an aromatic substrate.

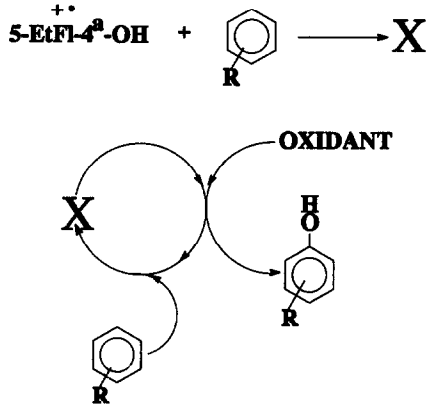
An unidentified side product was formed in 12.0 N H_2SO_4 (curve f, Fig. 3) showing a remarkable absorption maximum at long wavelength (762-764 nm) and increased absorbances near the absorption maxima of $\mathbf{5H^+}$. Therefore, the yields of $\mathbf{5H^+}$ could be overestimated by 5 - 10% if they are determined solely on the basis of the absorbances at 358-360 and 488-490 nm. Since the side product slowly arose from $\mathbf{5H^+}$, it is not considered to be of further relevance in the context of these studies.

Using very sensitive bio- and chemi-luminescent assay methods,[9e] we did not find any indication on a formation of either free O_2 or H_2O_2 to account for "the missing oxygen" [O] in the stoichiometry of the process (Eq. 11b). This implies that the "missing oxygen" has to be looked for in products derived from either the flavin or other compounds present. Aromatics like phenylalanine and benzoic acid were added as test substrates. The high yields of $\mathbf{5H^+}$ indicated that mainly the test substrates had been converted, possibly, to hydroxylated or dimeric derivatives or to products following an attack of the substrate side-chain. This paper deals with the search for the production of hydroxyphenylalanines.

When the spontaneous formation of $\mathbf{5H^+}$ had reached an optimal value, the pH of the reaction solution was adjusted to 3.0 - 3.5 to shift the equilibrium (Eq. 10) to the side of **5**. The flavosemiquinone **5**, which accounted for 85-95% of the total initial flavin, was completely removed by extraction with $CHCl_3$.

Subsequently, HPLC analyses of the flavosemiquinone-free solutions indeed revealed the presence and the amounts of tyrosine and its *m*- and *o*-hydroxyphenylalanine isomers as illustrated in Fig. 4. The formation of *m*- and *o*-hydroxyphenylalanine was also checked using both 2,6-dichloroquinone-4-chloro-imide[17, 18] and Folin Ciocalteu's reagent[18, 20] and of tyrosine with both 1-nitroso-2-naphthol[19] and Folin Ciocalteu's reagent.[18, 20] The results, expressed as "HOPhe/Fl" values (the totals of *p*-, *m*- and *o*-hydroxyphenylalanines per starting flavin molecule), fluctated from 0.01 to 0.09 for the reactions in 0.05 - 4.0 N H_2SO_4, increasing to the range of HOPhe/Fl= 0.08 - 0.20 for the process in 6 N H_2SO_4. These data were corrected with the analyses of the appropiate flavin-free controls. *It should be emphasized that these results were obtained anaerobically in aqueous solutions containing **4** and phenylalanine as the only starting compounds!* HOPhe/Fl= 0.5 is the theoretical value, based on the stoichiometry of the overall, anaerobic process (Eq. 11b). The experimental findings, in the range of HOPhe/Fl= 0.01 - 0.20, correspond to 2 - 40% yields of anaerobic hydroxylation, increasing with the acidity of the media.

Besides the hydroxyphenylalanines, formed anaerobically, the flavosemiquinone-free aqueous solutions contained an interesting intermediate which was accumulated in rather low yields (< 10%), consistent with the high recoveries of 5-EtFl$^{\bullet}$. This product **X**, which remains to be identified, acted as a key intermediate in a secondary, oxidative formation of hydroxyphenylalanines (Scheme3) by which it was first detected as described below.



Scheme 3

The solutions, obtained after the optimal formation of **5(5H$^+$)**, the subsequent adjustment of the pH to 3.0 - 3.5 and the removal of **5**, were aerobically stored at 4 °C and regularly subjected to HPLC analyses over a period of 3 - 4 weeks. Surprisingly, the contents of hydroxyphenylalanines slowly increased, in a particular experiment (following a radical formation in 6 N H_2SO_4 under N_2 at 23 °C) from HOPhe/Fl= 0.13 to 0.31 (Fig. 4) and, in another one, from HOPhe/Fl= 0.01 to 0.98 (following a radical formation in 0.1 N H_2SO_4 under N_2 at 23 °C). The final distribution of *p*- : *m*- : *o*-hydroxyphenylalanine isomers was 29 : 31 : 40.

The secondary, oxidative process has the nature of a chain reaction (Scheme 3), since the HOPhe/Fl findings could considerably surpass the maximal, theoretical value of 0.5 (Eq. 11b). This was achieved without any further supply of **2** or the starting flavins **1** and **4**. In the case where the HOPhe/Fl ratio changed to a value of 0.98, a 100-fold increase in the overall yield of hydroxylation was obtained.

The occurrence of aromatic hydroxylation through the protonated radical **2** implies that the latter undergoes a scission of the C^{4a}-OH bond. No indications have been found that such a bond cleavage would anaerobically give a free HO$^{\bullet}$ and, subsequently, a free hydroxycyclohexadienyl radical to disproportionate to the hydroxyaromatic and the aromatic in a 1 : 1 ratio. However, it is possible that a homolysis of the C^{4a}-OH bond in **2** concurs with a bonding of the substrate within a cage to result in a new type of flavin adduct **X**, structurally different from the one proposed in Eq. 5 (Scheme 1). An appreciable stability in acidic solutions and negative peroxide tests do not favor the possibility that **X** would be some 4^a-peroxyflavin species formed simultanously with the flavosemiquinone in a disproportionation of two 4^a-hydroxyflavin radicals. Although, for steric reasons, relatively higher percentages of *p*- and *m*- hydroxyphenylalanine might be expected to arise from a primary attack of the aromatic by **2**, the consecutive chain reaction probably causes the *p*- : *m*- : *o*-hydroxyphenylalanine isomer distribution to reach a value close to the ratio shown by flavin-free HO$^{\bullet}$ controls.

Since a subsequent oxidation of **X** can lead to a substantial secondary production of the hydroxylated aromatic, the nature of the oxidant is expected to greatly influence the course of this process. Further studies to demonstrate an effective role for H_2O_2 and $HO^{\bullet}$ as a secondary oxidant and to further characterize **X** are under way.

Conclusion

Flavinoxy and $HO^{\bullet}$ radicals are the products of homolysis of the O-O bond in a dihydroflavin hydroperoxide (Eq. 2). Early studies[4a,d-f] already suggested a primary role for the flavinoxy radical in the hydroxylation of aromatics, particularly, in acidic environment. For a further substantiation of this, such flavin species had to be generated without a simultaneous formation of $HO^{\bullet}$. This required a construction of a new flavin model system in which a dihydroflavin hydroperoxide is no longer involved. Alternatively, the one-electron oxidation of a 4^a-hydroxy-dihydroflavin pseudobase **1** to the flavin pseudobase radical cation **2** (Eq. 6), being the protonated form of a flavinoxy radical, was shown to fulfil the requirement.

N^5-alkylflavins can undergo a spontaneous conversion to the corresponding flavosemiquinone and the pseudobase radical cation **2** (Eq. 11a). Anaerobically, in the additional presence of phenylalanine, tyrosine and its *m*- and *o*-hydroxyphenylalanine isomers were formed in yields varying from 2 to 40%, increasing with the acidity of the solutions. Besides the formation of hydroxyphenylalanine, an intermediate **X** (Scheme 3) was detected which showed an ability to further react in an oxidative chain reaction considerably increasing the yields of hydroxylation. It is briefly noted that hydroxylation is not restricted to phenylalanine. Comparable results were obtained using other aromatics like benzoic acid, which is converted to a mixture of *p*-, *m*- and *o*-hydroxybenzoic acid.

A 4^a-hydroxydihydroflavin radical (cf. **2**) has already been proposed as a key intermediate in bacterial bioluminescence. Although acting differently from a mechanistic point of view, we believe that the same type of flavin radical, arising in a homolysis of the O-O bond in a dihydroflavin-4^a-hydroperoxide, plays a key role in flavin mediated monooxygenation of aromatics, primarily in a direct attack of the substrate. Aromatic hydroxylations should be distinguished from the monooxygenations of nucleophilic substrates such as secondary hydroxylamines and amines, tertiary amines and alkyl sulfides. These reactions, which do not appear to involve a flavin radical as an attacking intermediate, were succesfully mimicked by Bruice *et al.*[21] starting from a heterolysis of the O-O bond in 5-ethyl-4^a-hydroperoxy-3-methyl-4^a,5-dihydroflavin.

Experimental

Instrumentation. UV-spectra were recorded using a Milton Roy, Spectronic 3000 Array absorption

spectrophotometer. Anaerobic experiments were carried out by using a special apparatus made in this laboratory. It consisted of a 1 cm light path quartz cuvette fused to one or two compartments, provided with valves, allowing the reactants to be mixed at any moment after N_2 was flushed through for 1-3 h. The reaction volumes were 10 - 12 ml. Nitrogen was purified over a BASF R3-11 catalyst.

Tyrosine and its *m*- and *o*-hydroxyphenylalanine isomers were separated and determined using HPLC on a Waters μ Bondapak ™ C18 (39 x 390 mm) column; solvents A/B (9:1); flow rate= 0.6-1.0 ml/min; UV detection at 275 nm, absorption mode. Solvent A= 0.05 M CH_3COONH_4 in H_2O, pH was adjusted to 4.9 using CH_3COOH; solvent B= 0.1 M CH_3COONH_4 in H_2O (pH = 4.9) / CH_3OH (1:4 v/v). The results were corrected with the data from the appropiate controls (0.08 M solutions of phenylalanine in different acidic solutions without flavin and flavin solutions without phenylalanine) subjected to the same treatments. The determination of *m*- and *o*-hydroxyphenylalanine was also checked using both 2,6-dichloroquinone-4-chloro-imide[17, 18] and Folin Ciocalteu's reagent[18, 20] and of tyrosine with both 1-nitroso-2-naphthol[19] and and Folin Ciocalteu's reagent.[18, 20]

Chemicals and Reagents. 5-Ethyl-3-methyllumiflavinium perchlorate (**4**,ClO_4^-) and 5-ethyl-4^a-hydroxy-3-methyl-4^a,5-dihydrolumiflavin (**1**) were prepared as described in an earlier paper.[9d] The preparation of **4**,ClO_4^- was simplified as follows.

3-Methyllumiflavin (3.00 g; 11.1 mmol) was hydrogenated over 10% Pd-C (1 g) in a mixture of 96% ethanol (250 ml), H_2O (230 ml), concentrated HCl (20 ml) and acetaldehyde (20 ml) at room temperature and at atmospheric pressure. The catalyst was filtered off to give a light yellow-red filtrate, which was evaporated to dryness in *vacuo*. The residue was dissolved in 2 N $HClO_4$ (50 ml). Solid $NaClO_4$ (15 g) was added in small portions followed by $NaNO_2$ (5 g). The mixture was cooled at 10 °C with stirring for 2 h. The violet crystals were filtered off, washed with ice cooled water and dried in *vacuo* at room temperature over P_2O_5 to give a yield of 80%.

Phenylalanine and tyrosine (both in D-, L- and DL-forms) were purchased from Aldrich Chemical Co. DL-*m*- and DL-*o*-Hydroxyphenylalanine were bought from Sigma. D-Phenylalanine was preferred as a substrate because of the best purity in the HPLC-analyses (cf. curve a, Fig. 4). Stock solutions (0.08 M) were prepared in 0.05 - 12 N acidic solutions. The reaction volumes varied from 10 to 12 ml. The starting flavin concentration was in the range of 1 - 4 x 10^{-4} M.

The flavosemiquinone **5** was removed after the spectral changes under nitrogen had come to an end. A calculated amount of $NaHCO_3$ was added to adjust the pH to 3.0 - 3.5 using H_2O to dilute the reaction mixture. The dilution factor was kept as low as possible (≤ 2.5). The flavosemiquinone was removed by five extractions with 1-2 ml portions of $CHCl_3$ after which N_2 was blown through the aqueous layer to remove the $CHCl_3$-remnants. The aqueous solutions were aerobically stored at 4 °C.

The recovery of the flavinium cation **4** was spectrally determined in parallel experiments by injecting a 5 M $NaNO_2$ solution in H_2O (10 µl) to the acidic solutions of the flavosemiquinone causing its immediate oxidation.

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