KINETICS OF CUMENE HYDROPEROXIDE-DEPENDENT ANILINE HYDROXYLATION INVOLVING CYTOCHROME P-450 IN MICROSOMAL AND SOLUBILIZED FORMS

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Received April 26,1977

Summary: In the temperature range 15-37°C the kinetics of cumene hydroperoxide-dependent aniline hydroxylation with participation of rat liver microsomes and the cytochrome P-450 free of cytochrome b_5 and NADPH-cytochrome P-450-reductase has been studied. Catalytic constants of aniline oxidation have been found in a general form:

CHP+cytochrome P-450 - $1.70\ 10^{10}$ exp $(-15800/RT)$ sec^{-1},
CHP+microsomes - $6.80\ 10^{9}$ exp $(-15300/RT)$ sec^{-1}.

Cytochrome P-450 is fully responsible for an overall catalytic activity in the reactions of cumene hydroperoxide-dependent aniline oxidation. The integration level of the P-450 does not influence the P-450 reactivity in the aniline oxidation by cumene hydroperoxide.

The character of the limiting step in the complex enzymatic hydroxylating process is among fundamental problems in the study of mechanism of action of liver microsomal enzymatic hydroxylating system. It is important in this connection to compare kinetic and energetic parameters of hydroxylation for one and the same substrate involving microsomes and cofactors, NADPH and O_2, on the one hand, and microsomes and organic hydroperoxides, on the other.

In the first case, an electron transfer to the substrate-cytochrome P-450-O_2 complex occurs (1). In the second, there is no electron transport to the P-450-hydroperoxide complex, since hydroperoxide contains the oxygen reduced by two electrons. The microsome-hydroperoxide system efficiency in the hydroxylation

ISSN 0006-291X

of aromatics was studied using aniline (2,3), benzpyrene, cumarine (2) and naphthalene (4). Quantitative characteristics for aniline oxidation by three systems: i) microsomes-NADPH-O_2, ii) microsomes-cumene hydroperoxide, and iii) microsomes-tert butylhydroperoxide, were reported in our paper (3). We have obtained catalytic constants for aniline oxidation in a general form to be:

$$\text{microsomes} + \text{NADPH} + O_2 - 1.60\ 10^8 \exp(-13400/RT)\ \text{sec}^{-1},$$

$$\text{microsomes} + \text{CHP} - 6.83\ 10^9 \exp(-15300/RT)\ \text{sec}^{-1},$$

$$\text{microsomes} + \text{TBHP} - 1.66\ 10^9 \exp(-14500/RT)\ \text{sec}^{-1}.$$

The three systems show very near values of activation energies allowing to assume that the catalytic constants characterize one and the same act, an active oxygen insertion into the substrate molecule, likely to be a limiting step of the entire hydroxylating process.

It is important to compare quantitative characteristics of the hydroperoxide-dependent aniline hydroxylation involving cytochrome P-450 in purified and microsomal forms, since in the latter case a possible role of cytochrome b_5 and that of other enzymatic hydroxylating system components cannot be excluded. In this work we compare cumene hydroperoxide-dependent aniline oxidation catalyzed by microsomes and by purified cytochrome P-450 free of cytochrome b_5 and reductase.

Methods: Microsomes were isolated from the liver of male albino Wistar strain rats (150-180 g) by the method described previously(5). Protein was determined by the Lowry et. al. (6) method. Cytochrome P-450 was solubilized and purified by the methods (7-9). A fraction of P-450 with 40-50% ammonium sulfate saturation contained 4.4 nmoles/mg protein. Specific content of cytochrome b_5 free of P-450 was 7.80 nmoles/mg protein. Cytochrome P-450 was measured according to the method of Omura and Sato (10); cytochrome b_5 was determined by the method (11) using an extinction coefficient of 185 mM^{-1} cm^{-1}.

The reaction of aniline oxidation was carried out at 14-37°C and was characterized by the initial rates of p-aminophenol (pAP) formation. The pAP was determined colorimetrically

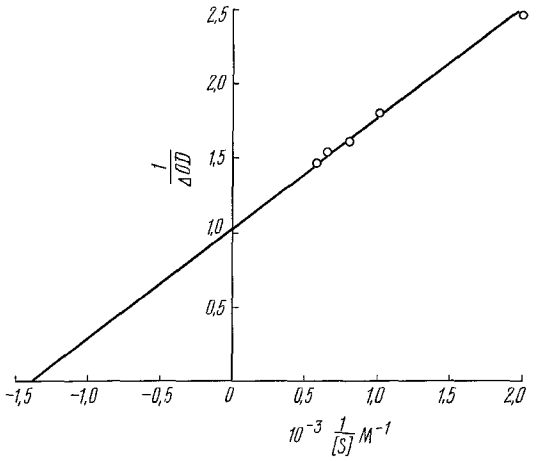

Fig 1 $1/\Delta OD_{431-395}$ vs reciprocal aniline concentrations at 20°c
$[P\text{-}450]_o = 1{,}06\ 10^{-6}$ M

on PhEC 56 M by the known method (12). The incubation mixture contained 0.05 M Tris buffer (pH 7.4), 0.001 M CHP and either P-450 (0.24 mg/ml) or microsomes (1mg/ml), corresponding to P-450 concentrations of $1.06\ 10^{-6}$ M and $7.1\ 10^{-7}$ M, respectively in 3 ml of final volume. The absorption spectra of the systems, cytochrome P-450-CHP and cytochrome P-450-aniline were recorded on Unicum SP-700 and Specord UV VIS at 20°C in sodium phosphate buffer (pH 7.4).

Results and Discussion: From the difference absorption spectra, reflecting the cytochrome P-450-aniline interaction, a spectral constant K_s was calculated to be $7.4\ 10^{-4}$ M at 20°c (Fig 1) whereas that of the microsomes-aniline interaction equaled $1.6\ 10^{-4}$ M (13). This difference implies that aniline is bound more tightly with microsomal cytochrome than with the solubilized one, which in turn confirms a significant role of the microsomal lipids in this process.

The difference spectra of P-450 and CHP are characterized by the time-increasing troughs (418 nm) and peaks (460 nm) as well as by the appearance of a 440 nm maximum immediately after combining the reagents; this peak rapidly disappears at 20°C

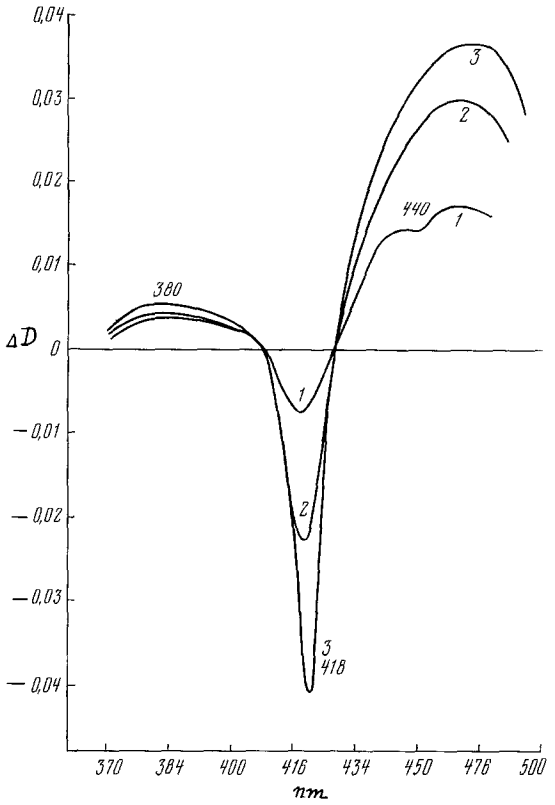

Fig 2 Absorption spectra of P-450 and CHP mixture: 1 - 0.5, 2 - 2, and 3 - 4 min after mixing $2{,}9\ 10^{-4}$ M of CHP and $3.6\ 10^{-6}$ M of P-450 in phosphate buffer pH 7.4

(Fig 2). An absorption maximum at 418 nm in the absolute P-450 spectrum decreases with time upon the addition of CHP. We believe the observed spectral changes to be due to cytochrome P-450 destruction under the action of CHP. Also, a maximum drop at 418 nm in the absolute spectrum was observed upon the action of tertbutylhydroperoxide on the oxidized cytochrome P-450. As to the 440 nm maximum, we assign it to the formation of an unstable complex I, well known for peroxidases in combination with hydrogen peroxide.

It is essential to note that a complex with absorption

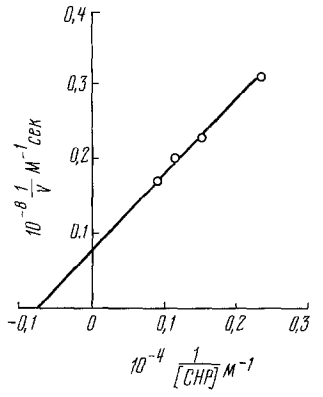

Fig 3 Aniline oxidation rate vs $[CHP]_o$ in reciprocal coordinates at 37°C: $[Aniline]_o$ = 0,005 M, microsomal protein (1mg/ml)

maximum at 440 nm was reported for the interaction of a homogeneously pure reduced cytochrome P-450 with molecular oxygen (14). In the presence of a number of substrates this complex decomposes with the rate constants near to catalytic constants of the same substrates oxidation by the liver microsomes (14). The formation of an oxygen-P-450 complex, stable at -45°C and decomposed at -20°C, has been recently shown (15). On the basis of the above data it can be assumed that the O_2 complexes with reduced P-450(14) and with microsomes at -45°C (15) are similar to that of oxidized P-450 with CHP. That in turn would suggest a similar active agent, complex I, analogous to the peroxidase complex with H_2O_2 (16), to act both in the system, oxidized P-450 + hydroperoxides and in the systems, reduced P-450 + O_2.

In Fig 3 the plot of the initial rate vs CHP concentration is shown. At 37°C K_m relative to CHP was 1.53 10^{-3}M.

The rate dependence of aniline oxidation on its initial concentrations has been investigated at CHP saturation. For all

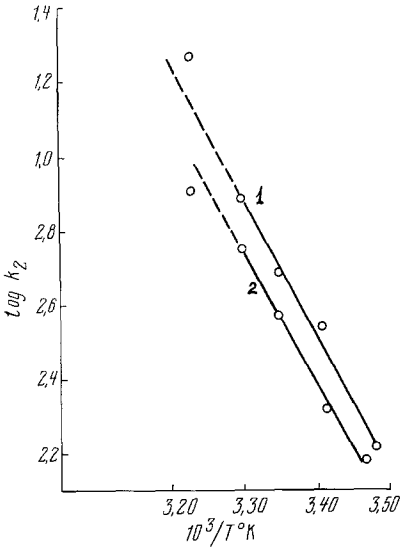

Fig 4 Temperature dependence of k_{cat} for aniline oxidation by CHP with P-450^{cat}(1), and with microsomes (2).

the temperatures studied the dependences are described with Michaelis-Menten equation. The maximal reaction rate, V, and K_m were calculated from the Lineweaver-Burk plots. From $V = k_{cat} E_o$ the values of catalytic constants at different temperatures were derived (E_o is the initial concentration of P-450 in the incubation mixture).

Table 1 shows kinetic characteristics of aniline oxidation both in the system of CHP-P-450 and CHP-microsomes system. The temperature course of k_{cat} for both systems is well described with the Arrhenius equation (Fig 4) by which the activation energies and k_{cat} in general forms were calculated :

CHP + P-450 - $1.76\ 10^{10}$ exp (-15800/RT) sec^{-1},

CHP + microsomes - $6.83\ 10^{9}$ exp (-15300/RT) sec^{-1}.

Similar values of E_{act} obtained in both cases confirm direct participation of cytochrome P-450 in the reaction of hydroxylation with liver microsomes.

Table I

Kinetic parameters of aniline oxidation

	CHP + microsomes			CHP + P-450		
t°C	10^8 V M sec^{-1}	k_{cat} sec^{-1}	10^4 K_m M	10^8 V M sec^{-1}	k_{cat} sec^{-1}	10^4 K_m M
14				1.70	0.016	15.40
15	1.22	0.016	8.33			
20	1.62	0.021	6.45	3.77	0.035	14.30
25	2.94	0.038	10.52	5.26	o.049	18.20
30	4.44	0.058	9.52	7.40	0.070	21.05
37	6.25	0.082	15.15	20.00	0.188	36.40

The experiments showed that cytochrome b_5-CHP system was not able to hydroxylate aniline at 37°C. The divergence of k_{cat} at 37°C from the Arrhenius plot (Fig 4) can be connected with the thermal denaturation of P-450 and the initiation of the chain oxidation process. P-450 destruction is likely to proceed via the following reactions:

$$Fe^{3+} + ROOH \longrightarrow Fe^{2+} + ROO^{\bullet} + H^{+}$$

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\bullet} + HO^{-}$$

Radicals ($RO^{\bullet}$ and $RO_2^{\bullet}$) participate in the destruction of either heme itself or its protein invironment.

Thus, in the cumene hydroperoxide-dependent aniline oxidation the microsomal catalytic activity is fully due to cytochrome P-450. Cytochrome b_5 does not directly participate in the reaction. Complex I formed by hydroperoxide and cytochrome P-450 is the most likely hydroxylating agent in the CHP + P-450 system, evidenced by an absorption maximum at 440 nm (20°C). The values of k_{cat} obtained for CHP-P-450 and CHP-microsomes systems are practically equal. This means that the integration

level of cytochrome P-450 does not influence the P-450 reactivity in the aniline oxidation by cumene hydroperoxide.

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