

## Mechanistic Investigation of the Oxidation of the Carbanion of Methyl 2-Methoxy-2-phenylacetate by an Isoalloxazine

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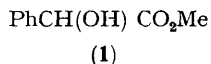
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**Summary** Evidence from product studies and radical trapping experiments indicates that the mechanism of the oxidation of the carbanion of methyl 2-methoxy 2-

phenylacetate by a model flavin compound in basic methanol is free radical in nature

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VARIOUS flavoenzymes, including lactic acid oxidase, amino acid oxidases, and succinic acid oxidase, catalyse dehydrogenation reactions which introduce unsaturation  $\alpha\beta$  to a carbonyl group.<sup>1</sup> In an effort to gain a better understanding of the mechanism(s) of these reactions we have investigated the dehydrogenation of alcoholic substrates and their corresponding methyl ethers mediated by the model flavin 10-(2,6-dimethylphenyl)isoalloxazine ( $\text{Fl}_{\text{ox}}$ ) in basic methanol solution. Our results with the model substrates 9-hydroxy- and 9-methoxy-fluorene have previously appeared.<sup>2</sup> We now report the results of our studies with methyl mandelate (**1**) and methyl 2-methoxy-2-phenylacetate (**2**) which compounds resemble the actual biological substrates of the flavoenzyme oxidases more closely.



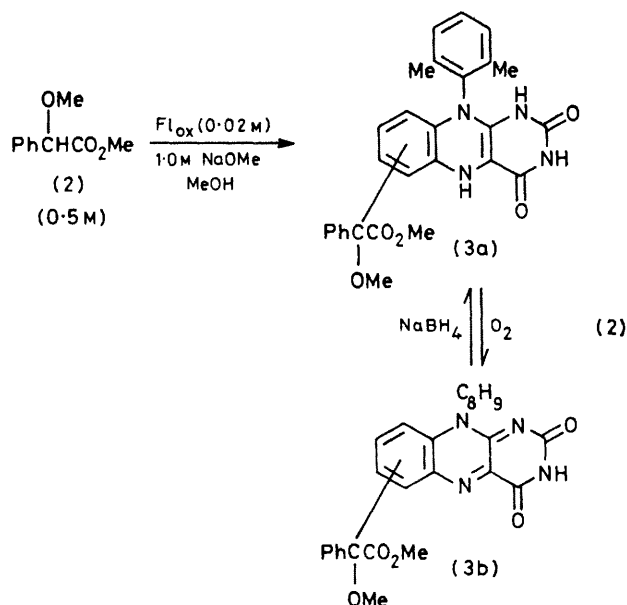
When (**1**) (0.10 M) and  $\text{Fl}_{\text{ox}}$  ( $1 \times 10^{-4}$  M) were combined in the dark in basic methanol (1.0 M NaOMe; 30 °C) in the absence of  $\text{O}_2$ , the characteristic visible spectrum of  $\text{Fl}_{\text{ox}}$  disappeared in a first-order manner ( $k_{\text{obs}} 2.0 \times 10^{-3} \text{ s}^{-1}$ ) and was replaced by a spectrum characteristic of a reduced flavin. Admission of  $\text{O}_2$  at the end of the reaction resulted in the restoration of the spectrum of the original oxidized flavin ( $\lambda_{\text{max}} 441 \text{ nm}$ ;  $\epsilon 8900 \text{ l mol}^{-1} \text{ cm}^{-1}$ ). Product studies carried out using higher  $\text{Fl}_{\text{ox}}$  concentrations showed that the oxidation product of methyl mandelate is methyl benzoylformate (isolated in 80% yield) as expected.<sup>2-4</sup> Kinetic studies carried out by following the disappearance of  $\text{Fl}_{\text{ox}}$  indicated that the rate law is of the form shown in equation (1), which is characteristic of the rate law observed

$$-d[\text{Fl}_{\text{ox}}]/dt = k[(1)] [\text{NaOMe}] [\text{Fl}_{\text{ox}}] \quad (1)$$

for other weak carbon acid substrates<sup>2,3a,c,e</sup> and is consistent with the previously demonstrated mechanism involving interaction of  $\text{Fl}_{\text{ox}}$  with the carbanion of the substrate in the rate determining step.<sup>2,3</sup>

When (**2**) (1.0 M) and  $\text{Fl}_{\text{ox}}$  ( $1 \times 10^{-4}$  M) were mixed under the same conditions as above the visible spectrum of  $\text{Fl}_{\text{ox}}$  disappeared at a much slower rate than with (**1**) ( $k_{\text{obs}} 4.5 \times 10^{-5} \text{ s}^{-1}$ ) to give a spectrum characteristic of a reduced flavin. Interestingly, (**2**) has previously been reported to be unreactive towards flavins under basic conditions.<sup>4</sup> At long reaction times (> 24 h) a subsequent reaction began to take place which resulted in an increase in absorbance at longer wavelengths ( $\lambda_{\text{max}} 620 \text{ nm}$ ). When  $\text{O}_2$  was admitted after 3 days a spectrum due to a new oxidized flavin ( $\lambda_{\text{max}} 445 \text{ nm}$ ) appeared in two phases. (i) Approximately 80% of the final absorbance at 445 nm appeared immediately upon addition of  $\text{O}_2$ . (ii) The remaining absorbance at 445 nm appeared more slowly over a period of about 30 min and was accompanied by the disappearance of the long-wavelength absorbance band. A product study was initiated at higher  $\text{Fl}_{\text{ox}}$  concentrations ( $2 \times 10^{-2}$  M). The reaction was quenched before completion (ca. 2 days) to avoid complications owing to the subsequent reaction noted above. The only product that could be

detected was a flavin adduct (**3**) [equation (2)] which could be separated from the changed starting flavin by column chromatography on silica gel using ethyl acetate- $\text{CH}_2\text{Cl}_2$  (5:95) as eluant.<sup>†</sup> The adduct was isolated in 90% yield after correction for recovered unchanged  $\text{Fl}_{\text{ox}}$ . The visible spectrum of (**3b**) was identical to that of the oxidized flavin recorded at the end of the kinetic experiment involving (**2**). The identity of the material with  $\lambda_{\text{max}} 620 \text{ nm}$  was not determined, but it could be shown that (**3a**) incubated in 1.0 M methanolic sodium methoxide slowly gave rise to the same long wavelength absorption observed in the kinetic experiment.



The radical trapping agents  $\text{O}_2$  and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl<sup>5</sup> ( $>\text{N-O}\cdot$ ) were capable of altering the course of the reaction as shown in the Table.

TABLE. % Yields of products obtained upon reaction of (**2**) (0.5 M) with  $\text{Fl}_{\text{ox}}$  (0.05 M) in methanol containing NaOMe (1.0 M) in the presence of an excess of  $\text{O}_2$  or  $>\text{N-O}\cdot$  (2.0 M).

Product	With $\text{O}_2$		With $>\text{N-O}\cdot$
	2 days <sup>a</sup>	7 days <sup>a</sup>	7 days <sup>a</sup>
$\text{PhCO}_2\text{Me}$	27	56	53
$\text{PhCO}_2\text{H}$	8	16	0
( <b>2</b> )	64	16	12
$\text{PhCOCO}_2\text{Me}$	0	0	26
$\text{Fl}_{\text{ox}}$	85	72	47
( <b>3b</b> )	0	18	27

<sup>a</sup> Incubated at  $25 \pm 2$  °C in the dark for the indicated length of time before products were isolated. <sup>b</sup> The only materials detected from product studies in the absence of radical traps were unchanged (**2**) and  $\text{Fl}_{\text{ox}}$ , and the adduct (**3b**). These studies were performed under conditions similar or identical to those involving the radical traps, except for the presence of the trapping agent, and incubation periods ranged from 2 to 5 days. The total yield of  $\text{Fl}_{\text{ox}}$  and (**3b**) recovered accounted for more than 90% of the original flavin in all cases in which radical traps were not present.

<sup>†</sup> Identification of the partial structure of (**3**) was based on the following spectroscopic data for (**3b**): n.m.r. ( $\text{CDCl}_3$ ),  $\delta$  8.60 (s, 1NH), 8.4-6.9 (m, 11H), 3.70 (s, 3H), 3.15 (s, 3H), and 1.87 (d,  $J$  3.5 Hz, 6H); mass spectrum (EI 70V)  $m/e$  496, 481, and 317; u.v.-vis. (in methanol)  $\lambda_{\text{max}} 435 \text{ nm}$  ( $\epsilon 11,300 \text{ l mol}^{-1} \text{ cm}^{-1}$ ), 340 (8000); u.v.-vis. (in 1.0 M sodium methoxide in methanol)  $\lambda_{\text{max}} 445 \text{ nm}$  ( $\epsilon 9,500 \text{ l mol}^{-1} \text{ cm}^{-1}$ ) 342 (9700). The u.v.-visible spectrum of (**3a**) was typical of a reduced flavin (in methanol)  $\lambda_{\text{max}} 360 \text{ nm}$  ( $\epsilon 6200 \text{ l mol}^{-1} \text{ cm}^{-1}$ ) (in 1.0 M sodium methoxide in methanol)  $\lambda_{\text{max}} 362 \text{ nm}$  ( $\epsilon 6700 \text{ l mol}^{-1} \text{ cm}^{-1}$ ). High resolution mass spectrum of (**3b**):  $m/e$  496; calc. for  $\text{C}_{28}\text{H}_{24}\text{N}_4\text{O}_6$ , 496.175; obs., 496.180. All data indicate that (**3**) is not a mixture of adducts, but is a single compound.

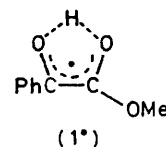
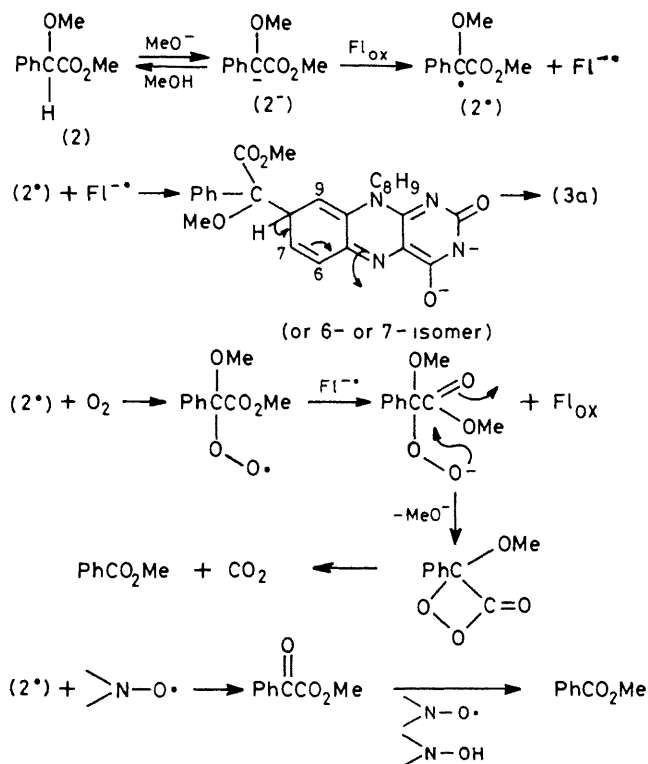
The yields reported are based on the original amounts of starting materials in the reaction mixture. Control experiments showed that (3b) is stable in basic methanol solution in the presence of  $O_2$ ,  $H_2O_2$ , and  $>N-O\cdot$  so that the products reported in the Table are not due to decomposition of (3b) under the reaction conditions. In all cases it could be shown that no reaction took place in the absence of  $Fl_{ox}$  or sodium methoxide.

The mechanisms shown in the Scheme are most consistent with the results of the product studies involving (2) and  $Fl_{ox}$ . As is true in all other cases studied<sup>2,3</sup> the carbanion, (2<sup>-</sup>), is the active form of the substrate. The radical, (2<sup>•</sup>), formed from the reaction of (2<sup>-</sup>) with  $Fl_{ox}$  cannot be oxidized to ketoester by dissociation of  $H^+$  followed by one-electron transfer or by  $H\cdot$  transfer as can occur in the oxidation of  $\alpha$ -hydroxycarbonyl compounds.<sup>6</sup> Instead (2<sup>•</sup>) is trapped by  $Fl^-$  to form (3a) as shown. The radical traps function by scavenging (2<sup>•</sup>) before it can react with  $Fl^-$ . The fact that some (3b) is formed in the presence of

$O_2$  or  $>N-O\cdot$  indicates that the relatively unstable (2<sup>•</sup>) is trapped quite rapidly by  $Fl^-$ . Our previous studies showed that the relatively stable 9-methoxyfluorene radical was not competitively trapped by  $Fl^-$  in the presence of large excesses of  $O_2$  or  $>N-O\cdot$ .<sup>2</sup>

The mechanism shown in the Scheme accounts for the production of methyl benzoate in the presence of  $O_2$ . The small amount of benzoic acid isolated in these cases could be due to hydrolysis of methyl benzoate by water produced from decomposition of  $H_2O_2$  which may result from the reaction of  $Fl^-$  or  $FlH_2$  with  $O_2$ .<sup>7</sup> It is unclear whether methyl benzoylformate isolated in the experiments involving  $>N-O\cdot$  is produced *via* an electron transfer mechanism or *via* an hydroxylamine type intermediate.<sup>8</sup> Control experiments do show that methyl benzoate is formed from methyl benzoylformate in basic methanol in the presence of a mixture of  $>N-O\cdot$  and the corresponding hydroxylamine.

Under comparable conditions the rate of disappearance of  $Fl_{ox}$  in the presence of (2) is *ca* 450-fold slower than in the presence of (1). It could be argued that this rate difference indicates that the oxidation of (1) by  $Fl_{ox}$  does not involve radical intermediates but that (1) is oxidized by an alternative mechanism not available to (2).<sup>2,3,7</sup> However, we believe that the differences in the rates of oxidation of (1) and (2) are a reflection of the greater stability of the resonance-stabilized 1,2-enediol radical (1<sup>•</sup>). Such a resonance-stabilized structure is not possible for the analogous radical derived from (2). In contrast, the carbanions of 9-hydroxy- and 9-methoxy-fluorene are oxidized by  $Fl_{ox}$  at comparable rates.<sup>2</sup> The radicals derived from the oxidation of these two carbanions would be of similar stability.<sup>2</sup> The results of this study provide convincing evidence for the radical nature of the oxidation of (2) by  $Fl_{ox}$ . This and the similar conclusions of our earlier study<sup>2</sup> suggest that the oxidations of other carbon acid substrates by flavins proceed *via* the same mechanism.



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