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Conference Paper

Differentiation Between Type I and Type II Substrate Binding to Cytochrome *P450* by Temperature Studies*

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The binding reactions of type I and type II substances to cytochrome *P450* solubilized from phenobarbital induced rat liver microsomes show different dependence on temperature. With increasing temperature the type II binding is lowered whereas the stability of type I substrate complexes increases. The binding enthalpies were calculated from the van't Hoff plots and discussed in connection with the entropy and Gibbs energy of substrate binding to cytochrome *P450*. Our data on temperature dependence provide further evidence pointing to a difference between the binding sites of the two classes of substrates, and support the view that the type I binding site is located in a hydrophobic part of the cytochrome *P450* molecule.

INTRODUCTION

In earlier studies on the binding of different organic compounds to cytochrome *P450* from phenobarbital induced rat livers in microsomal and solubilized forms it was shown that the stability and the type of the enzyme substrate complex are determined by the hydrophobicity, stereochemistry and the basic properties of the substrate[1]. Because of the capacity of cytochrome *P450* to combine with a great number of chemically different compounds, it was assumed that the binding site(s) are not specifically determined.

The numerous substrates may be divided into at least two classes according to the different spectral properties of the complexes formed[2]. Type I substrates produce a blue shift, while type II compounds show a red shift in the Soret region of the absorption spectra of cytochrome *P450*. Besides the different binding affinities for the substrates very little is known about the chemical nature of the binding sites. It has been pointed out that the different types of binding occur at different sites in the cytochrome *P450* molecule. The type I binding site is assumed to be located in a hydrophobic region of the hemoprotein, whereas the type II binding occurs at the heme iron. An analysis of the thermodynamic data of the complex formation between cytochrome *P450* and the substrates should be an appropriate approach to get more information on the binding sites.

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Measurements of the overall hydroxylation reaction in dependence on the temperature have been performed by several authors[3-5].

However, because of the complexity of the reaction, in which different reaction steps are involved, it is impossible to draw conclusions concerning the thermodynamic properties of a single reaction step. In order to get an insight into the first step of the cytochrome *P450* reaction sequence and to draw conclusions about the nature of the binding sites we have determined the binding of substrates to solubilized cytochrome *P450* from rat liver in dependence on temperature.

EXPERIMENTAL

Partially purified cytochrome *P450* was obtained from phenobarbital pretreated rat liver by microsome solubilization according to Lu *et al.*[6]. It contained 3.4 nmoles cytochrome *P450* per mg protein, and, in addition 25% cytochrome *P420* and 10% cytochrome b_5.

The concentrations of cytochrome *P450* and cytochrome *P420* were determined from the CO-difference spectra according to Omura and Sato[7,8]. The cytochrome b_5 content was assayed from the difference spectrum of NADH-reduced and oxidized forms[9].

Benzphetamine hydrochloride was prepared by extraction and crystallization in a pure form from Didrex[R] (Upjohn Comp., Kalamazoo, Michigan, USA) and checked by mass spectroscopy. Sodium hexobarbital was obtained from VEB Arzneimittelwerk Dresden, GDR. Commercial imidazole (99% pure) and cyanide were from Schuchardt, Munich, FRG. Aniline was distilled twice before use.

All spectrophotometric measurements were carried out using a Beckman Acta CV spectrophotometer with cuvettes of 1 cm light path. The cytochrome *P450* concentrations in the solutions used for the optical measurements were about 4 μM. The binding studies were performed in 0.1 M phosphate buffer, pH 7.4, with 20% glycerol (v/v).

The difference spectra were recorded after the addition of the substrate solution in steps of 2 μl to the sample cuvette. An equal volume of the solvent was put into the reference cuvette. The influence of temperature on substrate binding was investigated in the 10 °C to 30 °C range. During the experiments at 30 °C the concentration of cytochrome *P450* showed only a small decrease of about 3% after 3 hours. The temperature was measured within the cuvette holder by means of calibrated thermistors. The measurements below 15 °C were carried out under a stream of dry nitrogen.

The spectral changes after the addition of the substrates were evaluated according to EADIE because there are considerable uncertainties in the determination of the end point of substrate binding. A 1 : 1 stoichiometry of the binding of substrates to cytochrome *P450* was assumed, although two binding constants were determined. Possible reasons for the occurrence of two constants will be discussed later. The spectral dissociation constants (K') are not thermodynamic equilibrium constants but only apparent ones. This is due to the dependence of the actual substrate concentration on the lipid phase and on the limited substrate solubility in water.

RESULTS AND DISCUSSION

The difference spectra of the substances investigated with solubilized cytochrome *P450* are shown in Figure 1. Benzphetamine and sodium hexobarbital as typical type I substrates were used. As substances producing type II spectra aniline, imidazole and cyanide were applied. Cyanide was selected because it is the simplest type II compound with no possibility to interact with parts of the hemoprotein.

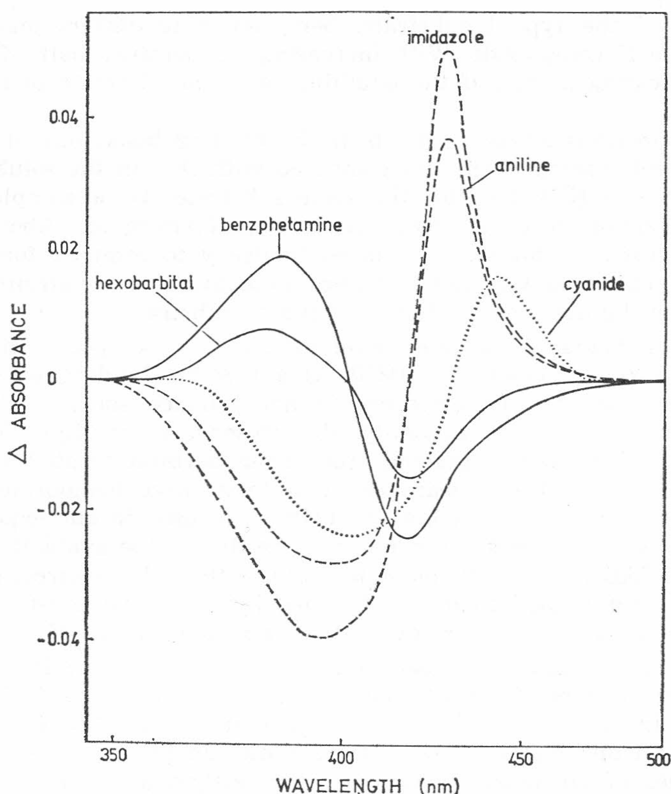

Figure 1. Difference spectra of solubilized cytochrome *P450* (concentration of *P450* = 4.5 μM in 0.1 M phosphate buffer, pH 7.4, with 20% (v/v) glycerol) from phenobarbital pretreated rat liver microsomes in dependence on different substrates. Concentration of each substrate = 0.5 mM.

In Table I the K' values are given for benzphetamine, aniline and cyanide in dependence on temperature. It can be seen that with increasing temperature the binding of cyanide as well as of aniline to cytochrome *P450* decreases. This behaviour has been observed for the two K' values. The temperature

TABLE I

Temperature dependence of spectral dissociation constants K' (mM) of cytochrome P450 substrate complexes

Temperature (°C)	benzphetamine		aniline		cyanide	
	K'_1	K'_2	K'_1	K'_2	K'_1	K'_2
10	0.070	0.17	0.37	0.71	0.75	1.40
15	0.050	0.12	0.40	0.75	0.89	1.60
20	0.037	0.10	0.45	0.89	0.97	1.05
25	–	–	0.49	0.95	1.00	2.50
30	0.028	0.053	0.57	0.98	1.05	2.90

dependence of the type I substrate, benzphetamine, differs markedly from those of type II compounds. With increasing temperature both K' values are lowered indicating a shift of the equilibrium in the direction of complex formation.

In our previous studies[1], in which the binding behaviour of cytochrome *P450* in the microsomal form was compared with that in the solubilized form, we observed two K' values for the same substrate. These samples show the same behaviour in the whole temperature range investigated. About 15 to 20% of the preparation exhibit an enhanced tendency to complex formation (K_1') whereas 80 to 85% show higher K' values indicating a lower affinity (K_2'). This behaviour can be observed with both types of substrates.

For an explanation of this heterogeneity several possibilities may be considered: Firstly, cytochrome *P420*, as a possible binding enzyme can be excluded because type I substrates do not produce spectral changes with this inactive cytochrome[10]. Secondly, the presence of multiple forms in the preparations which were obtained from phenobarbital treated rats. Several authors have shown the appearance of at least three hemoprotein forms in soluble cytochrome *P450* of a higher purity than used in our experiments[11,12]. Thirdly, the heterogeneous behaviour may be due to the evaluation procedure according to EADIE. Attempts made to evaluate the data by direct curve fitting have shown that a good fitting is obtained by using one constant only. This one constant obtained by such evaluation is similar to the K_2' values found.

The principal differences between the type I and type II substances in the temperature dependence of their binding to cytochrome *P450* were confirmed also in studies with hexobarbital and imidazole. The van't Hoff plots of the spectral dissociation constants are shown in Figure 2. The plots of the log K' values of all substrate complexes investigated against the reciprocal temperature yield straight lines for each substrate. It can be seen that different slopes are valid for type I and type II substrates. Besides the qualitative difference between the two types of substrates, Figure 2 clearly shows that there are also quantitative differences within the same group.

To compare the data quantitatively the binding enthalpy was calculated according to the van't Hoff equation. The Gibbs energy and the entropy of binding were determined by the use of standard equations. For the calculations of the thermodynamic values the association constants were used. In Table II the values given for a temperature of 25 °C are listed. The enthalpy of binding shows the basic difference between type I substrates and type II compounds. The binding enthalpy of type I substances studied so far is positive, indicating an endothermic reaction. The binding of the type II substrates is an exothermic one. These results reaffirm the assumption about two different binding sites for type I and type II substances[2].

Two further points should be discussed here. Firstly, comparing the imidazole complexes of different hemoproteins it may be established that the binding enthalpy of about — 2 kcal · mol^{-1} for cytochrome *P450* is significantly smaller than the value for human methemoglobin ($\Delta H' = -9.8$ kcal · mol^{-1})[13]. This finding might be due to the fact that in cytochrome *P450* the heme iron is in the low spin state. This means that the axial ligands are firmly bound to the iron. If type II ligands in cytochrome *P450* are bound more directly to the heme iron than type I substrates, it is necessary to replace one of the

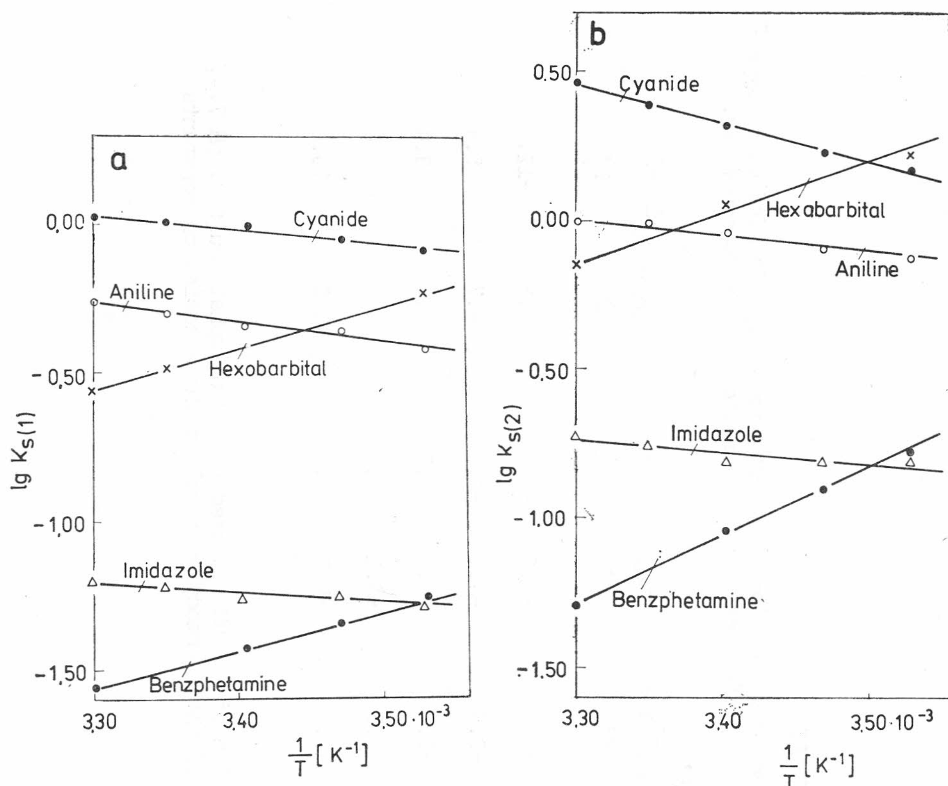

Figure 2. van't Hoff plots of the spectral dissociation constants K_1' (a) and K_2' (b) given in mM. Concentration of *P450* = 3.0 μM; 0.1 M phosphate buffer, pH 7.4, with 20% (v/v) glycerol.

firmly bound iron ligands in the course of the binding reaction. Since this replacement will consume energy, a lower heat of binding is observed for the reaction of imidazole with cytochrome *P450* than in the case of high spin methemoglobin.

Secondly, the higher entropy values of the type I substrates compared with type II ligands. The endothermic reaction of type I substrates is characterized by relatively high positive entropy values compared with smaller ones for the exothermic reactions.

Discussing this difference one has to take into account the fact that the system under investigation is a heterogeneous one containing an aqueous and a nonaqueous phase, and that the binding of each substrate involves different partial reactions in which changes in the protein conformation as well as changes in the ordered state of water molecules are involved. Therefore, calculations of the thermodynamic values of substrate binding reactions, have to take into account all these partial reactions. Nevertheless, it seems possible to draw certain conclusions from the entropy values with regard to the participation of hydrophobic interactions. For instance, type I substrates and aniline have about the same hydrophobic nature. The essentially higher entropy values

TABLE II

Changes of the binding enthalpy, the Gibbs energy and the binding entropy caused by the binding of type I and type II substances to solubilized cytochrome P450 from rat liver pretreated with phenobarbital.*

substrates	spectral type	$\Delta H'$ (kcal·mol^{-1})		$\Delta G'$ (kcal·mol^{-1})		$\Delta S'$ (cal·mol^{-1} K^{-1})	
		K_1'	K_2'	K_1'	K_2'	K_1'	K_2'
cyanide	II	-2.2	-7.0	-4.2	-3.6	6.3	-12.0
aniline	II	-3.1	-2.5	-4.6	-4.2	4.7	5.4
imidazole	II	-1.6	-2.3	-5.8	-5.1	14.0	9.4
benzphetamine	I	5.7	9.5	-6.1	-5.7	39.6	51.0
hexobarbital	I	7.3	7.3	-4.7	-4.2	40.2	38.6

of type I substrates compared with aniline would indicate the predominant participation of hydrophobic interactions which are not observed with the lipophilic aniline as a type II substrate. One may conclude that the main part of the high entropy changes in type I substrates results from the lipophilic nature of the type I binding site in the protein moiety of cytochrome *P450*.

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DISCUSSION

M. J. Coon:

Your results are interesting in that they show a clear difference between type I and type II compounds.

(*a*) Have you determined the effect of phospholipid on substrate binding?

(*b*) I am not certain as to whether you attribute the two K_s values for each substrate to the presence of the two different forms of *P450*. This would seem to me to be a likely explanation.

G.-R. Jänig:

(*a*) No, we have not investigated the effect of phospholipid. There is a certain amount of phospholipid still present in our partially purified cytochrome *P450* according to the preparative procedure used. In recombination experiments we found an increase in the turnover numbers after an addition of a lipid fraction.

(*b*) Yes, different forms of *P450* present in the material could be the reason for the occurence of the two K_s values.

P. Debey:

Have you investigated any variation of the binding thermodynamic parameters as a function of pH?

G.-R. Jänig:

All of our data were obtained from measurements in 0.1 M potassium phosphate buffer with 20% (v/v) glycerol adjusted to pH 7.4 at 20 °C. We have not checked the pH after the addition of the substrates. It should be justified to assume the buffer capacity to have been sufficient.

H. Schleyer:
Have you also tried other inducing agents such as benzo[*a*]pyrene or methyl-cholanthrene? If not, I might suggest this because such microsomes show relatively large deviations in »heme chemistry« — which may prove to be very interesting.

G.-R. Jänig:
No, we did not. The experiments were only performed with solubilized cytochrome *P450* from phenobarbital induced rats. Preparations obtained from untreated rats showed nearly the same behaviour.

R. H. Austin:
(*a*) Are all type I binding compounds of a hydrophobic nature?
(*b*) In your calculated ΔS changes for type I binding, can you differentiate entropy changes in protein structure from entropy changes due to transfer of a hydrophobic molecule to a non-polar environment?

G.-R. Jänig:
(*a*) Generally, all of the type I substrates known are hydrophobic. The type II compounds have hydrophobic as well as polar groups in the molecule.
(*b*) Further experiments with different substrates are necessary for differentiation of this kind.

SAŽETAK

Razlikovanje između supstrata tipa I i II temperaturnom zavisnošću vezivanja na citokrom *P450*

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Reakcije vezivanja tvari tipa I i II na citokrom *P450* solubiliziran iz jetara štakora tretiranih fenobarbitalom pokazuju suprotne zavisnosti o temperaturi. Povišenje temperature slabi vezivanje tipa II dok stabilnost supstrata tipa I raste. Termodinamičke veličine izračunane iz van't Hoffovih grafova diskutiraju se s obzirom na način vezivanja supstrata na citokrom *P450*. Rezultati potvrđuju razlike u mjestima vezivanja za dvije klase supstrata, i govore u prilog hidrofobnog dijela molekule citokroma *P450* za vezivanje supstrata tipa I.

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