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IRON-PORPHYRIN CATALYSIS OF THE OXIDATIVE DEALKYLATION OF PARA-NITRO-ANISOLE AND 7-ETHOXYCOUMARIN BY CUMYLHYDROPEROXIDE : a possible model for the corresponding cytochrome P 450-dependent reactions.

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SUMMARY

A biphasic system containing an iron porphyrin, Fe (TPP)(Cl)* or [Fe(TPP)]$_2$O, efficiently catalyzes the cumyl- or tertiobutyl-hydroperoxide-supported dealkylation of p-nitroanisole and 7-ethoxycoumarin to the corresponding phenol and formaldehyde. Stoichiometric amounts of iron porphyrin and hydroperoxide give a quantitative reaction. Catalytic amounts of iron porphyrin give reaction rates and yields which are proportional to substrate concentration. With increasing hydroperoxide concentrations, the rates level off to limit values and the yield rapidly decreases. The maximum rates obtained approach those of the reactions mediated by cytochrome P 450-dependent monooxygenases.

Cytochrome P 450-dependent monooxygenases are able to catalyze the hydroxylation of a wide variety of organic compounds, either by dioxygen in the presence of NADPH, or by a number of single oxygen donors such as hydroperoxides, peroxyacids, chlorite, periodate and iodosylbenzene (1-5). It has been proposed that a very reactive cytochrome P 450-FeO complex is involved in the oxygen-transfer process (2-7).

In the search for chemical model hydroxylating systems, recent results have shown that iron-porphyrins catalyze the iodosylbenzene-supported epoxidation of olefins (8) and hydroxylation of aliphatic hydrocarbons such as cyclohexane and adamantane (8).

As a part of our effort to devise heme model systems able to effect the hydroxylation reactions which are commonly catalyzed by cytochrome P 450-dependent monooxygenases, we here describe a biphasic system containing an iron-porphyrin which efficiently catalyzes the alkylhydroperoxide-supported dealkylation of para-nitroanisole and 7-ethoxycoumarin.

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*Abbreviations : CHP : cumylhydroperoxide ; THP : tertiobutylhydroperoxide ; TPP : tetraphenylporphyrin ; pNA : p-nitroanisole ; pNP : p-nitrophenol.

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MATERIAL AND METHODS

Solvents and reagents were Prolabo Analytical grade. Para-nitroanisole (Merck) was recrystallized from methanol-water then cyclohexane (mp = 54°C) ; 7-ethoxycoumarin (9) and iron tetraphenylporphyrin complexes, Fe(TPP)(Cl) and $[Fe(TPP)]_2O$ were prepared as previously described (10). Emulgen 911, a nonylphenoxypolyethyleneglycol was a gift of Kao-Atlas Japan.
Biphasic hydroxylation reaction : To 2.5 ml of NaOH 0.05 M in a 1 cm spectrophotometer glass cuvette was added a 250 µl solution in benzene of p-nitroanisole (0.1-3 M) and iron porphyrin (0.01-2 mM). The mixture was stirred at the interface with an electric microstirrer (500 rpm) without disturbing the aqueous layer in the light path. After recording a baseline , the hydroperoxide (0.05-200 mM) was added to the organic layer and the increase of absorption of the aqueous layer at 402 nm was recorded (ε_{402} = 21500) (11). For the deethylation of 7-ethoxycoumarin (0.7 M in benzene) the aqueous phase was potassium phosphate buffer (0.1 M, pH 8.5) and fluorescence of the phenolate was recorded according to a previously described method (9), (λ_{ex} = 366 nm, λ_{em} 420).

Monophasic hydroxylation reaction : The same reaction was also carried out in the absence of an aqueous layer. Quantitative analysis was performed by HPLC (Ultrasphere ODS, Methanol:water 55:45), in conditions where p-nitrophenol, cumylhydroperoxide, acetophenone, cumenol, p-nitroanisole and benzene were separated . pNP was also analyzed after extraction with NaOH and formaldehyde was quantified by the method of Nash (12) after extraction into 0.05 M HCl. When the reaction is performed in dry solvent, the reaction product seems to be the intermediate hemiformal, p-nitrophenoxymethanol. Accordingly, extraction of the reaction mixture with 0.05 M NaOH after complete disappearance of the hydroperoxide leads to a slow appearance of p-nitrophenolate (perfectly first order, t1/2 at 20°C ≃4 min), while p-nitrophenol added to the organic phase is extracted as p-nitrophenolate much more rapidly (t1/2 ≃10 sec.). Furthermore, treatment of the reaction mixture first with a small amount of trifluoroacetic acid (10 mM) leads to rapid extraction of the phenolate (t1/2 ≃ 10 sec.). It is noteworthy that similar hemiformals have been synthetized and shown to be stable in anhydrous organic solvents (13). In all reactions with the biphasic system we have found that hydrolysis of this hemiformal to p-nitrophenolate was not rate limiting.

RESULTS AND DISCUSSION

Formaldehyde and p-nitrophenol are formed upon oxidation of paranitroanisole by cumylhydroperoxide in the presence of catalytic amounts of the synthetic complex chloromesotetraphenylporphinato-iron III, Fe(TPP)(Cl), in benzene at room temperature. The phenol was identified by isolation after basic extraction and comparison of its visible and IR spectral characteristics with those of an authentic sample. Formaldehyde was extracted into 0.05 M HCl and identified by its reaction with the Nash reagent (12). The kinetics of the reaction were followed by direct spectroscopic quantitation of the liberated phenol continuously extracted in an aqueous alkaline phase (see experimental).

The data collected in table I show that the dealkylation proceeds only when both an iron porphyrin and an oxygen donor are present. Neither the porphyrin ligand (TPP H_2) by itself, nor the iron III salt $FeCl_3$ are able to catalyze the reaction indicating the key role of the porphyrinic

TABLE I : paranitroanisole demethylation by single oxygen donors in the presence of various catalysts.

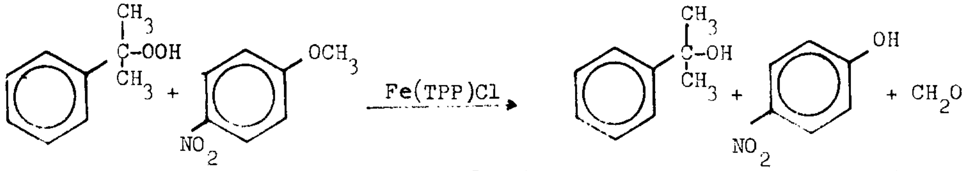
	Oxidizing agent		Catalyst		Yield of pnitrophenol formed (%) at :	
					20°C	60°C
a	CHP	1 mM	Fe(TPP)(Cl)	0 mM	0	0
	"	"	TPP H_2	1 mM	0	0
	"	"	$FeCl_3$	1 mM	0	0
	"	0 mM	Fe(TPP)(Cl)	1 mM	0	0
	"	1 mM	"	"	12	38
	"	"	$Fe(TPP)_2O$	1 mM	11	35
	THP	1 mM	Fe(TPP)(Cl)	1 mM	16	25
	"	"	$Fe(TPP)_2O$	1 mM	36	44
	Ar-IO*	1 mM	Fe(TPP)(Cl)	1 mM	10	
b	CHP	0.2 mM	Fe(TPP)(Cl)	0.2 mM	20	82
	"	"	$Fe(TPP)_2O$	0.2 mM	24	91
	THP	0.2 mM	Fe(TPP)(Cl)	0.2 mM	45	97
	"	"	$Fe(TPP)_2O$	0.2 mM	45	100
c	CHP	28 mM	Fe(TPP)(Cl)	1 mM	10	-
	"	51 mM	"	0.1 mM	3	4.1

Conditions : 0.5 ml of 1 M p-nitroanisole in benzene + 2.5 ml of 0.05 M NaOH magnetically stirred. Phenol yields are given in mole per mole of starting oxidizing agent. These results are the mean ± 5 % of at least 3 experiments. * : 2,4,6-trimethyl-iodosylbenzene.

Fe^{III} complex. When the reactions were performed in anaerobic conditions, similar yields and rates were observed. When the μ-oxo dimer $[Fe(TPP)]_2O$ is used in place of Fe(TPP)(Cl), the reaction proceeds but after a longer induction period. In the absence of an aqueous alkaline phase, conditions where accumulation of the intermediate hemiformal p-nitrophenoxymethanol seems to occur (see experimental), the phenol yields are lower, probably because of its partial further oxidation to a very polar yellow compound. Accordingly, in similar reaction conditions, p-nitrophenol gives also the same compound at the expense of the hydroperoxide, as shown by HPLC analysis.

Replacement of cumylhydroperoxide by other oxygen-donors such as tertiobutylhydroperoxide or 2,4,6-trimethyliodosylbenzene leads to similar phenol yields. Hydrogen peroxide on the contrary was found unable to produce detectable amounts of phenol, even in the presence of a phase-transfer agent to facilitate its access to the organic phase.

In the conditions of table Ib, nearly quantitative use of the oxidizing equivalents of the hydroperoxide to dealkylate substrate is observed. Respective yields of 80 and 90 % based on the starting hydroperoxide are determined for formaldehyde and cumenol (1-methyl,1-phenylethanol). Taking into account the decreased yield of the Nash reaction in the presence of cumylhydroperoxide (2, 14) and the fact that the latter yields small amounts of acetophenone and α-methylstyrene in the conditions of table Ib, the above results agree with the stoichiometry of the following equation :



In the conditions of table Ic, the iron porphyrin acts as a true catalyst and, accordingly, it is found unchanged at the end of the reaction. Moreover upon new additions of hydroperoxide, the dealkylation proceeds again to the same extent and with a similar kinetic but lacking the lag period. When Fe(TPP)(Cl) is the catalyst, it is slowly converted by the aqueous NaOH into $[Fe(TPP)]_2O$ with a much slower rate than that of the dealkylation reaction, and the total concentration of iron porphyrin remains constant during the reaction. Similar yields are obtained with both porphyrin complexes.

Influence of the reaction conditions on the p-nitrophenol yield : When various concentrations of p-nitroanisole (0.01-3 M) and cumylhydroperoxide (0.1-150 mM) are used, one observes that the phenol yield increases linearly with starting p-nitroanisole concentration (fig. 1A). It increases with tem-

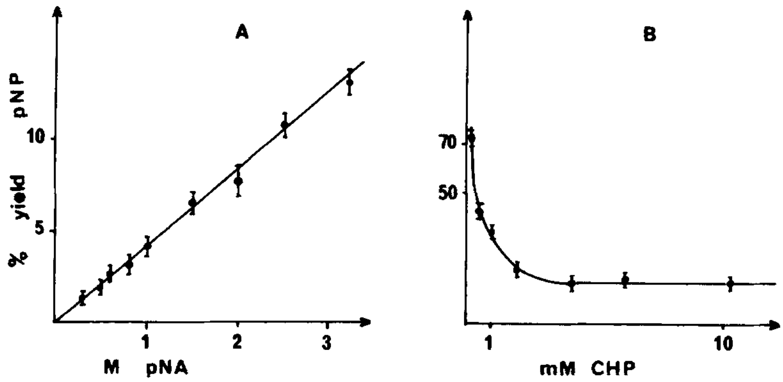
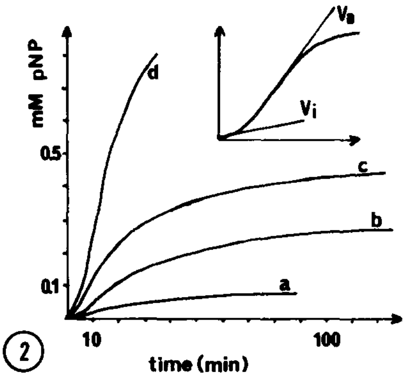


Fig. 1 : Yield of p-nitrophenol based on starting CHP as a function of : A) starting p-nitroanisole concentration (CHP : 28 mM) ; B) CHP concentration (pNA : 3 M). Conditions : Fe(TPP)(Cl) 0.2 mM in 0.5 ml benzene + 2.5 ml 0.05 M NaOH at 20°C ; values are mean ± S.D. of four experiments.



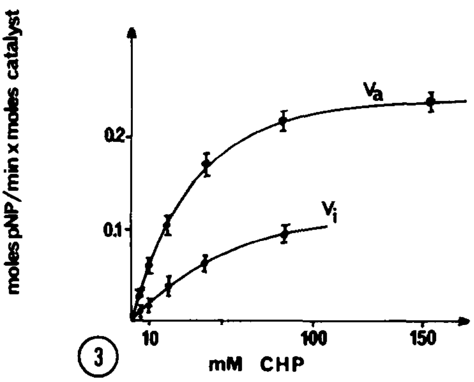


Fig. 2 : Formation of p-nitrophenol as a function of time with various CHP concentrations (a : 1 mM ; b : 5 mM ; c : 10 mM ; d : 51 mM). Conditions : 2 mM Fe(TPP) $_2$O, 1 M pNA in 0.25 ml benzene + 2.5 ml 0.05 M NaOH electrically stirred in a spectrophotometer cuvette at 30°C.

Fig. 3 : Initial (vi) and linear (va) PNP formation rates versus [CHP]. Conditions : 5o µl CHP diluted in benzene was added to 250 µl benzene containing 1 M pNA and 0.3 mM Fe(TPP)(Cl) in a spectrophotometer cuvette containing 2.5 ml 0.05 M NaOH electrically stirred at 30°C.

perature between 15-60°C and decreases with increasing concentration of cumylhydroperoxide (fig. 1B). The yield is approximately 10 % for 28 mM cumylhydroperoxide, 3 M p-nitroanisole, 0.2 mM Fe(TPP)(Cl) and 20°C, corresponding to 14 turnovers of the catalyst during the reaction (fig. 1A). The important decrease of the phenol yield upon raising cumylhydroperoxide concentration can be explained by the involvement of a competitive reaction of the oxidizing species, formed by interaction of iron-porphyrin and hydroperoxide, with hydroperoxide itself. This would correspond to a catalase-like dismutation of the hydroperoxide (15).

Influence of the conditions on the rate of the reaction : Some typical curves illustrating the formation of p-nitrophenol as a function of time are shown in fig. 2. The reaction rates corresponding to the initial (vi) and the quasi-linear (va) part of the curve increase linearly with p-nitroanisole concentration (data not shown). These rates tend to limit values when cumylhydroperoxide varies from 0.2 to 150 mM (fig. 3). A "pseudo Km" value of about 25 mM can be deduced from the plots of fig. 3. The maximum rate (va) obtained so far at 37°C and with 1 M p-nitroanisole was about 1 mole of p-nitrophenol formed per mole of Fe(TPP)(Cl) per min.

7-ethoxycoumarin dealkylation : The above described heme system also performs the deethylation of 7-ethoxycoumarin. The conditions of the reaction were not optimized : in stoichiometric conditions (1 mM Fe (TPP)(Cl) and CHP, 0.7 M 7-ethoxycoumarin) the yield of 7-hydroxycoumarin is 2.5 % at 20°C and 10 % at 40°C ; in catalytic conditions (1 mM Fe(TPP)(Cl) and 50 mM CHP) the yield is 1.6 % at 20°C. It is possible but difficult to follow phenol formation by measuring the fluorescence of the phenolate in aqueous buffer (9) because of its photodegradation during the long period of the reaction, which precluded good kinetic studies.

Micellar oxidizing system : We have also designed one other oxidizing system where the organic solvent is replaced by a non-ionic detergent. For instance the formation of p-nitrophenolate can be observed continuously by visible spectroscopy when cumylhydroperoxide (2.5 mM) is added to a micellar emulsion of Emulgen 911 (12 mM) in 0.05 M NaOH containing 3 mM p-nitroanisole and 10 µM Fe(TPP)(Cl). We did not refine this system because of the numerous parameters to take into account but in the above conditions the quasi-linear rates (va) observed respectively at 20°C and 38°C are 0.08 and 0.3 mole pNP formed per mole Fe(TPP)(Cl) per min.

The aforementioned results show that simple iron-porphyrins in an organic solvent efficiently catalyze the alkylhydroperoxide-supported oxidative dealkylation of ethers commonly used as substrates for cytochrome P 450-dependent monooxygenases. The above described heme system does not provide the proximity effect between the iron oxidizing species and the substrate, which is provided by the protein in cytochrome P 450 catalyzed reactions. However, if one extrapolates the observed maximum rates versus substrate concentration to pure substrate ($\simeq$7 M), in order to take into account the high effective substrate molarity which should be characteristic of the enzymatic reaction, the turnover number at 37°C is about 7 min^{-1} a value not so far from those reported for cumylhydroperoxide-supported cytochrome P 450 reactions (14). These results indicate that the presence of a thiolate axial ligand of the iron (16) is not an absolute requisite for the alkylhydroperoxide-supported dealkylation reactions to proceed. However a valid appreciation of the importance of this thiolate ligand in cytochrome P 450-dependent reactions requires further investigations of the mechanism of the above described iron-porphyrin-catalyzed reactions.

REFERENCES

1. Rahimtula, A.D. and O'Brien, P.J. (1974) Biochem. Biophys. Res. Commun. 60, 240-247.
2. Nordblom, G.D., White, R.E. and Coon, M.J. (1976) Arch. Biochem. Biophys. 175, 524-533.
3. Lichtenberger, F., Nastainczyk, W. and Ullrich, V. (1976) Biochem. Biophys. Res. Commun. 70, 339-346.
4. Hrycay, E.G., Gustafsson, J.A., Ingelman-Sundberg, M. and Ernster, L. (1975) F.E.B.S. Letters 56, 161-165.
5. O'Brien, P.J. (1978) Pharmac. Ther. A. 2, 517-536 and references therein.

6. Groves, J.T., Mc Clusky, G.A., White, R.E. and Coon, M.J. (1978) Biochem. Biophys. Res. Commun. 81, 154-160.
7. Chang, C.K., Shang Kuo, M. (1979) J. Am. Chem. Soc. 101, 3413-3414.
8. Groves, J.T., Nemo, T.E. and Myers, R.S. (1979) J. Am. Chem. Soc. 101, 1032-1033.
9. Ullrich, V. and Weber, P. (1972) Hoppe-Seyler's Z Physiol. Chem. 353, 1171-1177.
10. Fleischer, E.B., Palma, J.M., Srivastrava, T.S. and Chatterjee, A. (1971) J. Am. Chem. Soc. 93, 3163-3167.
11. Netter, K.J. and Seidel, G. (1964) J. Pharmacol. Exp. Ther. 146, 61-65.
12. Nash, T. (1953) Biochem. J. 55, 416-421.
13. Boehmke, G., Ruhlig, G. (1970) Brit. patent. 1, 185, 769 and Chem. Abs. 72, 121175 f.
14. Rahimtula, A.D. and O'Brien, P.J. (1975) Biochem. Biophys. Res. Commun. 62, 268-275.
15. Dunford, H.B. and Stillman, J.S. (1976)Coordination Chem. Reviews 19, 187-251.
16. Ullrich, V. (1979) Topics in current chemistry, 83, 68-119