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STUDY OF H_2O_2-SUPPORTED N-DEMETHYLATIONS CATALYZED BY CYTOCHROME P-450 AND HORSERADISH PEROXIDASE

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SUMMARY: H_2O_2-supported oxidative demethylation reactions catalyzed by cytochrome P-450 and horseradish peroxidase have been compared. In contrast to peroxidase catalyzed reactions no free substrate radicals could be detected by EPR stopped flow measurements in demethylation reactions catalyzed by highly purified cytochrome P-450 although the rate of product formation for both enzyme systems was identical. These findings cause doubts in a general peroxide dependent demethylation mechanism valid for all hemoproteins and in the hypothesis that free substrate radicals are principally formed during cytochrome P-450 catalysis.

INTRODUCTION: Liver microsomal P-450 has been shown to catalyze the oxidation of several classes of substrates supported by NADPH and molecular oxygen as well as by hydroperoxides (1 - 5). For this pathway common species of "activated oxygen" have been proposed. However, recently there are serious doubts in intermediary species common for both ways, e.g. striking differences between NADPH- and cumene hydroperoxide supported hydroxylation of benzo(a) pyrene have been found (6,7) indicating a dominance of an 1-electron-oxidation mechanism in the case of organic hydroperoxides. Contradictory a similarity between NADPH- and H_2O_2-supported reactions catalyzed by P-450 could be shown (8). The products of both NADPH- and H_2O_2-supported hydroxylation of benzo(a)pyrene indicate

Abbreviations: P-450, cytochrome P-450
HRP, horseradish peroxidase

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the dominance of an epoxidation pathway, i.e. an oxenoid 2-electron-mechanism. Despite these findings many authors up to now suggest that the H_2O_2-supported reaction of P-450 follows a peroxidase-like mechanism. Such a mechanism would involve the formation of free substrate radicals. A general peroxidase-like mechanism common for hemoproteins catalyzing hydroperoxide-supported N-demethylation reactions has been proposed by Griffin and coworkers (9 - 12) with loss of one electron and formation of alkylamine radical as first step.

According to these authors P-450 should catalyze demethylation reactions by this mechanism, too. White and Coon (13) have postulated a reaction scheme fo P-450 (the so called "quasi Fenton" mechanism) where a substrate carbon radical is generated by hydrogen abstraction from the substrate with the help of an oxygen radical (RO•), i.e. the radicalic splitting of CH-bond.

To prove these hypothesises and to characterize H_2O_2-supported reactions catalyzed by P-450 EPR stopped flow measurements were performed.

MATERIALS AND METHODS: Highly purified rabbit liver P-450 LM_2 was a kind gift of Prof. M.J. Coon (Ann Arbor, USA). It was prepared by the method described in (14). The sample solution contained 13.35 nM P-450 and 1.06 mg protein/ml in 0.01 M KPO_4 buffer with 20 % glycerin and 1 mM EDTA. Horseradish peroxidase (HRP) was obtained from VEB Arzneimittelwerk Dresden, GDR (RZ 0.3) and from Sigma (type VI, salt free powder, RZ about 3).

Aminopyrine was purchased from Aldrich. Hydrogen peroxide was obtained from Mallinckrodt Chemical Works (St. Louis, USA). The H_2O_2 concentration was determined by polarography.

The formation of formaldehyde was assayed by the Nash procedure (15) after the reaction had been quenched with 15 % trichloroacetic acid and filtrated to remove precipitated protein.

Electron paramagnetic resonance (EPR) spectra were recorded on a Varian E3 spectrometer on line with a computer KRS 4200 (VEB Robotron, Dresden, GDR).

The concentration of free radicals was determined by double integration of EPR spectra (taking into account the time dependence of radical concentration) and comparison with the known spin concentration of an aqueous solution of 2,2,5,5-tetramethyl-3-carboxamido-pyrrolidin-1-oxyl as standard.

For measurement of radical kinetics the EPR spectrometer was equipped with a novel stopped-flow apparatus the principle of which was described by Klimes et al. (16). The used device has a mixing efficiency of 95 % and allows the observation of a chosen amplitude of

the EPR signal as a function of time 1 ms after mixing. In order to achieve a higher spectral intensity in these kinetic studies the EPR spectrum was overmodulated with 2.0 mT to give a singlet. The kinetic curve was recorded on a fixed field position at the maximum at the maximum of the signal.
In cases when the x,y-recorder was used for registration, the time resolution was limited to 100 ms due to the time constant of the recorder. For detection of species with half life times shorter than 100 ms a storage ascilloscope (OG 2-31, VEB Messelektronik, Berlin, GDR) was used.

RESULTS: Aminopyrine was chosen as model substrate to compare substrate radical formation by P-450 and HRP because it is N-demethylated by both enzymes. However, the reaction rate of H_2O_2-supported aminopyrine demethylation catalyzed by HRP is approximately 3 orders of magnitude higher than for the P-450 reaction. Therefore first of all adequate reaction mixtures had to be chosen to reach the same rate of product formation (i.e. formaldehyde) in both the HRP and P-450 systems. That was done decreasing the HRP concentration used to 10 nM drastically in comparison to P-450 which was used in the concentration of 670 nM. Both enzymes showed a linear concentration dependence of formaldehyde formation per minute during the first 10 min of the reaction. Fig. 1 shows that with this low HRP concentration an EPR signal indicating an aminopyrine radical could be clearly detected. The used stopped flow technique makes sure that even transient free substrate radicals with half life times up to 1 ms would be detected. In control experiments if aminopyrine, HRP or H_2O_2 are omitted no EPR signal could be found. Kinetic experiments combined with rapid scan technique showed that the radical concentration in the reaction catalyzed by HRP reached a maximum after 4 min (Fig. 2). The maximal radical concentration was evaluated to be 25 µM.
In contrast no aminopyrine radicals could be detected in stopped-flow EPR experiments with highly purified P-450.
To be sure that there is no radical scavanger within the P-450 preparation, free aminopyrine radicals were first generated by the

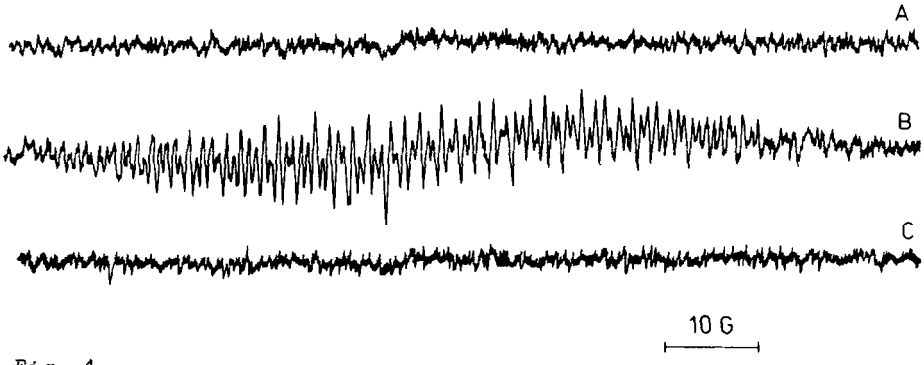

Fig. 1
Comparison of EPR spectra of aminopyrine/H_2O_2/HRP and aminopyrine/H_2O_2/P-450 LM2. All measurements were performed in 0.1 M Na-phosphate buffer (pH 6.5) at 25 °C with 2.4 mM H_2O_2, 2.5 mM aminopyrine.
(A) enzyme omitted, (B) 10 nM HRP, (C) 0.67 µM P-450.
Spectrometer settings: microwave power, 20 mW; modulation amplitude, 0.16 mT; scan time, 8 min; time constant, 0.3 s; receiver gain, $6.2\ 10^5$.

HRP/H_2O_2/aminopyrine system and after this P-450 was added. Highly purified P-450 did not show significant influence on the concentration of aminopyrine radicals.

DISCUSSION: The experiments reported here make it unlikely that P-450 does follow the general demethylation mechanism proposed by

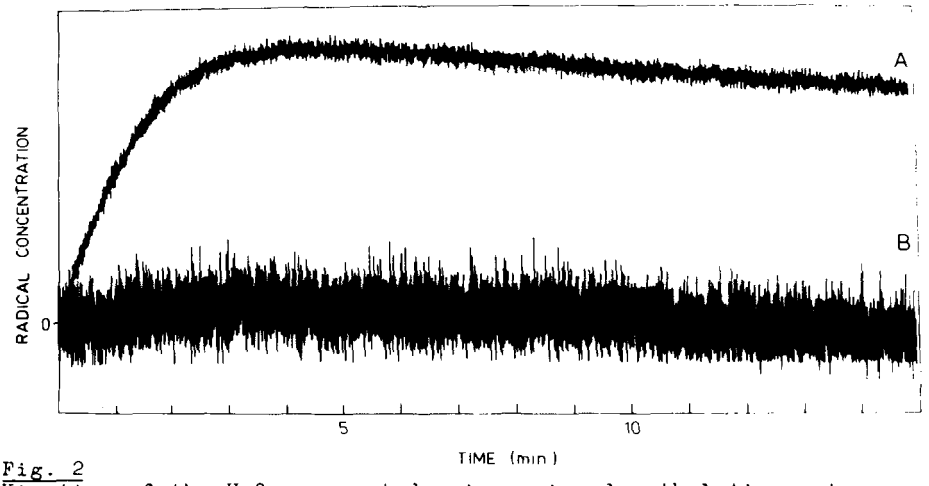

Fig. 2
Kinetics of the H_2O_2-supported aminopyrine demethylation catalyzed by HRP or P-450 LM2, respectively. The reaction was initiated by mixing of equal amounts of H_2O_2/aminopyrine solution and a corresponding enzyme solution with a stopped flow device at 25 °C. Spectrometer settings: microwave power, 20 mW; modulation amplitude, 2.0 mT; receiver gain, 2.5×10^5 (A) and 1×10^6 (B) respectively. The final concentrations of the reaction mixtures were 2.4 mM H_2O_2 and 2.5 mM in 0.1 M Na-phosphate buffer (pH 6.5) (A) with 10 nM HRP or (B) 0.67 µM P-450.

Griffin and Ting (9) because no free substrate radicals could be detected by EPR in P-450 catalyzed demethylation reactions. Therefore at least H_2O_2-supported demethylation reactions of P-450 do not reveal a peroxidase like mechanism*. The finding that highly purified P-450 does not form free substrate radicals even with such very easily oxidable substrates like N-alkylamines cause also doubts in the hypothesis of White and Coon (13) that generally substrate radicals have to be formed during P-450 catalysis. This assumption of radicals split of the substrate CH-bond was based on the finding (17) that in the hydroxylation of the exo-tetradeuterated norbornane 14 - 18 % of the exo-deuterium were converted to the endo position. The authors interprete this finding as indication of an intermediate norbornane radical produced by hydrogen abstraction. The preferred hydroxylation of the tertiary CH-bond in methylcyclohexan and the large intramolecular isotopic effect k_H/k_D for the hydroxylation of norbornane and substrates with equivalent CH- and CD-bonds (17,18) were taken as a further indication for a radicalic split of the substrate CH-bond.

The following facts seem to argue, in addition to our findings, for the nonradicalic mechanism:

(a) The tertiary carbon atom is not only a preferred position for the stabilization of the radical but also for a positive charge (carbenium ion) or partial positive charge (19).

(b) A large intramolecular isotopic effect is not a forcible evidence for a radicalic split of CH-bond but only an evidecne that the split of the CH-bond (radicalic, ionic or rearrangement including (CH-opening) is an elementary step (19).

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* This is supported by the results of Renneberg and Estabrook (in preparation) analyzing more than 10 different amines converted both by HRP and P-450. A reverse proportionality was found, i.e., substrates very fast demethylated in H_2O_2-supported reactions by HRP were only poorly demethylated by P-450, and vice versa.

(c) Epoxidations, one of the most characteristic reactions of P-450, and the NIH shift are not explainable by a radicalic mechanism. No epoxidation product is known which results from radicalic reaction. Quantum chemical calculations according to the MINDO/3 method (20) show that the theoretically possible attack of the oxygen radical on ethylene does not lead to the epoxide but yields an assymmetric biradical. The activation barrier for this radical reaction is about 14 kcal/mole higher than for the attack of an oxygen atom in the singlet state yielding the epoxide as product.

(d) If free substrate radicals are essential intermediates they could interact at least partly with other radicals and form dimers or other recombination products. For example in the H_2O_2-supported demethylation of N,N-dimethylaniline catalyzed by HRP the appearance of the dimerization product N,N,N',N'-tetramethylbenzidine was shown (22) which is in contrast to N-methylaniline and formaldehyde formed by P-450. Recombination products have not been shown to our knowledge up to now for P-450 catalyzed reactions. The often discussed "cage effect" of the active site could hinder the interaction of hypothetical radicals but in this case at least an interaction with molecular oxygen should occur (substrate radical $R\bullet + \bullet\underline{O} - \underline{O}\bullet \longrightarrow R - \underline{O}-\underline{O}\bullet$). However, none of such substrate peroxides formed by P-450 are detected.

Summarizing one can draw the conclusion that at least H_2O_2-supported reactions of P-450 do not follow a peroxidase or Fenton like mechanism which are based both on the formation of substrate radicals However, substrate radicals could be formed in reactions of P-450 supported by organic hydroperoxides, e.g. a heme initiated decomposition of cumene hydroperoxide was found with formation of highly reactive free cumyloxy radicals responsible for 1-electron-oxidations (10). In addition a very high yield of 1-electron-oxidation products could be shown in the cumene hydroperoxide supported hy-

droxylation of benzo(a)pyrene catalyzed by microsomal P-450 (6). Furthermore was shown that oxo-radicals are formed both during NADPH- and cumene hydroperoxide supported hydroxylation of benzo(a)pyrene (6) and in NADPH-supported oxidation of 2-naphtylamine (22). These oxo-radicals, however, are not primary free substrate radicals resulting from the radicalic split of a CH-bond but secondary products formed after hydroxylation. Recently with several 4-substituted 3,5-bis(ethoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridine substrates radicals could be detected by spin-trapping (23). However, the P-450 used was not highly purified and was destroyed rapidly in the reaction.

There seems to be a strong difference in reactions supported by NADPH/O_2 or H_2O_2 resp.(mainly oxenoid mechanism) on the one hand and by organic hydroperoxides (1-electron-oxidation) on the other hand It could well be that nonradicalic (oxenoid) and radicalic (1-electron-oxidation) mechanisms coexist in P-450 reactions (24). In dependence on the used cofactor or oxidant the ratio of these two mechanism could be altered. Thus both the H_2O_2- and NADPH-supported reactions would be catalyzed in a rather similar (oxenoid) way in contrast to radicalic peroxidase like reactions supported by organic hydroperoxides.

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