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PRIMARY ARYLAMINE OXIDATION BY A FLAVINHYDROPEROXIDE

A STUDY OF THE BASIS FOR THE SUBSTRATE SPECIFICITY OF THE FLAVOPROTEIN MONOOXYGENASE

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Abstract—4a-Hydroperoxy-5-ethyl-3,8,10-trimethylisoalloxazine (FlEtOOH) was prepared as a chemical model for the flavin-containing monooxygenase enzyme of mammalian liver. FlEtOOH was found to oxidize a series of para-substituted primary arylamines to the corresponding arylnitroso compound. The rates of arylamine oxidation were found to correlate with the Hammett substituent constant, σ, as well as with amine basicity. These results suggest that amine nucleophilicity should be an important determinant of flavin monooxygenase reactivity toward primary arylamines; nevertheless, the enzyme demonstrates considerable substrate preference based on other factors.

Enzymatic N-oxidation of aromatic amines is accepted as an obligatory step in the production of genotoxic metabolites *in vivo* [1]. Arylhydroxylamines, arylnitroso compounds and arylhydroxamic acids are considered to be proximate genotoxic metabolites [1, 2]. The enzymes responsible for N-oxidation in mammals are the cytochrome P-450 (Cyt P-450) dependent oxygenases and the hepatic FAD containing monooxygenase (FMO) [3], although prostaglandin endoperoxide synthetase may also be involved in the oxidation of arylamines [4]. In general, the more basic nitrogenous compounds are substrates for FMO and less basic amines and amides are oxidized by Cyt P-450 oxygenases [5].

The catalytic mechanism of the FMO was elucidated by spectral studies and was shown to involve the formation of a hydroperoxy-FAD derivative in an NADPH and O_2 dependent reaction [6]. In subsequent studies, Bruice and co-workers demonstrated the transfer of oxygen equivalents from a synthetic hydroperoxyflavin model compound (4a-hydroperoxy - 5 - ethyl - 3,7,8,10 - tetramethylisoalloxazine) to secondary and tertiary amines at rates $\sim 10^4$ times greater than hydrogen peroxide. The reaction products were identical to those obtained from incubations with FMO [7, 8]. The results were consistent with a mechanism that involves nucleophilic attack of a secondary or tertiary nitrogen on the terminal oxygen of the hydroperoxyflavin. The products of the reaction were the corresponding 4a-hydroxyflavin and either a secondary hydroxylamine or tertiary amine N-oxide respectively [7, 8]. Primary aliphatic amines were not observed to be oxidized by the model hydroperoxyflavin and were not observed to be substrates for FMO. Primary aromatic amines have not been investigated in such a model system, although peroxyacetic acid oxidation of para-substituted anilines was studied by Ibne-Rasa and Edwards [9]. In general, few primary arylamines are known to be substrates for FMO [10].

From previous work in this laboratory on the N-oxidation of primary arylamines by chloroperoxidase [11] and pea seed peroxygenase [12], we thought the substrate specificity of FMO as described by Ziegler and co-workers to be rather unusual. Particular interest was given to the report that primary arylamines containing only a single aromatic ring were not substrates for N-oxidation by FMO [10]. The purpose of the work now described was to determine if selected primary arylamines react chemically with a model hydroperoxyflavin. Since we now report that primary arylamines do readily react with such a model hydroperoxyflavin (Fig. 1), it would appear that specific structural features at the active site of FMO preclude some of these compounds from serving as FMO substrates.

MATERIALS AND METHODS

All solvents were dried and purified by redistillation according to standard methods [13]. Anhydrous methanol was prepared by refluxing reagent grade methanol over magnesium turnings with a few iodine crystals followed by distillation [13]. Arylamines were obtained from Aldrich (4-phenetidine, 4-toluidine, 4-chloroaniline), Sigma (4-fluoroaniline), and Eastman (aniline hydrochloride). Arylamines were purified by distillation at reduced pressure, recrystallization, or silica gel chromatography. Arylnitroso compounds were synthesized as chromatographic standards by methods previously described [11]. 3,8,10-Trimethylisoalloxazine was synthesized by the procedure of Yoneda *et al.* [14]. The method of Ghisla *et al.* [15] was used to prepare 5-ethyl-3,8,10-trimethylisoalloxazinium perchlorate

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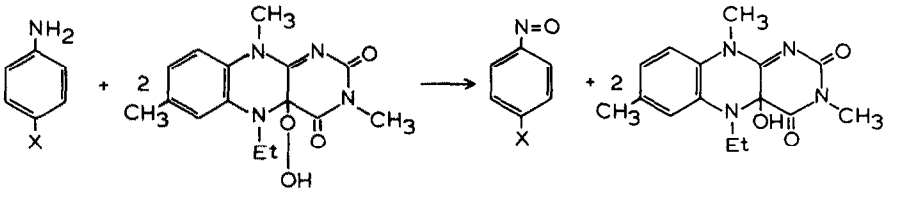

Fig. 1. Reaction of FlEtOOH with *p*-substituted aniline

($FlEt_{ox}$) which was reacted with H_2O_2 in a procedure similar to that of Kemal and Bruice [16] to form 4a-hydroperoxy - 5 - ethyl-3,8,10-trimethylisoalloxazine (FlEtOOH). FlEtOOH (m.p. 118–9°) was characterized by its electronic spectrum ($\lambda_{max}^{MeOH} = 367\,\mu m$; $\varepsilon = 8700\,M^{-1}\,cm^{-1}$, chemical ionization mass spectrum (319, M + 1; 302, M-15) and elemental analysis performed by Galbraith Laboratories (Found: C, 56.47; H, 5.81; N, 17.63. $C_{15}H_{18}N_4O_4$ requires C, 56.58; H, 5.70; N, 17.61).

Kinetic measurements were conducted under pseudo-first-order conditions in anhydrous methanol. Solutions of FlEtOOH were prepared in anhydrous methanol, and the peroxidative equivalent was determined iodometrically [16]. FlEtOOH was added to methanolic amine solutions at 30° such that the final concentration was 1.5 to 2.5×10^{-4} M. Kinetics were determined at 370 nm using a Beckman model 35 recording spectrophotometer in stoppered cuvettes.

Arylamine oxidation products were analyzed by high pressure liquid chromatography (HPLC) on a μBondpak C-18 column (Waters Associates) using the solvent systems listed in Table 1. The solvent flow rate was 1.5 ml/min, and component detections were made by u.v. absorbance at the wavelengths listed in Table 1. Quantitative calculations were made on the basis of component peak heights compared to peak heights generated by known amounts of authentic standards.

The flavin-derived reaction product, FlEtOH, was determined in anhydrous *t*-butanol. The excess arylamine concentrations used precluded a direct HPLC analysis of the FlEtOH; therefore, this product was quantitated following conversion to $FlEt_{ox}$ by reaction with 1 N HCl [8]. To 0.1 ml of a *t*-butanol solution was added 0.9 ml of 1 N HCl, and the product was quantitated spectrophotometrically at 555 nm ($\lambda_{max} = 555$ nm; $\varepsilon = 6500\,M^{-1}\,cm^{-1}$).

RESULTS

The reactions of aromatic amines with FlEtOOH were studied in anhydrous methanol at 30° by monitoring FlEtOOH disappearance at 370 nm. The kinetic determinations were made under the conditions $[ArNH_2] \gg [FlEtOOH]$ (>100-fold excess). The reaction was first order in FlEtOOH concentration, and psuedo-first-order rate constants (k_{obs}) were determined from plots of ln $[FlEtOOH]_t/[FlEtOOH]_o$ vs time. All plots showed good linearity to at least 90% completion of reaction. Plots of k_{obs} were linear with respect to arylamine concentration, and bimolecular rate constants were determined from the linear least squares slopes of plots typified by Fig. 2. These plots showed a positive non-zero intercept whose value agreed within experimental error to the independently determined rate constant for the spontaneous decomposition of FlEtOOH in anhydrous methanol ($k_o = 1.4 \times 10^{-4}\,sec^{-1}$). This relationship is shown in equation 1.

$$-\frac{d}{dt}[FlEtOOH] = (k_o + k_{obs})[FlEtOOH]$$

Product yields were consistent with this scheme of two concurrent competing paths for FlEtOOH

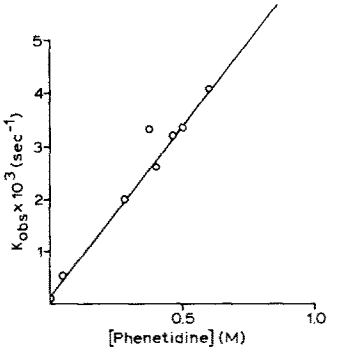

Fig. 2. Plot of pseudo-first-order rate constants vs *p*-phenetidine concentration.

Table 1. HPLC parameters for *p*-substituted arylnitroso products*

p-Substituent	Solvent	RT (min)	Analytical wavelength (nm)
OEt	40% CH_3CN	11.3	340
CH_3	40% CH_3CN	10.5	313
F	30% CH_3CH, pH 3.5	7.5	313
H	35% MeOH, pH 3.5	11	313
Cl	40% CH_3CN	10.5	313

* Performed under the conditions described in Materials and Methods.

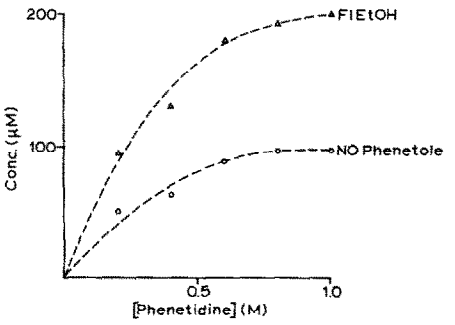

Fig. 3. Plot of product concentrations vs *p*-phenetidine concentration.

decomposition. Figure 3 shows the parallel increases in both the arylnitroso product and FlEtOH, which were the only products observed. These products were formed in a ratio of 1 mole arylnitroso product: 2 moles FlEtOH, and both were produced in quantitative yield at high concentrations of arylamine. The ratio of FlEtOH: ArNO was constant under aerobic and anaerobic conditions in *t*-butanol. The lack of an oxygen effect on the 2:1 stoichiometric relationship precludes air oxidation of arylhydroxylamine to the arylnitroso derivative following the initial FlEtOOH oxidation of arylamine.

Formation of intermediary arylhydroxylamines from the reaction of FlEtOOH with arylamine could not be demonstrated by HPLC utilizing chromatographic conditions which readily separated arylamine, arylhydroxylamine and arylnitroso compound. Only arylnitroso products were observed even under conditions where $[FlEtOOH] \ll [ArNH_2]$.

Arylhydroxylamines were converted to arylnitroso compounds by FlEtOOH under the conditions described in Materials and Methods. The reaction proceeded at a rapid rate, but the high reactivity of arylhydroxylamines with arylnitroso products and atmospheric oxygen under the conditions employed prevented a detailed kinetic analysis. Arylnitro compounds were not observed as products during the reaction of FlEtOOH with arylamine or arylnitroso derivatives.

Ibne-Rasa and Edwards [9] showed that phenylhydroxylamine was oxidized six times faster than aniline by peroxyacetic acid. For the oxidation of aniline, phenylhydroxylamine concentration was calculated from experimentally-derived rate constants and was shown to be at low, approximately steady-state, concentrations [9].

Table 2. Bimolecular rate constants for FlEtOOH oxidation of *p*-substituted anilines

p-Substituent	$k_1(\times 10^3\ M^{-1}\ sec^{-1})$
OEt	3.2
CH_3	0.79
F	0.39
H	0.45
Cl	0.12

Based on these product studies, equation 2 presents the information obtained and is the basis for the kinetic determinations.

$$ArNH_2 + FlEtOOH \xrightarrow{k_1} ArNHOH + FlEtOH \quad (2)$$

$$ArNHOH + FlEtOOH \xrightarrow{k_2} ArN{=}O + FlEtOH$$

where $k_2 \gg k_1$

Under these conditions, [ArNHOH] is at steady state and

$$-\frac{d}{dt}[FlEtOOH] = 2k_1[ArNH_2][FlEtOOH] = k_{obs}[FlEtOOH].$$

Values of k_1 were determined from the slope of the k_{obs} vs $[ArNH_2]$ and are listed in Table 2.

DISCUSSION

Primary arylamines are converted to arylnitroso compounds upon reaction with two equivalents of FlEtOOH. The kinetics of reaction are consistent with a two-step mechanism in which oxygen equivalents are sequentially transferred. The intermediate arylhydroxylamine oxidation state was not observed due to its high reactivity. The results are completely explained by the nucleophilic attack of nitrogen on the terminal peroxy-oxygen of FlEtOOH. A nucleophilic mechanism was demonstrated previously by Bruice and co-workers [7, 8] for the oxidation of secondary and tertiary amines by a closely related hydroperoxyflavin (8-methyl-FlEtOOH).

A Hammett structure–reactivity study was undertaken to gain further mechanistic data. The plot of $-\log k_1$ vs σ is shown in Fig. 4 (correlation coefficient = 0.97, $\rho = -2.6$). The structure–reactivity relationship seen in Fig. 4 is similar to that observed for the oxidation of para-substituted anilines by peroxyacetic acid ($\rho^- = -1.9$) [9]. The reaction parameter determined in this study is also

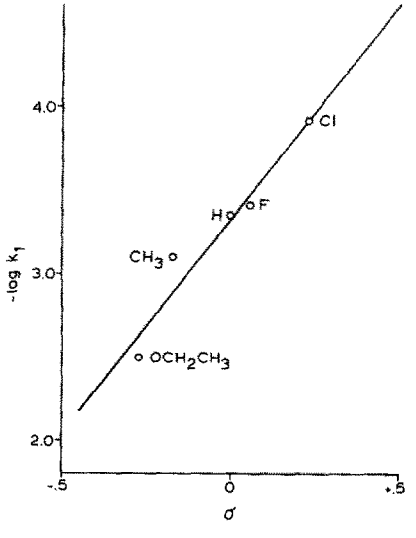

Fig. 4. Hammett plot of $-\log k_1$ vs σ.

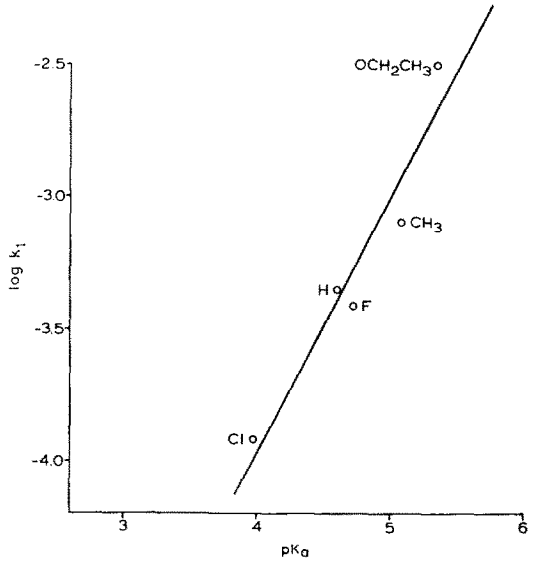

Fig. 5. Bronsted-type plot of log k_1 vs *p*-substituted arylamine pK_a (25°, H_2O).

similar to the values determined for the reactions of 8-methyl-FlEtOOH with para-substituted *N*-methylanilines ($\rho = -1.2$) [17] and 8-methyl-10-*m*-xylyl-FlEtOOH with para-substituted thioanisoles ($\rho = -1.5$) [18]. We interpret the observed rate enhancement by electron donating substituents to reflect the influence of charge localization on nitrogen nucleophilicity.

Figure 5 shows a Bronsted-type plot of log k_1 vs pK_a [19] of para-substituted anilines (correlation coefficient = 0.97). It can be concluded from the slope of the line ($\beta_{nuc} = 0.96$) that, within this series of substituted anilines, reactivity toward FlEtOOH is highly dependent on basicity. The value of β_{nuc} is similar to that seen in the nucleophilic displacement reaction of para-substituted anilines with *p*-nitrophenyl acetate ($\beta_{nuc} = 0.7$) [20].

Huckel molecular orbital calculations have shown a correlation between total amino-nitrogen charge and the pK_a for aminonaphthalenes and aminoazulenes [21, 22]. These studies predict an increased nucleophilicity of 2-aminonaphthalene relative to 1-aminonaphthalene. 2-Aminonaphthalene has been shown to be oxidized at a greater rate than 1-aminonaphthalene by porcine FMO. A requirement for the participation of an imino-tautomer in FMO catalyzed oxygenations, as advanced by Ziegler *et al.* [23], would predict 1-aminonaphthalene to be the preferred substrate. The relative contribution of imino-tautomers for these two isomeric aminonaphthalenes as estimated by stretching frequencies for the N—H bond [24] and by resonance theory is consistent with a larger fraction of 1-aminonaphthalene in the imino-form. This inconsistency suggests that amino-group nucleophilicity is a more relevant parameter for predicting aromatic N-oxidation by FMO than is imino-tautomer contribution to a substrate.

A comparison of the results from our study of arylamine oxidation by a hydroperoxyflavin model

Table 3. pK_a values for arylamines (25°, H_2O)

Arylamine	pK_a
Aniline	4.6*
1-Aminonaphthalene	3.9†
2-Aminonaphthalene	4.2†

* Ref. 17.
† Ref. 22.

of FMO to the reported results with actual FMO systems suggests that, although amine nucleophilicity is the predominant determinant of reactivity, hydrophobic interactions modify the reactivity at the enzyme active site. A comparison of published values of k_{cat}/K_m for arylamine substrates of FMO shows a preference for the polynuclear substrates such as the naphthylamines relative to monoaromatics [23]. Basicity considerations alone would predict aniline to be a substrate for FMO (cf. Table 3), yet it is not [10]. A slight preference of porcine FMO for polyaromatic substrates is also seen in the thiourea series [25]. 1-Naphthylthiourea oxidation by FMO is characterized by higher k_{cat}/K_m relative to phenylthiourea.

The products from the reaction of porcine FMO with arylamines have been described as mixtures of arylhydroxylamines and arylnitroso compounds [10, 26]. In one study, the presence of an additional reductive enzyme system (NADPH–cytochrome *c* reductase) was utilized to convert 2-nitrosonaphthalene to 2-hydroxylaminonaphthalene for quantitative measurements [10]. 2-Nitrosonaphthalene was the major product in the absence of the reductive enzyme system [10]. Published high pressure liquid chromatograms from FMO-mediated oxidation of 2-aminofluorene showed small amounts of 2-hydroxylaminofluorene, larger amounts of 2-nitrosofluorene, and small amounts of 2-nitrofluorene [26]. The exclusive formation of aromatic nitroso compounds from both FlEtOOH and peroxyacetic acid [9] mediated oxidation of aromatic amines suggests that arylhydroxylamine intermediates must be rapidly oxidized under the conditions of the reaction. This has been the usual observation for reactions that are peroxidative in nature, including enzymatic reactions [11, 12]. On the other hand, monooxygenase-dependent oxidations containing a reduced nicotinamide cofactor (e.g. NADPH) generally give the corresponding hydroxylamine as the principal product [10, 27]. It is not clear from the literature whether arylhydroxylamines are recalcitrant to oxidation by O_2 and NADPH in the presence of a monooxygenase, or whether the expected nitroso product from such an oxidation is reduced back to the hydroxylamine state by NADPH or a similar reducing agent [28, 29].

By comparison of the results of this study with FMO substrate data from the literature [23], it appears that enzyme–substrate binding is very important in determining reactivity and that these binding forces favor the oxidation of polynuclear aromatic amines. Nevertheless, the results of this study predict that basic monoaromatic amines like *p*-phenetidine should also be substrates for FMO.

The once popular analgesic *N*-acetyl-*p*-phenetidine (phenacetin) is metabolically converted to *p*-phenetidine [30]. *p*-Phenetidine was found in our study to be oxidized by FlEtOOH at rates approximately three times faster than the rate reported by Ball and Bruice for the 8-methyl-FlEtOOH oxidation of *N*-methylaniline [8], which is a known substrate for porcine FMO [23]. However, the oxidation of *p*-phenetidine by FMO has not been investigated.

Metabolic conversion of primary arylamines to arylhydroxylamines and arylnitroso derivatives by FMO is a potentially significant pathway in the production of proximate genotoxic metabolites *in vivo*. The contribution of FMO to the N-oxidation of primary arylamines could be particularly important in humans in view of the report that FMO-mediated N-oxidation of 2-aminofluorene by human liver microsomes is a major pathway not observed in most species of laboratory animals [26]. Further studies of primary arylamine oxidation by synthetic and enzymatically generated hydroperoxyflavins are under investigation.

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