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Conference Paper

Substrate Binding Kinetics and its Role in the Cytochrome *P450* Hydroxylation Sequence*

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The kinetics of the binding of type I and type II substrates to cytochrome *P450*LM has been investigated. Type I substrates are preferentially bound compared to type II compounds. The rate constants range between 10^3 and 10^5 [M^{-1} s^{-1}], indicating possible interference with rate limiting steps. Substrates bind to the reduced cytochrome with considerably lower rate constants. The results are in favour of the sequential reaction mechanism. The solubilized enzyme preparation shows properties similar to the microsomal enzyme system.

INTRODUCTION

Cytochrome *P450* serves as the terminal oxidase in the activation of molecular oxygen by complexing the oxygen to overcome the high energy barrier of about 120 kcal/mol for splitting the molecule.

The first step in the hydroxylation sequence of the enzyme system is the binding of the respective substrate to the cytochrome. This reaction is the main topic of this paper.

The questions under investigation may be classified as follows:

1. Substrate receptor site: What is the relationship between the kinetics of the substrate binding reaction and the dependence of the spectral and dissociation constants on the type of substrate?
2. Enzyme system: Are the rate constants relevant for the function of the enzyme system (integration state of the preparation, interaction between the system components)?
3. Hydroxylation mechanism: Can the substrate binding reaction interfere with rate limiting steps and is there any iron state reflected in the reaction mechanism?

EXPERIMENTAL

The investigations were performed using two preparations from male Wistar rats pretreated with sodium phenobarbital. The main experimental material was a purified solubilized *P450* fraction (*P450* (sol)), prepared by the Lu *et al.* method[1]. The *P450* content amounted to 4—5 nmoles/mg protein; and there was about 15—20% *P420* and 10% cytochrome b_5; the mean molecular weight was 600 000. For sake of comparison a microsomal fraction was investigated additionally. Representative type

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I and type II substrates were selected with respect to their spectral characteristics. The reagents were mainly commercial products, purified by further crystallization or distillation.

The kinetic investigations were performed in 0.1 M phosphate buffer solutions with 5% (v/v) glycerol at pH 7.4 and room temperature. The analytical concentration was 2.5 μM *P450*. Stopped flow investigations were performed with the DURRUM D-100 rapid kinetics device, data converting and processing by means of a DATALAB DL 905 transient recorder and the KRS 4200 and R 300 computer systems from Kombinat VEB ROBOTRON.

RESULTS AND DISCUSSION

1. The receptor site functional behavior was first studied for the CO binding reaction. It is generally accepted that this ligand binds in position 6 of the iron complex structure. The binding process exhibits 1st order behavior with respect to protein as well as to the ligand. The reaction proceeds inhomogeneously with about 70/30% phase distribution (Figure 1). The rate constant of the main fraction was determined to be $6.6 \cdot 10^5$ [M^{-1} s^{-1}]. The reaction has been followed, at 5 different wavelengths between 400 and 460 nm: neither the rate constant nor the fractional composition changed.

The rate constant determined is in line with data of other authors[2,3]. Furthermore, it is of the order of other hemoproteins CO-binding reaction[4,5]. Therefore, the accessibility of the ligand to the iron should not be restricted by membrane components. On the other hand the inhomogeneous binding behavior, as demonstrated previously by other authors[6-7], indicates the existence of either isoenzymes or some inhomogeneity of the reaction species. Differences in *P450*/*/P420* composition are excluded by the wavelength independence as well as by the control reaction of *P420*, the latter being obtained by deoxycholate treatment of the *P450* species. Since the microsomal fraction also shows the same binding behavior, no preparative artifact seems to be involved.

The overall binding behavior of the substrates (type I and type II) is like that of CO. Second order kinetics and inhomogeneity have been observed.

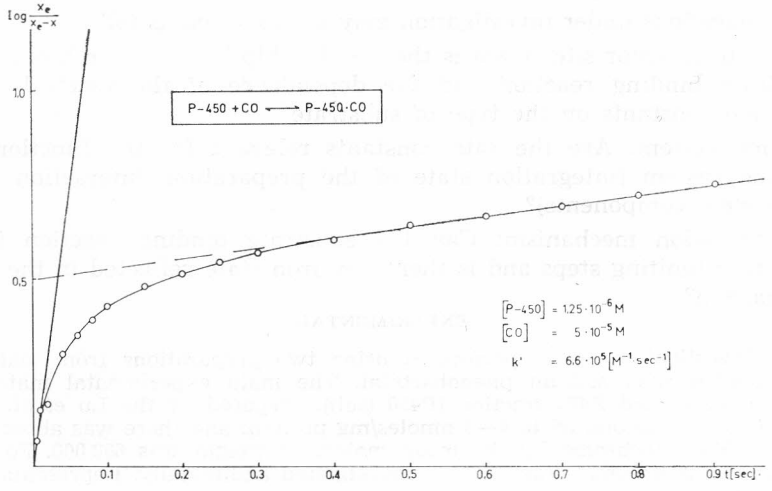

Figure 1. CO binding reaction to the reduced cytochrome *P450*. pH 7.4, T = 22 °C, phosphate buffer, $Na_2S_2O_4$ reduction. [*P450*] = $1.25 \cdot 10^{-6}$ M, [CO] = $5 \cdot 10^{-5}$ M. x = complex concentration at time t, x(e) = complex concentration at equilibrium.

Figure 2 shows the aniline reaction as an example. The inhomogeneity again is surprising and points to the existence of isoenzymes or structural inhomogeneities. As already indicated by the apparent dissociation constants of the respective enzyme substrate complexes, they differ distinctly with respect to the binding rate constants as well (Table I). The type I representatives, bound to the protein by more hydrophobic interactions, are favoured by about one order of magnitude. This may reflect the hydrophobic binding mode in general, but increased phospholipid fluidity[9,10] induced by the hydrophobic substrates may be also important.

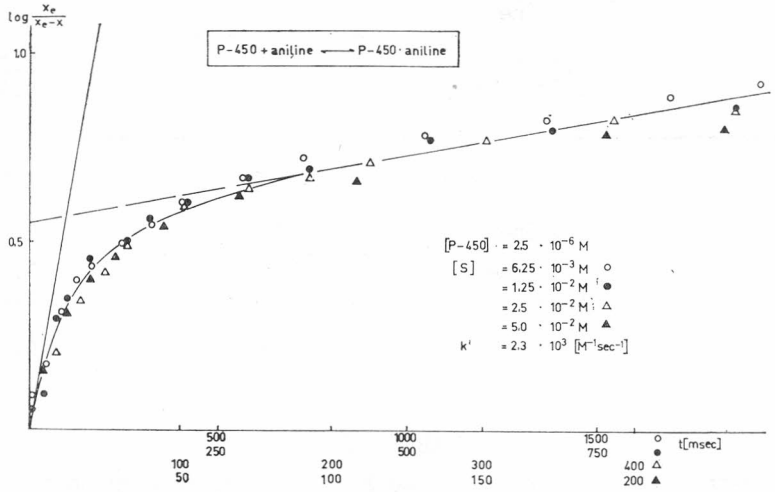

Figure 2. Aniline binding reaction to the oxidized cytochrome *P450*. pH 7.4, T = 22 °C, phosphate buffer. [*P450*] = $2.5 \cdot 10^{-6}$ M, [S] = $6.25 \cdot 10^{-3}$ M (○), $1.25 \cdot 10^{-2}$ M (●), $2.5 \cdot 10^{-2}$ M (△), $5.0 \cdot 10^{-2}$ M (▲). x, x(e) *cf.* Figure 1.

2. The enzyme system — being composed of the cytochrome, the reductase and phospholipid as the main components is regulated by their distinct interactions. The enzyme reduction does not only depend on the reductase functional state but is additionally sensitive to phospholipid interaction[11]. Therefore, this reaction and its substrate steering function[12,13] were investigated with regard to the integration state of the soluble cytochrome fraction, *i. e.* to the relevance of our investigations for the enzyme system.

The mean molecular weight of the preparation (600 000) indicates that more than one cytochrome molecule should be incorporated in the associates. In Table II the reduction behavior is shown. The reduction rate as well as the substrate steering function is retained. Therefore we conclude that this preparation represents an equivalent of the microsomes. The same CO and aniline binding kinetics in both preparations support this statement (Table III).

3. The substrate binding data, evidently relevant for the enzyme system, allow further interpretation with respect to the hydroxylation mechanism as formulated by Gillette[14] and shown in Figure 3.

TABLE I

Rate constants, $k' / M^{-1}s^{-1}$, of cytochrome P450 substrate binding reactions

iron state	substrate	type	k'
Fe(III)	benzphetamine	I	$9.3 \cdot 10^4$
	benzylephedrine	I	$4.2 \cdot 10^4$
	hexobarbital	I	$1.8 \cdot 10^4$
	clobenzorex	I	$1.1 \cdot 10^4$
	aminopyrine	II	$6.4 \cdot 10^3$
	aniline	II	$2.3 \cdot 10^3$
	pyridine	II	$1.2 \cdot 10^4$
	imidazole	II	$4.3 \cdot 10^2$
Fe(II)	benzphetamine	I	$1 \cdot 10^3$
	aniline	II	$1 \cdot 10^0$
	pyridine	II	$1.2 \cdot 10^4$
	imidazole	II	$2.1 \cdot 10^1$
	carbonmonoxide	-	$6.6 \cdot 10^5$

TABLE II

NADPH-reduction of cytochrome P450 - substrate complexes

Substrate	Type	Reaction half time s
none	-	4.5
benzphetamine	I	2.2
cyclohexane	I	2.0
aniline	II	6.2

Two questions arise with respect to the substrate binding kinetics: 1. does the substrate binding interfere with rate limiting steps, and 2. do the experiments favor a sequential or branched mechanism (both cases are possible from substrate conversion data).

It is widely accepted that substrate binding does not interfere with the first reduction step. This conclusion is based on the binding data for camphor, metyrapone, CO and O_2 to different *P450* species where the binding rate constants range from $10^5 - 10^7$ [M^{-1} s^{-1}]. Our results show that the liver system

TABLE III

Rate constants k_s $[M^{-1}sec^{-1}]$ of substrate binding reactions to cytochrome P450 in different microsomal preparations

Preparation	Iron state	Substrate	k_s
microsomal suspension	Fe(II)	CO	$6.8 \cdot 10^5$
	Fe(III)	aniline	$2.9 \cdot 10^3$
solubilized microsomal fraction	Fe(II)	CO	$6.6 \cdot 10^5$
	Fe(III)	aniline	$2.3 \cdot 10^3$

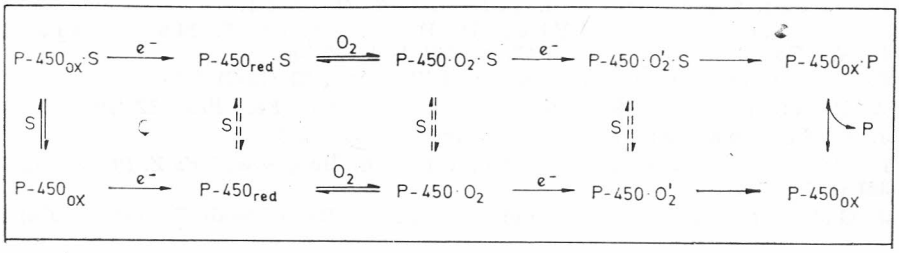

Figure 3. Cytochrome *P450* reaction sequence (Gillette *et al.*[14]) *P450*(ox, red) = cytochrome *P450* in the oxidized, reduced state. O_2' = activated oxygen, e^- = reduction step.

rate constants are lower by about 2 orders of magnitude. This applies especially to type II substrates. Thus, substrate binding at low substrate concentrations (10^{-3} — 10^{-4} M) takes place within seconds, *i. e.* it may interfere with rate limiting steps. The relatively low turnover values of type II substrates could be related to this fact.

Table I presents data about substrate binding to the reduced cytochrome. Some of the rate constants, as compared with the oxidized species, are lower by orders of magnitude. Therefore, the competing equilibria in the enzyme mechanism should be shifted very much to the sequential mechanism. This sequence is further supported by the favorable reduction of the enzyme substrate complex compared with that of the enzyme.

The results of our investigations may be summarized as follows:

1. Type I substrates are bound to the cytochrome receptor site in preference to type II representatives.
2. The apparent complex dissociation constants are mainly dependent on the on-rate constants.
3. The relevance of the *P450*(sol) data for the enzyme system is verified by the retained enzyme reduction function as well as by the equivalence of the CO and aniline binding behavior in the solubilized and microsomal enzyme preparation.

4. The substrate binding rate constants ($10^3 - 10^5$ [M^{-1} s^{-1}]) indicate possible interference with the rate limiting processes in the hydroxylation sequence.
5. The substrates are preferentially bound by the oxidized cytochrome, which favors the sequential reaction mechanism.

REFERENCES

1. A. Y. H. Lu, R. Kuntzman, S. West, M. Jakobson, and A. H. Conney, *J. Biol. Chem.* **247** (1972) 1727.
2. P. Rösen and A. Stier, *Biochem. Biophys. Res. Commun.* **51** (1973) 603.
3. P. Debey, G. Hui Bon Hoa, and P. Douzou, *FEBS Lett.* **32** (1973) 227.
4. R. H. Austin, K. Beeson, L. Eisenstein, H. Frauenfelder, I. C. Gunsalus, and V. P. Marschall, *Science* **181** (1973) 541.
5. R. H. Austin, K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus, *Biochemistry* **14** (1975) 5355.
6. C. Balny and P. Debey, *FEBS Lett.* **62** (1976) 198.
7. P. Debey, C. Balny, and P. Douzou, *FEBS Lett.* **35** (1973) 86.
8. P. Debey and P. Douzou, *FEBS Lett.* **39** (1974) 271.
9. J. A. Peterson, R. E. Ebel, D. H. O'Keeffe, T. Matsubara, and R. W. Estabrook, *J. Biol. Chem.* **251** (1976) 4010.
10. E. Oldfield and D. Chapman, *FEBS Lett.* **23** (1972) 285.
11. A. P. Autor, D. P. Ballou, and M. J. Coon, *Fed. Proc.* **32** (1973) 762.
12. J. B. Schenkman, *Molec. Pharmacol.* **8** (1972) 178.
13. H. Diehl, J. Schädelin, and V. Ulrich, *Hoppe-Seyler's Z. Physiol. Chem.* **351** (1970) 1359.
14. J. Gillette, H. Sasame, and B. Stripp, *Drug Metab. Dispos.* **1** (1973) 164.

DISCUSSION

R. H. Austin:

(*a*) Did you observe the biphasic CO-*P450* kinetics in all your preparations of *P450*, *i. e.*, whether solubilized or bound to microsome?

(*b*) Have you tried fitting the above CO-*P450* kinetics to a continuous power law, of the form $N = N_0 t^{-n}$ as opposed to 2 exponentials?

J. Blanck:

(*a*) The biphasic binding behaviour is already exibited by the microsomal fraction. Since the solubilized sample contained the main components of the enzyme system the same behaviour may be expected. A further purified preparation (8 nmole/mg protein) again shows the biphasic kinetics.

(*b*) The two binding rates are clearly separated by about the factor 20, which therefore indicated a fitting with two exponentials.

T. G. Traylor:

What was the other rate constant in your biphasic plot for CO reaction?

J. Blanck:

30% of our *P450* preparation binds CO with a rate constant about 20 times slower compared to the first, rapid one (6.6×10^5 [M^{-1} s^{-1}]).

H. Schleyer:

Did you investigate possible effects of O_2 on the rates of *P450*(Fe^{2+}) + compound?

J. Blanck:

Part of the *P450* may be very sensitive to oxygen, as was for instance shown by Gillette in the NADPH reduction reaction, where the rate of a second phase was decreased on increasing the oxygen concentration. We reduced with $Na_2S_2O_4$, but prior deoxygenation was not investigated.

SAŽETAK

Kinetika vezivanja supstrata i njezina uloga u sekvenciji hidroksilacije citokroma *P450*

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Ispitivana je kinetika vezivanja supstrata tipa I i II na citokrom *P450* iz mikrosoma jetara pacova, na solubiliziranim i mikrosomskim uzorcima. Konstante brzina vezanja su između 10^3 i 10^5 M^{-1} s^{-1}, što znači da može doći do ometanja kritičkih faza reakcije (rate limiting steps). Protein preferira supstrate tipa I, a oba se tipa vežu sa znatno manjim konstantama brzine na reducirani citokrom. Rezultati idu u prilog sekvencijskog reakcijskog mehanizma nasuprot shemi reakcije s grananjem. Solubilizirani preparat relativne molarne mase 600 000 pokazao je svojstva slična enzimskom sustavu mikrosoma.

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