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

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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

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.[1] 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 (Fl_{ox}) in basic methanol solution. Our results with the model substrates 9-hydroxy- and 9-methoxy-fluorene have previously appeared.[2] 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.

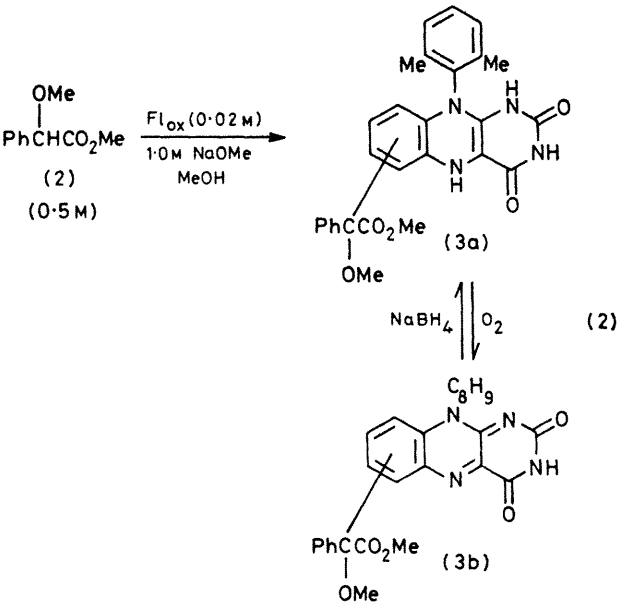
$$PhCH(OH)\ CO_2Me$$
(**1**)

When (**1**) (0·10 M) and Fl_{ox} (1×10^{-4} M) were combined in the dark in basic methanol (1·0 M NaOMe; 30 °C) in the absence of O_2, the characteristic visible spectrum of Fl_{ox} disappeared in a first-order manner (k_{obs} $2{\cdot}0 \times 10^{-3}$ s^{-1}) and was replaced by a spectrum characteristic of a reduced flavin. Admission of O_2 at the end of the reaction resulted in the restoration of the spectrum of the original oxidized flavin (λ_{max} 441 nm; ϵ 8900 l mol^{-1} cm^{-1}). Product studies carried out using higher Fl_{ox} concentrations showed that the oxidation product of methyl mandelate is methyl benzoylformate (isolated in 80% yield) as expected.[2-4] Kinetic studies carried out by following the disappearance of Fl_{ox} indicated that the rate law is of the form shown in equation (1), which is characteristic of the rate law observed

$$-d[Fl_{ox}]/dt = k[(\mathbf{1})]\ [NaOMe]\ [Fl_{ox}] \qquad (1)$$

for other weak carbon acid substrates[2,3a,c,e] and is consistent with the previously demonstrated mechanism involving interaction of Fl_{ox} with the carbanion of the substrate in the rate determining step.[2,3]

When (**2**) (1·0 M) and Fl_{ox} (1×10^{-4} M) were mixed under the same conditions as above the visible spectrum of Fl_{ox} disappeared at a much slower rate than with (**1**) (k_{obs} $4{\cdot}5 \times 10^{-5}$ 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.[4] At long reaction times (> 24 h) a subsequent reaction began to take place which resulted in an increase in absorbance at longer wavelengths (λ_{max} 620 nm). When O_2 was admitted after 3 days a spectrum due to a new oxidized flavin (λ_{max} 445 nm) appeared in two phases. (i) Approximately 80% of the final absorbance at 445 nm appeared immediately upon addition of 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 Fl_{ox} concentrations (2×10^{-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–CH_2Cl_2 (5 : 95) as eluant.† The adduct was isolated in 90% yield after correction for recovered unchanged Fl_{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 λ_{max} 620 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 O_2 and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl[5](>N–O•) 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 Fl_{ox} (0·05 M) in methanol containing NaOMe (1·0 M) in the presence of an excess of O_2 or >N–O• (2·0 M).

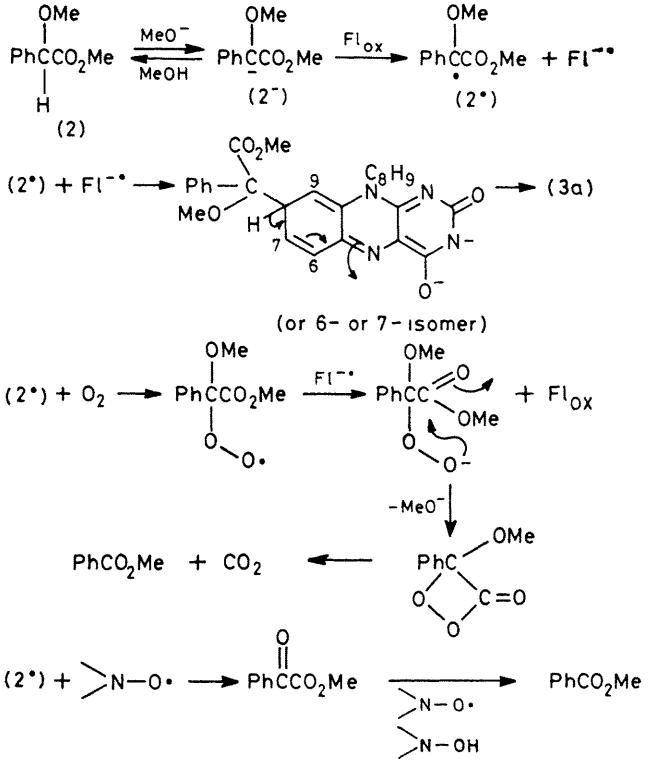
Product	With O_2 2 days[a]	With O_2 7 days[a]	With >N–O• 7 days[a]
$PhCO_2Me$	27	56	53
$PhCO_2H$	8	16	0
(**2**)	64	16	12
$PhCOCO_2Me$	0	0	26
Fl_{ox}	85	72	47
(**3b**)	0	18	27

[a] Incubated at 25 ± 2 °C in the dark for the indicated length of time before products were isolated. [b] The only materials detected from product studies in the absence of radical traps were unchanged (**2**) and Fl_{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 Fl_{ox} and (**3b**) recovered accounted for more than 90% of the original flavin in all cases in which radical traps were not present.

† Identification of the partial structure of (**3**) was based on the following spectroscopic data for (**3b**): n.m.r. ($CDCl_3$), δ 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) λ_{max} 435 nm (ϵ 11,300 l mol^{-1} cm^{-1}), 340 (8000); u.v.–vis (in 1·0 M sodium methoxide in methanol) λ_{max} 445 nm (ϵ 9,500 l mol^{-1} cm^{-1}) 342(9700). The u.v.–visible spectrum of (**3a**) was typical of a reduced flavin (in methanol) λ_{max} 360 nm (ϵ 6200 l mol^{-1} cm^{-1}) (in 1·0 M sodium methoxide in methanol) λ_{max} 362 nm (ϵ 6700 l mol^{-1} cm^{-1}). High resolution mass spectrum of (**3b**): *m/e* 496; calc. for $C_{28}H_{24}N_4O_5$, 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• 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[2,3] the carbanion, (**2**$^-$), is the active form of the substrate The radical, (**2**•), formed from the reaction of (**2**$^-$) with Fl_{ox} cannot be oxidized to ketoester by dissociation of H^+ followed by one-electron transfer or by H• transfer as can occur in the oxidation of α-hydroxycarbonyl compounds[6] Instead (**2**•) is trapped by Fl^{-}• to form (**3a**) as shown The radical traps function by scavenging (**2**•) before it can react with Fl^{-}• The fact that some (**3b**) is formed in the presence of

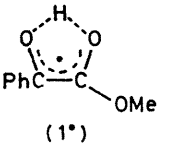


SCHEME

O_2 or >N–O• indicates that the relatively unstable (**2**•) 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•[2]

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[7] It is unclear whether methyl benzoylformate isolated in the experiments involving >N–O• is produced *via* an electron transfer mechanism or *via* an hydroxylamine type intermediate[8] Control experiments do show that methyl benzoate is formed from methyl benzoylformate in basic methanol in the presence of a mixture of >N–O• 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**)[2,3,7] 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**•) 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[2] The radicals derived from the oxidation of these two carbanions would be of similar stability[2] 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[2] suggest that the oxidations of other carbon acid substrates by flavins proceed *via* the same mechanism



(1•)

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