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OXIDATION OF MONOSUBSTITUTED OLEFINS BY CYTOCHROMES P-450 AND HEME MODELS : EVIDENCE FOR THE FORMATION OF ALDEHYDES IN ADDITION TO EPOXIDES AND ALLYLIC ALCOHOLS

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Aldehydes RCH_2CHO are formed in addition to epoxides and allylic alcohols upon oxidation of the monosubstituted olefins $RCH=CH_2$, styrene and 6-phenoxy-hex-1-ene, either by liver microsomal systems in the presence of NADPH and O_2 or C_6H_5IO, or by iron-porphyrin- C_6H_5IO model systems. These aldehydes do not derive from rearrangement of the corresponding epoxides suggesting that they are formed by an hydrogen migration within an interdiate formed by reaction of the olefin with the active oxygen-iron complex. Heme model studies show that the competition between the three possible routes of oxidation of these olefins is greatly dependent upon the environment of the iron.

The cytochromes P-450 of hepatic microsomal monooxygenases catalyze the oxidation of olefins (1-3). Generally, epoxides are the main primary products deriving from these oxidations and several studies have been devoted to the understanding of the mechanism and stereochemistry of the epoxidation reaction (4-7). Hydroxylation of the reactive allylic C-H bonds of olefins generally competes with the epoxidation reaction (6, 8), and, in the particular case of non-hindered terminal olefins, a third oxidative pathway is involved which leads to cytochrome P-450 inactivation by N-alkylation of the prosthetic heme (7,9). However, only very few detailed studies on the nature of all the possible reactions involved during cytochrome P-450 dependent oxidation of olefins, as well as on the factors influencing the relative importance of these reactions, have been so far reported. Most of them are concerned with cyclohexene, the oxidant used being either O_2 in the presence of NADPH or an oxygen atom donor such as iodosobenzene or cumylhydroperoxide (6,8).

This communication reports preliminary results on the regioselectivity of the oxidation of monosubstituted olefins by microsomal cytochromes P-450 and heme models. In particular, it gives a first evidence that aldehydes RCH_2-CHO are formed in addition to epoxides and allylic alcohols upon cytochrome P-450-dependent oxidation of terminal olefins $RCH=CH_2$. It also shows that these aldehydes are formed upon iron-porphyrin-cataly-

Abbreviations : PA = phenylacetaldehyde ; SO = styrene oxide ; 6-PH = 6-phenoxy-hex-1-ene ; TPP = meso-tetraphenyl porphyrin ; BH = "Basket handle" porphyrin (see Fig. 1) ; TCPO = 3,3,3-trichloro-propene oxide ; NADPH = nicotinamide adenine dinucleotide phosphate (reduced form ; G6P = glucose-6-phosphate ; G6PDH = glucose-6-phosphate dehydrogenase ; CHP = Cumylhydroperoxide.

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zed oxidation of the same olefins by C_6H_5IO. The present results establish that these aldehydes do not derive from rearrangement of the corresponding epoxides and suggest that they derive from an hydrogen migration within an intermediate formed by reaction of the olefin with the active oxygen-iron complex.

MATERIALS AND METHODS

Products : Styrene was purchased from PROLABO, phenyl-acetaldehyde (PA) and styrene oxide (SO) from Fluka, TCPO from ALDRICH, NADP, G6P and G6PDH from SIGMA chemicals Co, St Louis Mo. Iodosobenzene (10), 6-phenoxyhexanal 3 (11), iron-tetraphenylporphyrin Fe^{III}(TPP)(Cl)(12) and iron- "Basket-handle" porphyrin Fe^{III}(BH)(Cl)(13) were prepared as previously described. 6-phenoxy-hex-1-ene(6-PH) was prepared by reaction of 6-bromohexene (from FLUKA) with an excess of sodium phenate in DMSO (3h reflux), extracted into hexane and distilled (75% yield), bp 69°C (0.2 mmHg). 6-phenoxy-hex-1-ene oxide 1, was prepared from the corresponding alkene with m-chloroperbenzoïc acid by a standard procedure (14) (80% yield), bp 125°C (10 mmHg). 6-phenoxy-hex-1-en-3-ol, 2, was prepared by reaction of 6-PH with 0.5 eq of SeO_2 (from PROLABO) in the presence of tertiobutylhydroperoxide (from MERCK) in dichloroethane (48 h at 40°C) (15).
All these new products gave satisfactory elemental analysis, ^1H NMR and mass spectra in agreement with the indicated structures.

Typical procedure for microsomal oxidations of styrene and 6-PH : Liver microsomes were prepared (16) from male Sprague-Dawley rats which have been pretreated with sodium phenobarbital (PB)(80 mg/kg in saline, I.P., 3 days). NADPH-dependent aerobic oxidations : The olefin (1 mM) was incubated at 37°C with 1 mg of microsomal proteins (2 nmoles cytochrome P-450 per mg protein) in 1 ml of 0.1 M phosphate buffer pH 7.4 containing 0.5 mM TCPO and a NADPH generating system (1 mM NADP, 10 mM G6P and 2U G6PDH). Previous studies had shown that the Km for 6-PH epoxidation was about 0.01 mM (17). Aliquots were removed after 1,5,10 and 30 min. and treated by 1 ml CH_2Cl_2 to stop the reaction and extract the products. C_6H_5IO dependent-oxidations : conditions identical to the previous one except that the NADPH generating system was replaced by C_6H_5IO (1 mM) and that products analysis was done after 0.5 and 1 min. (reaction stopped by addition of 1 ml CH_2Cl_2 and 0.5 ml sodium bisulfite 1 M) as for longer incubation times there is a too important oxidative degradation of microsomal enzymes. The organic extracts were analyzed by g.l.c. and mass spectrometry after addition of phenylethylketone and nonadecane as internal standards respectively for styrene and 6-PH. No oxidation product (SO, PA, 1, 2 or 3) was detected in incubations performed in the absence of NADPH or C_6H_5IO. The formation of SO, PA, 1, 2, 3 were found linear as a function of time for about 10 min. with NADPH and O_2 but only for about 1 min. with C_6H_5IO.

Typical procedure for iron-porphyrins-dependent oxidation of olefins by C_6H_5IO : 4.5 μmol of C_6H_5IO was added to 1 μmol of Fe(porphyrin)(Cl) and 0.1 mmol of olefin in 0.1 ml C_6H_6. After all C_6H_5IO was consumed (1h reaction at 20°C), the internal standard was added and the mixture analyzed as above described. No oxidation took place when either C_6H_5IO or the iron-porphyrin was omitted.

Analysis conditions : Styrene oxidation was followed by g.l.c. with an INTERSMAT IG 120 FL (hydrogen flame ionization detector, glass column packed with 5% w/w Carbowax on Anachrom SD, column temperature 130°C, injector and detector temperature 210°C, N_2 carrier gas pressure 1 bar). For 6-PH oxidation we used a capillary column CPSyl5 chrompack, 25m (column temperature from 120° to 250°C, 4°C/min. injector and detector temperature 290°C, H_e carrier gas pressure 0.4 bar). For combined gas-chromatography mass spectrometry, a GIRDEL chromatograph 30 (equipped with a capillary column 50 m CPSyl 5 chrompack, operating with a 5°C per min. temperature programming from 80°C to 250°C and a carrier gas pressure of 1 bar) was coupled with a RIBER 1010 B mass spectrometer (electron ionization 70eV, trap current 2 mA) and PDP 8 computer.

RESULTS

Oxidation of 6-phenoxy-hex-1-ene (6-PH) by NADPH and O_2 or C_6H_5IO catalyzed by rat liver microsomes 6-PH was recently used as an hydrophobic polyfunctional substrate in order to study the intrinsic reactivities of different microsomal cytochromes P-450 (17). It has

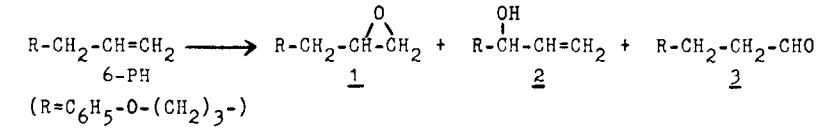
Table 1 : Oxidation of styrene and 6-phenoxy-hex-1-ene by rat liver microsomes with various oxidants (a)

Oxidants	Formation rates (nmol x nmol P-450^{-1} x min^{-1})(b)							
	SO	PA	R(c)	1	2	3	R(c)	R' (c)
NADPH/O_2	3.3±0.3	0.10±0.01	33±3	3.4±0.3	0.65±0.03	nd(d)		5.2±0.7
C_6H_5IO	52.5±4.7	2.0±0.2	26±4	8.8±0.9	3.2±0.2	0.9±0.1	10±2	2.7±0.5

a) The assays were performed as described in Materials and Methods. b) The results are given as mean values (±SD) from three experiments. Products formation was linear with time at least for 10 min. with the NADPH/O_2 system and for 1 min. for the C_6H_5IO system. c) R is the epoxide : aldehyde ratio and R' the epoxide : allylic alcohol ratio. d) not detected

been shown that the major oxidation product of 6-PH when incubated with liver microsomes from PB-treated rats in the presence of NADPH and O_2 was 6-phenoxy-hexan-1,2-diol (17). Adding the microsomal epoxide hydrolase inhibitor, trichloropropene oxide (TCPO, 0.5 mM) completely abolished the formation of the diol, which was replaced by 6-phenoxy-hex-1-ene oxide 1 without changing the rates of 6-PH oxidation to a significant extent.

The oxidation products of 6-PH(1 mM) by rat liver microsomes (1 mg protein/ml) incubated with NADPH (1 mM) in aerobic conditions or with C_6H_5IO (1 mM) in the presence of TCPO (0.5 mM) were analyzed by g.l.c. and mass spectrometry by comparison with authentic samples prepared by non ambiguous methods. The epoxide 1 and the allylic alcohol 2 (Scheme 1) were always found as major metabolites of 6-PH (Table 1), their formation ra-



Scheme 1

tes being markedly higher with C_6H_5IO as oxidant. The epoxide : allylic alcohol ratio, R', was found slightly lower in the case of the oxygen atom donor C_6H_5IO. A third product, the aldehyde 3 was formed in oxidations by C_6H_5IO but could not be detected in NADPH + O_2-dependent reactions. This aldehyde does not derive from an isomerization of epoxide 1 since incubation of 1 at various concentrations from 10 μM to 1 mM with the complete microsomal system (with TCPO and NADPH or C_6H_5IO, but not 6-PH) under identical conditions failed to give 3. Moreover, one found that aldehyde 3 (10 μM) is stable under incubation conditions identical to those previously used for 6-PH.

Oxidation of styrene by O_2 and NADPH or C_6H_5IO catalyzed by rat liver microsomes. Under conditions identical to those used for 6-PH, styrene is converted by TCPO-containing microsomes into two metabolites, styrene oxide (SO) and phenyl-acetaldehyde (PA)(table 1), which have been detected by g.l.c. and mass spectrometry. With both oxidants, O_2 and C_6H_5IO, the epoxide : aldehyde ratios (R) were similar (~ 30), although the rate of styrene oxidation was much greater with C_6H_5IO than with NADPH + O_2. In the absence of TCPO, SO was rapidly hydrolyzed to the corresponding diol while the kinetics of PA formation remained identical. Upon incubation of SO (at concentrations ranging from 10 μM to

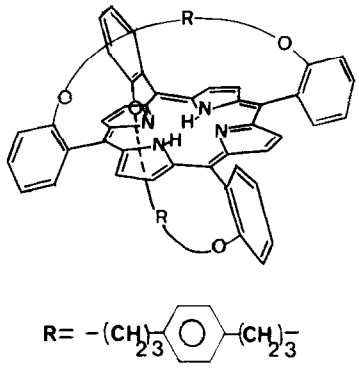


Figure 1 : "Basket-handle" porphyrin (BH) BH=TPP with an alkyl chain on both sides of the porphyrin plane branched in ortho position of the phenyl rings by either linkage (13).

1 mM) with the complete microsomal system (containing TCPO and NADPH or C_6H_5IO but not styrene) under identical conditions, no trace of PA could be detected by g.l.c. and mass spectrometry. This shows that phenyl-acetaldehyde does not derive from a rearrangement of styrene-oxide which could have been catalyzed by cytochrome P-450Fe(III) (18). It is noteworthy that PA (5 or 10 μM) is fully recovered after its incubation with the microsomal system under conditions identical to those of its formation except the presence of styrene and after identical extraction and analysis, showing that the aldehyde is stable under the conditions of its formation.

Oxidation of 6-PH and styrene by heme-C_6H_5IO model systems. It has been previously shown that iron-porphyrins were able to catalyze the transfer of the oxygen atom from C_6H_5IO to olefins (19-21) whereas they failed to catalyze the same reaction with CHP as an oxidant (22). We have thus studied the oxidation of 6-PH and styrene by C_6H_5IO in the presence of catalytic amounts of either Fe(III)(tetraphenylporphyrin=TPP)(Cl) or Fe(III)(BH)(Cl), BH being a "basket-handle" porphyrin derived from TPP by the presence of an alkyl chain on both sides of the porphyrin ring (13)(Fig. 1).

As the microsomes-C_6H_5IO system, these model systems led to epoxides as major products but also to significant amounts of aldehydes, phenylacetaldehyde and 3 respectively from styrene and 6-PH (Table 2). It has also been verified that the epoxides did not rearrange into the corresponding aldehydes under the reaction conditions. It is noteworthy that

Table 2 : Oxidation of styrene and 6-phenoxy-hex-1-ene by iodosobenzene catalyzed by various iron-porphyrins (a)

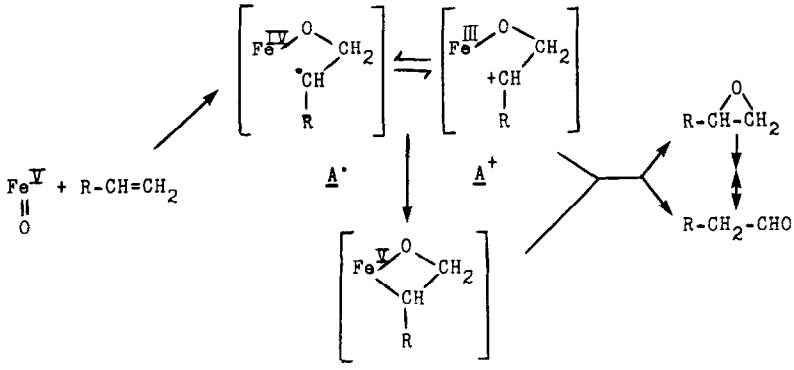
Catalyst	Products % yield (b)							
	SO	PA	R(c)	1	2	3	R(c)	R'(c)
Fe(TPP)(Cl)	75	3	25	40	48	1.4	28	0.8
Fe(BH)(Cl)	44	15	3	40	11	8.5	4.7	3.6

a) The assays were performed as described in Materials and Methods. b) % yields are based on the oxidant. These results are the mean ± 5 % of at least 3 experiments. c) see note c in Table 1.

the epoxide : allylic alcohol ratio R' greatly depends on the nature of the porphyrin catalyst, R' passing from 0.8 for Fe(TPP)(Cl) to 3.6 for Fe(BH)(Cl) (Table 1). The epoxide : aldehyde ratio, R, which is similar for both olefins, is even much more dependent on the nature of the porphyrin catalyst.

DISCUSSION

Epoxidation of the double bond was always observed as the major route of oxidation of 6-PH and styrene either by rat liver microsomes with NADPH + O_2 or C_6H_5IO, or by iron-porphyrin-C_6H_5IO systems. The mechanism generally admitted for epoxidation of olefins by C_6H_5IO in the presence of cytochrome P-450 (6,23,24) or iron-porphyrins (20,21) involves the addition on the double bond of a high-valent iron-oxo-species having a free radical character (Scheme 2). The high stereospecificity (20,24) of these epoxidations suggests that the intermediate free radical is very efficiently controlled by the iron. This control could be explained by a very fast oxidative transfer of the oxygen ligand of the Fe(IV) intermediate to this free radical leading eventually to the epoxide (6,7,20-24), or by a fast combination of the radical with the iron (25) leading to an intermediate four-membered metallocycle (26) that finally gives the epoxide by reductive elimination of two cis ligands of the iron.



Scheme 2

The aforementioned results establish that upon oxidation of 6-PH and styrene by C_6H_5IO catalyzed either by rat liver microsomes or by iron-porphyrins, linear aldehydes are also formed. These aldehydes are not derived from an isomerization of the corresponding epoxides similar to that observed for phenol formation from arene-oxides (NIH shift) (27). Very recently, PA has been shown to be formed upon oxidation of styrene by C_6H_5IO in the presence of iron-porphyrins (28), the proposed mechanism for this transformation involving an hydrogen migration inside the intermediate complex $\underline{A}^+$ (Scheme 2). Moreover the formation of trichloroacetaldehyde upon cytochrome P-450-dependent oxidation of trichloroethylene has been shown to occur by a chlorine atom migration within intermediate complexes analoguous to those indicated in scheme 2 (23). Finally, a reaction similar to that described in this paper, the formation of 2,2-dichloro-ethanal upon microsomal oxidation of 1,1-dichloro-ethylene has been very recently observed and found to derive from a 1,2-hydrogen migration, the epoxide being not involved as an intermediate (29). Taken

altogether these results point to possible 1,2-migrations of certain atoms within cytochrome P-450 intermediates involved in olefins oxidations. They suggest that the aldehydes formed upon the above described microsomal and chemical oxidation of styrene and 6-PH could derive from an hydrogen migration inside either the intermediate radical $\underline{A}^{\cdot}$ (or the cation $\underline{A}^{+}$) or the metallocycle indicated in scheme 2, these intermediates being thus the common precursors of epoxides and aldehydes.

Concerning the competition between the three possible routes of 6-PH oxidation leading to compounds 1, 2 and 3, the heme model studies showed that it is greatly dependent upon the environment of the heme (Table 2). With the BH porphyrin where the access of the olefin was made difficult because of the alkyl chains, the relative amount of allylic alcohol greatly decreased compared to Fe(TPP)(Cl). This is certainly related to a more difficult approach of the internal allylic CH_2 group of 6-PH, the approach of the terminal vinylic CH_2 group being less disfavored. Interestingly, the epoxide : allylic alcohol ratio obtained with Fe(BH)(Cl)($R' = 3,6$) is very different from that obtained with Fe(TPP)(Cl)($R' = 0.8$) and closer to those observed for the enzymatic reactions ($R' \sim 3$-5)(Table 1). The protein chains present in the iron vicinity in the active site of cytochromes P-450 should play a role similar to the BH chains for controlling the access of the olefin to the iron (22) and determining the epoxide : allylic alcohol ratio.

The access to the iron also plays a key role in determining the epoxide : aldehyde ratio (Table 2). The introduction of alkyl chains above the iron without changing to a significant extent the electronic characteristics of the porphyrin leads to a considerable increase of aldehyde formation. The R ratio is thus not only dependent on the olefin nature but also on the nature of the iron environment, and one should expect that it could vary with the nature of the cytochrome P-450 used. Such studies on the competition between the different routes of olefin oxidation should be important for determining the specific reactivities of the different forms of microsomal cytochrome P-450 and for building selective heme models for them.

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