Evidence of the Existence of a High Spin Low Spin Equilibrium in Liver Microsomal Cytochrome *P450*, and its Role in the Enzymatic Mechanism*

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In rabbit liver microsomal cytochrome *P450* a high spin (S = = 5/2) low spin (S = 1/2) equilibrium has been proved to exist by recording temperature difference spectra in the Soret and in the visible region of the absorption spectrum of solubilized cytochrome *P450*. In the presence of type II substrates the predominantly low spin state of cytochrome *P450* is maintained, only a very small shift to lower spin is observed. Ligands of the heme iron, such as cyanide and imidazole, produce a pure low spin state and therefore in the presence of these ligands no temperature difference spectra can be obtained. In the presence of type I substrate, however, the spin equilibrium is shifted to the high spin state. The extent of this shift (1) depends on specific properties of the substrate and (2) it is generally relatively small, up to about 8⁰/o in the case of substrates investigated so far.

INTRODUCTION

The first step in the reaction cycle of cytochrome *P450* is the binding of the substrate to the enzyme. The binding is connected with changes in the absorption spectrum especially in the Soret region from which the binding constant can be evaluated[1]. Moreover, in the case of the so far best known cytochrome *P450* from *Pseudomonas putida* it has been established that at substrate binding the low spin state of the heme iron is changed into the high spin state[2]. In the presence of camphor, a specific substrate, bacterial cytochrome *P450* exhibits in the EPR spectrum g values of 8, 4, and 1.8, typical of high spin ferric heme iron in a strong distorted rhombic field. But in the EPR spectrum of liver microsomal cytochrome *P450* such changes in the spin state are not induced at substrate binding.

The following possibilities may be considered in order to determine why in the presence of substrate (type I) no high spin signal is observed in the EPR spectrum of microsomal cytochrome *P450*:

1. The binding of substrate to microsomal cytochrome *P450* is not connected with a spin change. 2. A change in the spin state occurs, but this transition cannot be seen in the EPR spectrum. Although the EPR is an appropriate method to observe changes in the electronic structure of the heme iron, in the case of a very short electronic relaxation time of a heme complex the

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line broadening makes the signals unobservable. In this case EPR signals may be observed only at very low temperatures. 3. A further possibility is the existence of a mixture of two spin states which are in a temperature dependent equilibrium. Such temperature dependent equilibria between the high spin state with a total spin of $S = 5/2$ and the low spin state with a total spin of $S = 1/2$ have been discovered by George, Beetlestone and Griffith in metmyoglobin- and methemoglobin-hydroxide[3] and have been described by Blanck and Scheler to exist in certain other complexes of metmyoglobin and methemoglobin[4].

If in such a complex the ground state is low spin, then apparently in the EPR spectrum a high spin signal cannot be seen, because at low temperatures the equilibrium is shifted to the pure low spin state. An example of this behaviour is methemoglobin azide existing at room temperature in the two spin states (predominantly low spin) and showing a pure low spin EPR spectrum at 77 K[5].

Assuming that a spin equilibrium exists also in cytochrome P450, then the EPR method is not suitable to give evidence of its spin state at room temperature because EPR measurements have to be performed usually at low temperatures.

<div align="center">EXPERIMENTAL</div>

The EPR measurements on the microsomes, ~ 300 µM in cytochrome P450, were performed with an X-band superheterodyne spectrometer[5]. The microwave frequency was 9.4 GHz. The amplitude of modulation was 120 Gauss and the temperature 77 K. The amplitudes of the absorption lines in Figure 1 are not to scale because of the different gain settings.

The optical measurements were performed with cytochrome P450 solubilized from phenobarbital induced rabbit liver microsomes by the slightly modified method of Lu et. al.[6] as described in ref. 1. The concentrations of the hemoproteins in the solubilized sample were: 64.2 µM P450; 6 µM P420; 3 µM b_5. All optical measurements were carried out with a Beckman Acta C V spectrophotometer. The optical path was 0.2 cm unless indicated otherwise.

In microsomal cytochrome P450 of phenobarbital induced rabbit liver several enzyme fractions are present[7], but the main fraction (LM2) is enzymatically most active[8]. Therefore, it is assumed here that the change in the spin equilibrium of cytochrome P450 effected by substrates was mainly due to this fraction. Rather high substrate concentrations were used because it is known that at higher concentrations of the microsomal cytochrome P450 the binding affinity for substrates is diminished[9].

<div align="center">RESULTS AND DISCUSSION</div>

EPR vs. *Absorption Spectroscopy in the Determination of Spin-Equilibria*

Figure 1 shows the EPR spectra of phenobarbital induced rabbit liver microsomes without and in the presence of a substrate and in the presence of cyanide. All EPR spectra mainly have three absorption lines in the high field part, typical for low spin heme iron. The microsomes measured at 77 K also exhibit in the EPR spectra small absorption lines with g values of 4, 6, and 8, which are abolished in the presence of cyanide. In the presence of benzphetamine ,a typical type I-substrate, no characteristic change of the EPR spectrum is observed, apart from a small shoulder in the high field with a g value of 2.03. On the other hand in the presence of cyanide, the EPR spectrum of cytochrome P450 with g values typical for a low spin ferric heme compound with sulfur as one of the axial ligands, is partially changed into a new low spin spectrum with the g values of $g_z = 2.54$ and
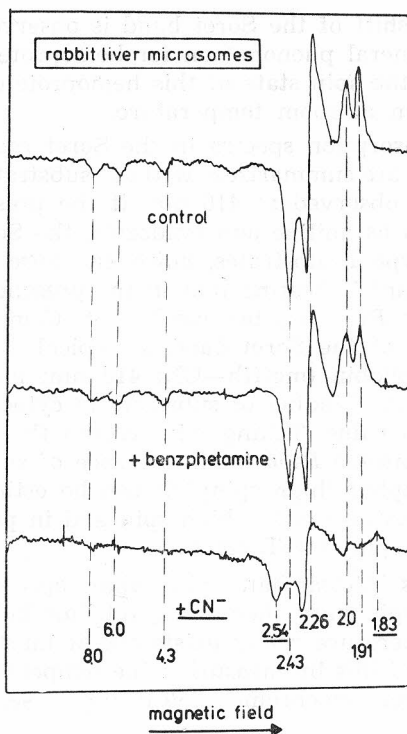
Figure 1. EPR spectra of rabbit liver microsomal suspension without substrate, with benzphetamine (about 10 mM final concentration), and with cyanide (more than 100 mM final concentration).

$g_\lambda = 1.83$. The third g value of this rhombic complex was calculated to amount to $g_y = 2.28$.

The observable EPR spectrum of the cyanide complex of cytochrome *P450* at a temperature of 77 K means that the electronic relaxation time of this complex may be comparable with that of catalase-CN[10]. T_{1e} of these cyanide complexes should be longer than that of the cyanide complexes of hemoglobin and myoglobin for which the EPR spectra at 77 K are not observable. From cytochrome *P450·CN* the crystal field parameters were calculated according to ref. 11. Because of the intermediate values of the rhombic distorsion R = 3.6 and the axial distorsion $\mu = 4.2$ no conclusion can be drawn about the trans ligand of the cyanide — sulfur or imidazole — as the postulated axial ligands of the heme iron in cytochrome *P450*.

As discussed in the Introduction, the lack of a characteristic change in the EPR spectrum of cytochrome *P450* in the presence of a type I substrate, benzphetamine, may be explained by a high spin low spin equilibrium in this complex with a low ground state. Therefore, only at room temperature the real spin state can be measured. This cannot be done with EPR but it is possible to determine the spin state indirectly by optical absoprtion spectroscopy.

As demonstrated in a classical paper of Scheler, Schoffa and Jung[12] the magnetic susceptibility of certain methemoglobin complexes is linearly correlated with the wavelength of the Soret band. With increasing high spin values the Soret band is shifted to shorter wavelengths and conversely with increasing

low spin values a red shift of the Soret band is observed. If we assume that this correlation is a general phenomenon for hemoproteins and valid also for cytochrome *P450* then the spin state of this hemoprotein can be derived from the absorption spectrum at room temperature.

In Figure 2 the absorption spectra in the Soret region of different cyto-chrome *P450* complexes are summarized. Without substrate, the Soret maximum of cytochrome *P450* is observed at 416 nm. It the presence of characteristic type II substrates, such as aniline and imidazole, the Soret band is shifted to longer wavelengths. Type I substrates, however, cause a blue shift which is very small but significant indicating that in the presence of type I substrates the spin of cytochrome *P450* has become higher. Comparing this small blue shift with the position of the Soret band of typical low spin and high spin complexes of methemoglobin (metHb—CN: 419 nm; metHb—F: 403 nm)[12] it can be concluded that the binding of substrate to cytochrome *P450* gives rise to an intermediate spin value. Taking into account the Soret maxima of *Pse-udomonas putida* cytochrome *P450* in the absence of substrate (low spin) and in the presence of camphor (high spin)[13] it can be estimated that the micro-somal *P450* without substrate is 8% high spin and in presence of type I sub-strate about 16% high spin (see Table I).

Assuming that this intermediate spin value represents a mixture of the high spin and the low spin state, then an equilibrium between these two states in dependence on temperature really exists and it must be possible to deter-mine it. We have proved this by measuring the temperature difference spectra of solubilized rabbit liver cytochrome *P450* in the presence and in the absence of substrates.
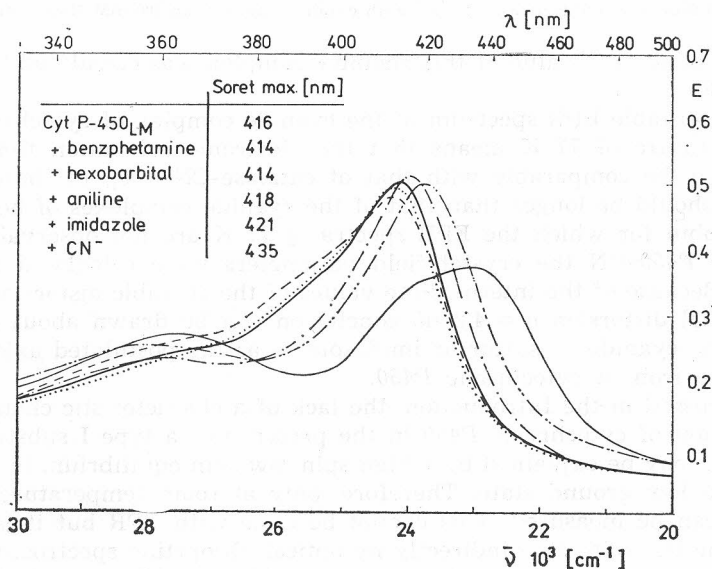

Figure 2. Optical absorption spectra in the Soret region of solubilized rabbit liver cytochrome *P450* in the presence of various substrates (the concentration values of substrates are given as final concentrations); ——, without substrate; — — —, with benzphetamine 3.4 mM; ......, with hexobarbital 20 mM; —.—.—., with aniline 18 mM; —..—..—..—, with imidazole 3.2 mM; ——, with cyanide, added as solid KCN, with the pH of the solution of about 10.

TABLE I

*Comparison of the Soret maxima in the absolute absorption spectra of oxidized cytochrome P450 without and in the presence of substrate, originating from Pseudomonas putida (P450*CAM*) and rabbit liver (P450*LM*)* [*]

	Soret maxima	low spin [**]	high spin	literature
$P450$CAM	418 nm	100%	–	13
+ camphor	392 nm	–	100%	
$P450$LM	416 nm	92%	8%	calculated ac-cording to [12]
+ benzphetamine	414 nm	84%	16%	from the Soret maxima in re – lation to $P450$ CAM[13]

[*] The data of the liver cytochrome $P450$ are from a solubilized sample (see Figure 2).

[**] The calculated amounts of the high and low spin state are rough data and may represent only a small change in the spin state of $P450$LM on substrate binding. At this Conference (see footnote p.251) I.C. Gunsalus reported that $P450$CAM without substrate and in the presence of substrate is not in the pure low spin or high spin state, respectively. Compared with 92% low spin without substrate and 6% low spin in the presence of camphor, for $P450$CAM, the values calculated for microsomal $P450$ are 14% high spin and in the presence of benzphetamine 20% high spin, respectively.

Figure 3 shows the original temperature difference spectra of cytochrome $P450$ in the presence of benzphetamine in the Soret region. A titration with a temperature difference of a maximum of only $\Delta T = 18$ K is necessary to record the well resolved temperature difference spectra which show maxima at 418 nm and minima at 386 nm indicating the low spin band and the high spin band, respectively. The visible spectral region of hemoproteins exhibits typical low spin and high spin bands[14]. These bands are well titratable in dependence on temperature in the difference spectrum of cytochrome $P450$ in the presence of benzphetamine as shown in Figure 4. Low spin bands show maxima at 538 and 568 nm and the high spin bands show minima at 495 and 641 nm. In the presence of another type I substrate, methphenethamine, cytochrome $P450$ exhibits temperature difference spectra in the Soret region and in the visible region, which are very similar to those of the cytochrome $P450$-benzphetamine complex. However, the absorption differences between the low spin maxima and the high spin minima at the same ΔT values of the benzphetamine complex are larger then those of the other one (see Figure 5).

Figure 6 shows that the amplitude of the high spin and low spin bands of the temperature difference spectra depends on the substrate indicating a specific influence of the substrate on the spin state. In accord with the blue shift of the Soret band in the presence of type I substrates, the spin equilibrium of cytochrome $P450$ is shifted to higher spin values and therefore a large dependence on temperature is observed.

The temperature dependence of the dissociation constants of ES complexes is very small[15] and it can be neglected. Without substrate, the temperature dependence of the spin state of cytochrome $P450$ is small, but significant This is in agreement with our estimation of a small high spin content of cytochrome $P450$ without substrate.
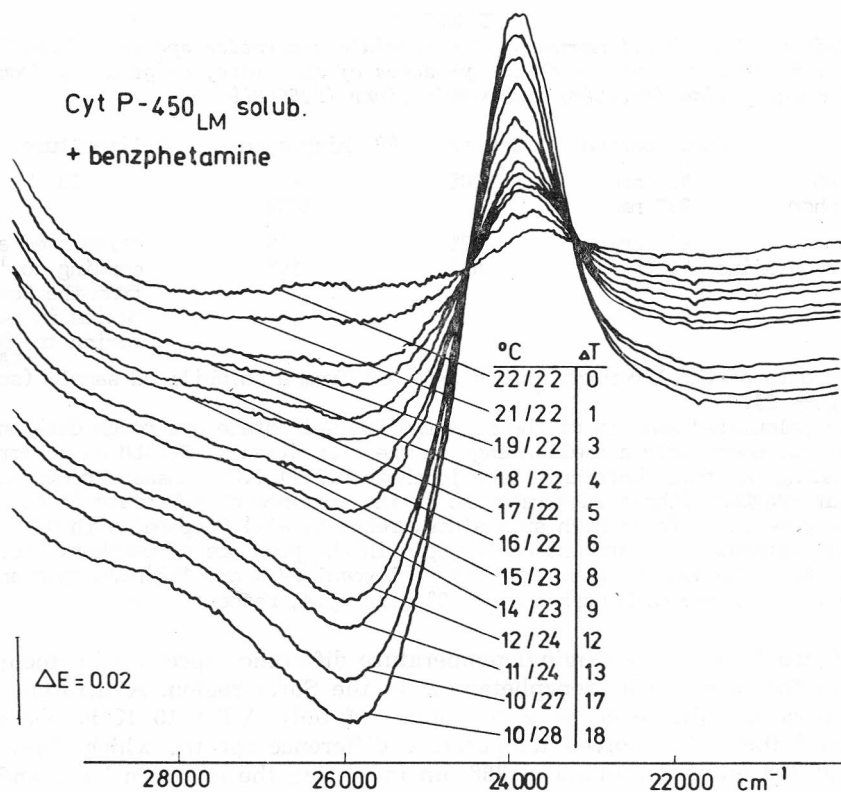
Figure 3. Temperature difference titration in the Soret region of solubilized rabbit liver cyto-
chrome *P450*, 64.2 µM in the presence of benzphetamine, 3.4 mM, layer thickness 0.2 cm (original
titration curves).

In the presence of type II substrates, such as aniline, the temperature
dependence of the spectrum of cytochrome *P450* is likewise small, but differs
significantly from the control in the small temperature difference range of
$\Delta T = 20$ K. No significant temperature difference spectra of cytochrome *P450*
are observed in the presence of imidazole or cyanide.

According to the sign of the high spin and the low spin bands, respectively,
it can be stated that with higher temperature the high spin state increases
and conversely with lower temperature the low spin state increases. This
means that the ground state of cytochrome *P450* is low spin. Since this is valid
also in the presence of substrates, it can be suggested that the axial ligands
of the heme iron at type I substrate binding are the same as without sub-
strates.

For a quantitative estimation of the spin state changes in dependence
on temperature the temperature difference spectra were simulated by means
of difference spectra with definite amounts of high spin and low spin portions.
Therefore, acid methemoglobin (predominantly high spin) was titrated with
sodium azide to produce methemoglobin azide (predominantly low spin). The
difference spectra were obtained by recording the different mixtures metHb/
/metHb·N$_3$ *versus* pure metHb·N$_3$ in the reference beam. With regard to the
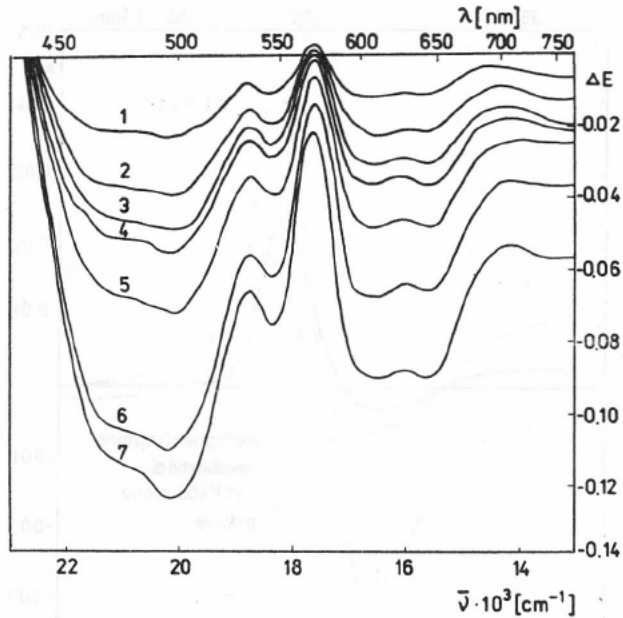
Figure 4. Temperature difference titration in the visible region of solubilized rabbit liver cytochrome *P450* in the presence of benzphetamine. The temperature differences were 1=3; 2=6; 3=8; 4=10; 5=12; 6=18; and 7=21 K. The concentrations were the same as in Figure 3; layer thickness 1.0 cm.
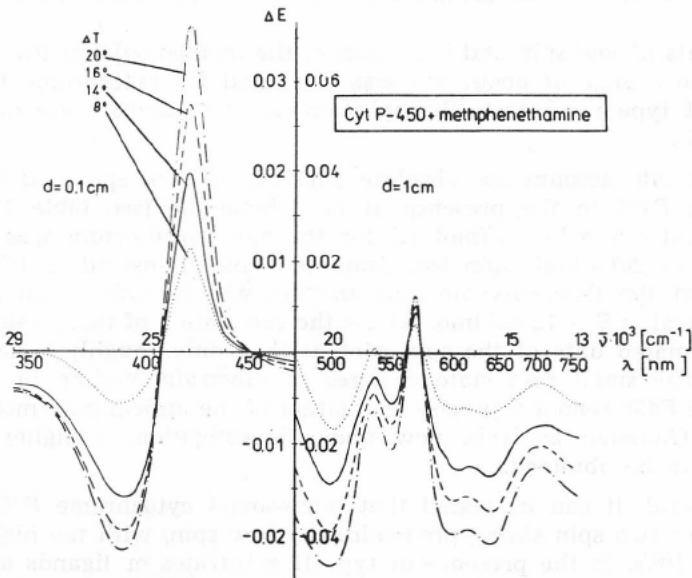

Figure 5. Temperature difference titration in the Soret and in the visible region of solubilized rabbit liver cytochrome *P450* in the presence of 4.5 mM methphenethamine.
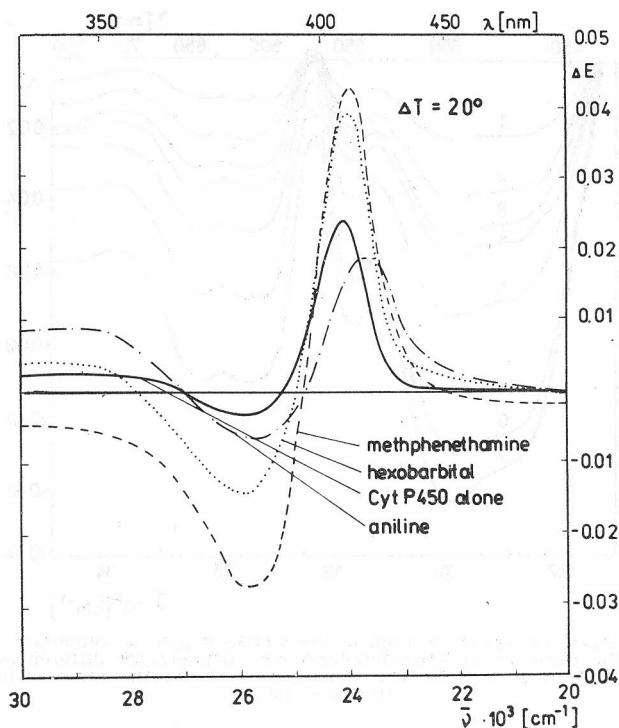
Figure 6. Temperature difference spectra in the Soret region of solubilized rabbit liver cyto-
chrome *P450* with various substrates (the concentration values of substrates are given as final
concentrations): methphenethamine 4.5 mM; hexobarbital 20 mM; aniline 18 mM; the temperature
difference of all spectra was 20 K and the layer thickness 0.1 cm.

real amounts of low spin and high spin in the methemoglobin titration curves
a spin state change of about 5% was estimated for cytochrome *P450* in the
presence of type I substrate (benzphetamine) at a temperature difference of
$\Delta T = 20$ K.

Taking into account the absolute amounts of high spin and low spin of
cytochrome *P450* in the presence of benzphetamine (see table 1) $\Delta H = 3.4$
kcal/mol and $\Delta S = 14$ cal/(mol K) for the spin equilibrium was calculated.
If a value of 20% high spin (see footnote Table I) instead of 16% is taken
into account the thermodynamic parameters will be only slightly different:
$\Delta H = 2.8$ kcal, $\Delta S = 12$ cal/(mol K). As the calculation of these values is based
on the estimated data of the spin content they only roughly reflect the real
situation. The small spin state changes at substrate binding in microsomal
cytochrome *P450* render a precise evaluation of the optical data more difficult,
but by a Gaussian analysis, now under investigation, a higher degree of
accuracy can be obtained.

In general, it can be stated that microsomal cytochrome *P450* exists in
a mixture of two spin states, predominantly low spin, with the high spin part
of only 8—10%. In the presence of type II substrates or ligands of the heme
iron an almost pure low spin state exists, but in the presence of type I sub-
strates the spin equilibrium is shifted to the high spin state. The extent of

this shift (*i*) depends on the specific properties of the substrate and (*ii*) in general it is relatively small; only up to about 8% for the substrates investigated so far.

The Importance of the Spin Equilibrium in Microsomal Cytochrome P450 for the Enzymatic Mechanism

The binding of substrates to cytochrome P450 is connected only with a small gain in energy[15]. In agreement with this, circular dichroic investigations of cytochrome P450 have shown that the predominantly hydrophobic binding of the substrates at the active site of the enzyme does not significantly affect the interaction between the prosthetic group and the protein[16]. On the other hand, the Tanabe-Sugano-diagram[17] shows that in transition metal complexes with ligand field strength near the crossing-over point of the spin pairing, only a small amount of energy is necessary to shift one spin state into another.

In d^5 complexes the low spin term t_{2g} reacts to small changes in the ligand field strengths and a new quality of the spin state (high spin or low spin) is produced. Since the spin state of cytochrome P450 is influenced by a relatively small influx of energy (binding of a variety of substrates thus excluding a high degree of stereospecifity; small changes of temperature are sufficient to produce the new spin state) the ligand field strength of the ferric heme complex in cytochrome P450 should be near the crossing-over point. This special property of the heme in cytochrome P450, presuming a weak low spin ligand, is supported by the small enthalpy value of the high spin low spin equilibrium. This property provides further evidence showing why the small energy gain at substrate binding producing only local conformational changes at the substrate binding site is sufficient to convert the low spin state into a high spin one.

A high spin complex is characterized by a weaker ligand field and longer bonding distances between the metal and the ligands than in a low spin complex (iron out of the heme plane)[18]. It is obvious that in such a complex it is much more easier to separate one of the axial ligands necessary for the binding of oxygen to the heme iron than in a low spin complex.

In our opinion the shift of the spin equilibrium to high spin caused by binding of substrates to the enzyme is the trigger for the energy consuming process of the heme iron reduction. This second step in the reaction cycle of cytochrome P450 showing an enhanced rate in the presence of substrates[19] is connected with the loosening of one of the axial ligands producing the 5-coordinated ferro heme complex. The existence of such a complex ready for oxygen binding is proved by the spin value of S = 2 (found for P450CAM [20]). Since spin equilibria have very short relaxation times of about 5 µs.[21] the only partially high spin state effected by substrates cannot be a limiting factor for the overall reaction rate of cytochrome P450.

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DISCUSSION

D. L. Williams-Smith:

How do you account for the apparent loss of EPR signal intensity in rabbit liver microsomes on binding cyanide?

H. Rein:

The amplitudes of the EPR spectra are not comparable because different gain settings were used. On the other hand it is true that the EPR spectrum of the *P450*-cyanide complex is of lower intensity than that of *P450* without an external ligand. This may be explained by (1) some reduction of the heme iron by cyanide in the presence of large quantities of this salt and (2) by line broadening due to a relatively short electronic relaxation time.

I. C. Gunsalus:

The microsomal *P450*LM type contains several heme species that may not bind substrate or may contain a ligand that promotes Type II spectral formation. Second, with a very high K_D (dissociation constant) for substrate, the fraction of *P450* as substrate complex is very much less than 1. Thus with improved enzyme purification and the best bound substrates, *i. e.* lowest K_D, one may expect to obtain more reliable data on spectral behavior.

H. Rein:

We assume that the change of the spin equilibrium of *P450* effected by substrates comes mainly from fraction LM2 which in microsomal preparations from phenobaroital induced rabbit liver is enzymatically the most active one. Moreover, the used substrates type I, benzphetamine and methphenetamine, have a high affinity to the enzyme so that with the substrate concentrations in our measurements about 95% saturation of the ES-complex is obtained.

SAŽETAK

O postojanju ravnoteže između visoko- i niskospinskog stanja u citokromu *P450* jetrenih mikrosoma i uloga te ravnoteže u enzimskom mehanizmu

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Ravnoteža između visokospinskog (S = 5/2) i niskospinskog (S = 1/2) stanja željeznog iona u citokromu *P450* iz mikrosoma jetre zeca potvrđena je mjerenjem diferencijalnih spektara u Soretovu i vidljivom području apsorpcije solubiliziranog *P450*. U prisustvu supstrata tipa II niskospinsko stanje dominira s laganim pomakom prema potpuno niskospinskom u odnosu na originalni *P450*. Ligandi hema željeza poput cijanida i imidazola dovode do potpuno niskospinskog stanja pa se u njihovu prisustvu diferencijalni spektri ne mogu niti dobiti. U prisustvu, pak, supstrata tipa I spinska je ravnoteža pomaknuta prema visokospinskog stanju. Veličina tog efekta (1) zavisi o specifičnim svojstvima supstrata, i (2) ona je uglavnom relativno mala, najviše do 8⁰/o u ispitivanim slučajevima.

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