PEROXIDATIC ACTIVITY OF LIVER MICROSOMAL CYTOCHROME *P*-450

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1. Introduction

In the mixed function oxidase reaction molecular oxygen is activated by an as yet unknown mechanism [1]. The stoichiometry suggests that activated oxygen exists at the redox level of peroxide or atomic oxygen (oxene). Although hydrogen peroxide was detected in a microsomal hydroxylation system [2] it was excluded as active species, because H_2O_2-generating systems were not able to substitute NADPH in drug hydroxylation [3]. In contrast cytochrome *P*-450 in presence of organic hydroperoxides supports the C-oxidation of several amines and hydroxylation of aromatic substrates and steroids [4–8]. Inability of H_2O_2 to support hydroxylation was ascribed to the presence of contaminating catalase in microsomal preparations [8].

The aim of the present paper is to analyze the catalytic properties of microsomal and partially purified cytochrome *P*-450 in presence of electrochemically generated H_2O_2 and HO_2^- and to show that H_2O_2 is capable to support the demethylation of several substrates in absence of NADPH. This reaction shows an absolute requirement for cytochrome *P*-450.

2. Material and methods

The microsomes used for experiments were obtained from the livers of phenobarbital pretreated male rabbits by established methods [9]. Partially purified cytochrome *P*-450 was prepared according to [10]. Demethylation of benzphetamine, aminopyrine, and *p*-notroanisole was assayed by measuring the formaldehyde formed [11]. *P*-nitroanisole demethylase activity was additionally assayed according to Netter and Seidel [12] and 4-amino antipyrine was measured as described in [13]. The typical reaction mixture contained 10 ml of 0.1 M phosphate buffer (pH 6.5), 5 μM cytochrome P–450 contained in 1.5 mg protein per ml, 1 mM semicarbacide and 2 mM substrate. The electrolysis was carried out with a PS 2.3 potentiostat (F.S. Meinsberg, DDR), oxygen was reduced at a mercury pool electrode (3 cm^2) in the potential region between −0.2 and −0.8 V. The sample volume was continuously stirred at 15°C. The current was chosen between 1–4 mA corresponding to 5–20 nmol H_2O_2 generated per second.

3. Results

Liver microsomes from phenobarbital-induced rabbits metabolize a variety of substrates in the absence of NADPH by cathodically reduced molecular oxygen. Benzphetamine, aminopyrine, and *p*-nitroanisole are demethylated by this system. The reaction products formaldehyde, 4-amino antipyrine, and *p*-nitrophenole were identified. The rate of benzphetamine demethylation is constant within 1 h. In *p*-nitroanisole demethylation a saturation is found after 1 h. The reaction rate depends linearly up to a protein concentration of at least 3 mg/ml. Total conversions of substrates ranged from 10–90%.

Increase of cytochrome *P*-420 content during electrolysis does not occur as proved by CO-difference spectra.

3.1. *Specificity of cytochrome* P-450

When electrolysis is carried out in the absence of

microsomes or with microsomes heated at 70°C for 5 min no benzphetamine is demethylated, giving evidence for the enzymatic nature of the reaction. Figure 1 shows that sodium deoxycholate inhibits the reaction indicating the requirement for native cytochrome *P*-450 in demethylation. In accordance with these results the CO-difference spectra show the maintenance of cytochrome *P*-450 during electrolytic oxygen reduction. Partially purified cytochrome *P*-450 catalyses these hydroxylation reactions as well. Cytochrome *c* reductase is not required.

In contrast to the NADPH dependent demethylation CO has no influence on the H_2O_2 supported reaction thus excluding the participation of ferrous cytochrome *P*-450 in this reaction. Horse radish peroxidase cannot substitute the microsomes in benzphetamine demethylation (fig.1). Typical inhibitors of peroxidase as azide (1 mM) and cyanide are ineffective in the microsomal system. These results show that contamination of peroxidase is not responsible for the metabolism of various substrates by cytochrome *P*-450 and electrochemically reduced oxygen.

Under these conditions also catalase, methemoglobin and hemin complexes were not able to substitute the microsomes demethylating substrates by cathodically reduced molecular oxygen. In contrast catalase catalyzes the cumene hydroperoxide dependent demethylation of aminopyrine and benzphetamine more effectively than microsomes [4].

3.2. *Variation of H_2O_2 generation*

Molecular oxygen is reduced at mercury to superoxide ion O_2^- in the potential region between −0.2 and −0.8 V. This species is immediately involved in very fast reactions leading to the anion O_2H^- or H_2O_2 [14]. In contrast to the hydrophobic films of triphenylphosphinoxide or quinoline [14,15] the transfer of the second electron during oxygen reduction is not inhibited by protein [16] or the microsomal fraction: In presence of microsomes the d.c. polarographic limiting current reaches the same value as in pure buffer solution, indicating the accordance of the number of transferred electrons per oxygen molecule. In addition the current yield of O_2^- generation at pH 6.5 is considerably lower than the value in benzphetamine demethylation of 40–60%. During the demethylation experiments H_2O_2 is accumulated in solution corresponding to the charge which is not consumed in demethylation. The H_2O_2 is not decomposed in the presence of microsomes and absence of substrate as proved by the polarographic analysis. In contrast the demethylation reaction is inhibited by addition of catalase, showing the essential role of peroxide in the system under investigation (fig.2).

As well as by electrochemically generated H_2O_2, by addition of H_2O_2 benzphetamine in the presence of microsomes is demethylated. The reaction rate increases with rising generation current or increasing peroxide concentration. The rate of benzphetamine demethylation with a starting concentration of 2 mM

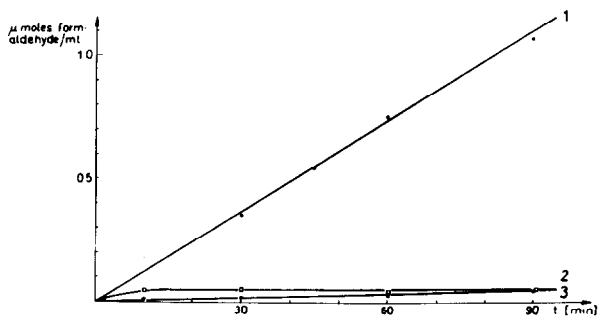

Fig.1: Specificity of cytochrome *P*-450 in demethylation of benzphetamine. 2 mA, 1.8 mM benzphetamine. (1) 5 µM cytochrome *P*-450. (2) 2 µM peroxidase. (3) 5 µM cytochrome *P*-450; 0.5% w/v sodium deoxycholate.

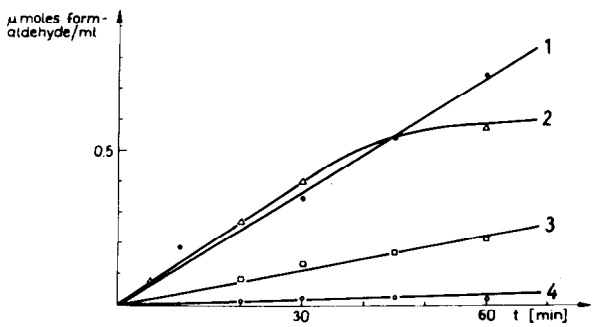

Fig.2: Variation of H_2O_2 generation. 5 µM cytochrome *P*-450; 1.8 mM benzphetamine. (1) Cathodic reduction (2 mA). (2) Addition of 2 mM H_2O_2. (3) Glucose oxidase (1 I.U.) and glucose. (4) Cathodic reduction at 2 mA in presence of catalase (1000 units according to Bergemeyer).

H_2O_2 agrees nearly with that value of the cathodic oxygen reduction at a current of 2.0 mA. It has to be considered that the initial H_2O_2 concentration in electrolysis is 0. Only after an electrolysis of 33 min the transferred charge corresponds to the peroxide concentration of 2 mM in the sample. These results suggest that the continuous reduction of oxygen gives a more active form of peroxide e.g. HO_2^-. In accordance with this the generation of H_2O_2 by the system glucose oxidase and glucose effects a slower demethylation of benzphetamine than the generation of the same amount of H_2O_2 by cathodic reduction of oxygen.

3.3. *Influence of substrate concentration*

The demethylation rate of *p*-nitroanisole or benzphetamine does not reach the saturation even at concentrations corresponding to the ten-fold K_m value of the NADPH dependent reaction [6,17]. These results show that the K_m of benzphetamine and *p*-nitroanisole are considerably higher than in the NADPH supported reaction. They may be in the range of the K_m values of the cumene hydroperoxide dependent system [6].

In the case of continuous oxygen reduction the exact determination of K_m is complicated because the H_2O_2 concentration rises with time.

4. Discussion

The role of H_2O_2 in the activation of molecular oxygen by the microsomal cytochrome *P*-450 system is as not yet clear. The involvement of peroxide anions in the reaction cycle is proposed on the basis of the following results:

- The oxidation of NADPH by microsomal fractions yields equimolar amounts of H_2O_2 [2],
- In the absence of O_2 and NADPH, cytochrome *P*-450 catalyses the O- and N-dealkylation of several substrates by organic hydroperoxides [4–6]. In this reaction H_2O_2 was unable to substitute NADPH [4–6]. The reason for these differences between hydroperoxides and H_2O_2 has not yet been elucidated [7,8].

The results presented in this paper show that liver microsomes catalyzed the demethylation of benzphetamine, aminopyrine, and *p*-nitroanisole by cathodically reduced oxygen. The mechanism may be similar to the hydroperoxide dependent reaction as indicated by the great difference from the K_m values of the NADPH dependent *p*-nitroanisole demethylation.

Oxygen is reduced at the electrode in the potential region investigated via O_2^- to HO_2^- which forms H_2O_2 by combination with one proton [13]. The pK of this reaction is 11.6. Alternatively HO_2^- may react with the Fe^{3+} of cytochrome *P*-450, which is electrostatically more favoured than the interaction of H_2O_2 with the heme iron atom. The essential role of peroxide species is demonstrated by the inhibition of the reaction by catalase. Both reactions may proceed in parallel as indicated by the accumulation of H_2O_2 and demethylation product during electrolysis. This accumulated H_2O_2 is less effective, similar to the system containing glucose oxidase and glucose. The generation of the reactive HO_2^- at the electrode and/or the high protein concentration at the same interphase may be the reasons of the higher reaction rate found in the system with continuous cathodic reduction of molecular oxygen.

The specificity of cytochrome *P*-450 is demonstrated by the inhibition of substrate demethylation of sodium deoxycholate as well as by the ineffectivity of peroxidase and hemoglobin in the reaction.

The results give evidence that peroxide species can take part in the reaction cycle of cytochrome *P*-450. It may be the prestate of the oxene form or the active oxygen species in hydroxylation.

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