NADPH AND H_2O_2-DEPENDENT REACTIONS OF CYTOCHROME P-450_{LM} COMPARED WITH PEROXIDASE CATALYSIS

R. RENNEBERG, F. SCHELLER, K. RUCKPAUL, J. PIRRWITZ and P. MOHR

Zentralinstitut für Molekularbiologie der Akademie der Wissenschaften der DDR, Bereich Biokatalyse und Bereich Molekularbiophysik, 1115 Berlin-Buch, GDR

Received 10 October 1978

1. Introduction

Cytochrome P-450 has been shown not only to act as monooxygenase by activating molecular oxygen but also to catalyze substrate hydroxylation supported by various organic hydroperoxides as well as by H_2O_2 as cosubstrates [1–5]. This behaviour was ascribed to peroxidatic activity of P-450 [6–8]. According to [9] a peroxidatic reaction is characterized by the transfer of hydrogen atoms from the substrate molecule to H_2O_2 coupled with the oxidation and dehydration of the substrate, respectively. The peroxide-dependent reaction of P-450 provides spectra analogous to compound I of HRP (Fe^{4+}–O^-) [4] thus indicating a ferryl structure also in this reaction. Up to now, however, the subsequent activated species of oxygen in the P-450 reaction cycle has not yet been clarified in detail. Neither spectra analogous to compound II of HRP nor free substrate radicals have been detected in the P-450 system.

In order to get more insight into the mechanism determining HRP and P-450-catalyzed reactions H_2O_2 supported substrate conversions and NADPH-dependent reactions of P-450 were compared with H_2O_2 supported reactions of HRP. Assuming that identical reaction products and similar kinetics should reflect an analogous reaction mechanism the conversion of aniline by P-450 with respect to kinetics, reactions rates and reaction products was compared with those data obtained by the HRP catalyzed reaction. In spite of different reaction rates in NADPH- and H_2O_2-dependent reactions of P-450 identical reaction products were found. In the HRP-catalyzed reaction, however, pAP as hydroxylation product was only intermediately formed and subsequently converted to higher oxidized products thus indicating enzyme-dependent mechanisms of oxygen activation leading to different activated oxygen species and consequently to different reaction products.

Abbreviations: P-450, cytochrome P-450; HRP, horseradish peroxidase; pAP, p-aminophenole

2. Materials and methods

The microsomes used for experiments were obtained from the livers of phenobarbital-pretreated male rabbits by established methods [10]. The P-450 content was determined by CO-difference spectra according to [11,12] using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ ($A_{450\ nm}-A_{490\ nm}$).

HRP was obtained from Boehringer (RZ 3.0). The concentration of HRP was calculated from $A_{403\ nm}$ using an extinction coefficient of 100 $mM^{-1}\,cm^{-1}$ [13]. The standard reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM NaN_3, either 0.8–8.0 mM H_2O_2 or NADPH, and aniline hydrochloride (0.2–16 mM). The reaction was initiated by addition of microsomal P-450 or HRP to 1 μM final conc.

All samples were continuously stirred at 37°C. The hydroxylation of aniline was followed by means of a rotating disc electrode of paraffined spectral carbon. The pAP formed was directly determined in the reaction mixtures by the limiting current of the

anodic oxidation at 250 mV versus a saturated calomel electrode (SCE) [14] using a GWP 563-polarograph. Calibration was carried out with defined amounts of pAP in the given experimental solution, the current depends linearly on its concentration. At the same time, the rotating electrode ensured a defined convection. The H_2O_2 concentration was measured continuously using a modified oxygen electrode, recording the anodic current of the platinum electrode, polarized to +500 mV versus SCE. At this potential, oxidation both of pAP and H_2O_2 is accomplished, so that the current is proportional to the sum of the concentrations of the two substances.

3. Results and discussion

3.1. *Aniline hydroxylation by cytochrome* P-*450*

To compared the reaction products and rates of NADPH-dependent reactions with those supported by H_2O_2 parallel hydroxylation experiments using identical enzyme and substrate concentrations have been carried out. The reaction mechanism was characterized by the app. K_m and app. V_{max} values derived from Lineweaver-Burk plots assuming Michaelis-Menten kinetics.

3.1.1. NADPH–dependent reaction

By use of rabbit liver microsomes aniline was hydroxylated to pAP (fig.1.2). The concentration of pAP linearly increases with time at concentrations of NADPH (2.5 mM) which can be regarded as remaining constant as the reaction proceeds. The pAP formed yields less than 1% of the starting amount of aniline. An app. V_{max} of 0.2 nmol pAP (nmol *P*-450)$^{-1}$ min^{-1} and an app. K_m of 0.25 mM were calculated for the NADPH-dependent aniline hydroxylation.

3.1.2. H_2O_2-dependent reaction

On direct addition of 8 mM H_2O_2 saturation is reached within 20 min (fig.1.1). The occurrence of saturation shows that pAP is not further oxidized.

We found the optimum of the aniline hydroxylation rate to be shifted to pH 9.0 as compared with a pH 7.4 in the NADPH-dependent reaction. From the shift of the pH-optimum of the H_2O_2-supported

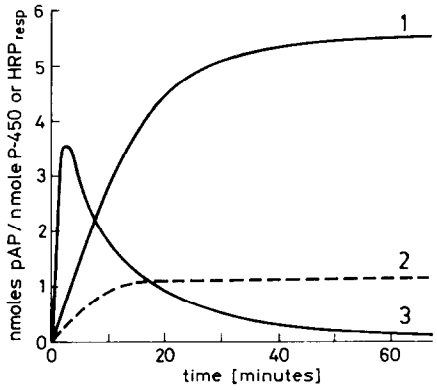


Fig.1. Time dependence of *p*-aminophenole formation at hydroxylation of aniline 0.1 M phosphate buffer (pH 6.5), 1 mM NaN_3, 37°C. (1) 1 μM cyt. *P*-450, addition of 8 mM H_2O_2 and 8 mM aniline hydrochloride. (2) 1 μM cyt. *P*-450, addition of 2.5 mM NADPH and 8 mM aniline hydrochloride. (3) 1 μM HRP, addition of 0.8 mM H_2O_2 and 0 8 mM aniline hydrochloride.

aminopyrine demethylation the participation of HO_2^-species was postulated [3]. At constant concentrations of H_2O_2 and aniline the initial rate linearly increases with increasing *P*-450 to 5 μM.

In the aniline hydroxylation an app. K_m of 18 mM for H_2O_2 as cosubstrate was determined. The app. K_m of H_2O_2 was found to be independent of the aniline concentration thus suggesting different binding sites for H_2O_2 and aniline. Our finding that imidazole more strongly inhibits the H_2O_2-dependent aniline hydroxylation than the NADPH-dependent reaction suggests H_2O_2 to be bound to the hemin-iron atom. The app. K_m of aniline at 3 different H_2O_2 concentrations (fig.2) consistently yields a value of 0.6 mM. This value is similar to the app. K_m of aniline in the NADPH-dependent reaction, which is reported [15–17] to vary from 0.1–6 mM. A similar app. K_m value in the H_2O_2- and NADPH-dependent demethylation reaction of benzphetamine has been obtained [3]. In the H_2O_2-dependent aniline hydroxylation an app. V_{max} of 1.3 nmol pAP (nmol P-450^{-1} min^{-1}) was calculated.

The lower app. V_{max} value of the NADPH-dependent reaction indicates that the reaction rate is determined by the interaction between reductase and terminal oxidase.

Decomposition of H_2O_2 by catalatic contamina-

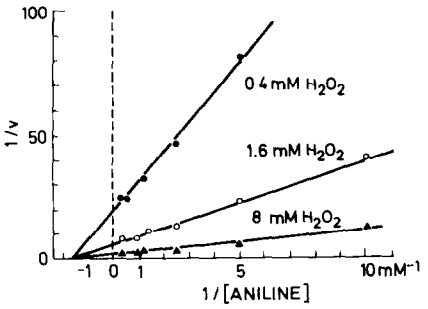
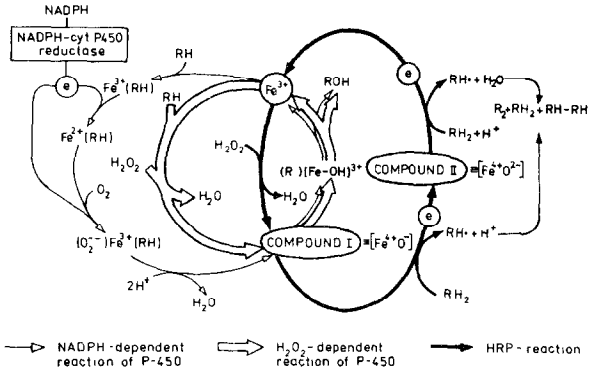


Fig.2. Effect of H_2O_2 and aniline concentration respectively on the initial rate of *p*-aminophenole formation in Lineweaver-Burk plots. Velocity is expressed as nmol pAP/nmol *P*-450/min.



Scheme 1. Comparison of reaction cycles of cytochrome *P*-450 (NADPH- and H_2O_2-dependent reactions) and horseradish peroxidase (RH, RH_2-substrates)

tions of the microsomes also occurs in the absence of substrate. This interference could be eliminated by addition of 1 mM NaN_3. On addition of aniline the sum of concentrations of pAP and H_2O_2 is not altered during the reaction indicating the same stoichiometry. Hence, pAP is the final product of the H_2O_2-dependent *P*-450 catalyzed aniline hydroxylation.

Both in NADPH- and H_2O_2-dependent aniline hydroxylation competitive inhibition by the product pAP has been found.

3.2. *Aniline hydroxylation by HRP*

In the H_2O_2 supported HRP catalyzed conversion of aniline pAP appears as a short-living intermediate (fig.1.3) which is rapidly oxidized to red coloured products. This is reflected in the stoichiometry of H_2O_2 consumption and pAP formation of about 30:1 and represents the fundamental difference between the H_2O_2-dependent reaction of HRP and *P*-450 which utilizes equimolar amounts of H_2O_2 in the hydroxylation reaction of aniline.

It is shown here that cytochrome *P*-450 in addition to several H_2O_2 supported demethylation reactions also catalyzes the hydroxylation of aniline by means of H_2O_2. This fact provides further evidence that the NADPH-dependent reaction of *P*-450 also proceeds with H_2O_2 as the co-substrate.

Comparing NADPH- and peroxide-dependent reactions of *P*-450 a number of common characteristics have been analyzed suggesting the involvement of the same form of activated oxygen (scheme 1):

(i) Identical reaction products (aniline is converted to pAP, steroids are hydroxylated in the same position [18].
(ii) Similarity of K_m values for the same substrate
(iii) Correlation of activation parameters [19].
(iv) The entering hydroxyl group displaces a deuterium substituent to an adjacent position ('NIH-shift') [20].
(v) Stimulation by lipid [21].
(vi) No free substrate radicals could be found by EPR investigations [22].

By the so-called oxidase activity of *P*-450 [23] H_2O_2 is also formed in NADPH-dependent substrate hydroxylation. Furthermore by the disproportionation of O_2^- formed in autoxidation of ferrous *P*-450 or the interaction of oxygen with NADPH–*P*-450-reductase H_2O_2 [24,25] is generated, which in vivo immediately is destroyed by catalase. The level of H_2O_2 found in microsomal hydroxylation is relatively low [26] with regard to the high app. K_m value of H_2O_2 in *P*-450-catalyzed reactions. Therefore the role of H_2O_2 in NADPH-dependent hydroxylation reactions of P-450 may be neglected.

4. Conclusion

The results presented raise the question what detailed differences in reaction mechanism characterize P-450 and HRP catalyzed reactions, respectively.

In spite of some similarities of *P*-450 and HRP, however, the HRP reaction significantly differs from the *P*-450 reaction in:

(i) Different reaction products (pAP – only intermediate).
(ii) Inability to metabolize several *P*-450 substrates (e.g., benzphetamine).
(iii) Increased H_2O_2 consumption for the oxidation of intermediates.
(iv) Formation of free substrate radicals which can disproportionate or dimerize [9], formation of substrate radicals was shown by EPR measurements in the H_2O_2-dependent reaction of HRP with aminopyrine [27]. Contrary to these results no radicals could be found in H_2O_2-dependent aminopyrine demethylation by *P*-450 [28].

The fundamental difference is the lower rate of formation of compound I in the *P*-450 system compared with HRP. The higher stability of compound II in the HRP reaction [29,30] leads to the separation of the two 1-e⁻steps. Therefore free radicals and compound II can be detected in the HRP reaction. The abstraction of 1 hydrogen atom gives formally also in *P*-450 system an oxidation state of compound II. In this complex the oxygen atom – called 'oxenoid' – should have one negative charge and may be able to react in electrophilic manner. The transient carbon radicals formed may exist only in the enzyme–substrate cage [31]. The transfer of the oxenoid species into the substrate radical may be fast in comparison with the hydrogen abstraction since no compound II analogue could be detected in the *P*-450 reaction.

All these findings indicate that in spite of the occurrence of the compound I form in both systems the oxidation of the substrate is accomplished in different ways.

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