EVIDENCE FOR THE EXISTENCE OF A HIGH SPIN–LOW SPIN EQUILIBRIUM IN LIVER MICROSOMAL CYTOCHROME *P*-450

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1. Introduction

From cytochrome $P\text{-}450_{cam}$ it is known that at substrate binding the Soret absorption band is shifted to shorter wavelengths (418 nm → 392 nm) [1]. Moreover, by electron spin resonance measurements it was shown that in the presence of camphor the low spin-state of heme iron in $P\text{-}450_{cam}$ is changed into the high spin form [2]. Contrary to these results the Soret absorption band of $P\text{-}450_{LM}$ exhibits only a small blue-shift at substrate binding (type I) (416 nm → 414 nm) fig.1, and by electron spin resonance measurements no significant change of the spin-state can be observed [3].

In met-hemoglobin and met-myoglobin the position of the Soret band is linearly correlated with the spin-state of the complex [4]. A shift of the Soret band to shorter wavelengths implies an increase of the high spin form and conversely a shift to longer wavelengths means that more low spin is formed. This dependence of the Soret band position on the spin-state is accounted for by the existence of mixtures of a high spin and a low spin component in different proportions [5].

If this correlation existing for hemoglobin and myoglobin is also valid for *P*-450, then from the small shift of the Soret band of $P\text{-}450_{LM}$ to shorter wavelengths at substrate binding (type I) the formation of a compound with an intermediate spin-value can be assumed. Compounds with such intermediate spin-values represent mixtures of the two spin-states $S = 5/2$ and $S = 1/2$ which exist in a temperature-dependent equilibrium [6,7]. These equilibria are to be determined by studying temperature difference spectra in the visible region titrating the typical high spin and low spin bands [5] and also the overlapping high and low spin bands in the Soret region.

By temperature difference spectra from solubilized *P*-450 from phenobarbital induced rabbit liver microsomes a high spin/low spin equilibrium has been indicated. In the presence of type I substrates the equilibrium is shifted to the high spin-state. The extent of this shift depends on specific properties of the substrate and is in general relatively small. In the presence of type II substrates the predominantly low spin-state of *P*-450 is maintained which by typical heme-iron ligands is shifted to the pure low spin-state.

2. Materials and methods

The studies were performed with solubilized cytochrome *P*-450 from phenobarbital induced rabbit liver microsomes [8]. The solubilization was performed with slight modifications according to the method of Lu et al. [9,10]. All measurements were performed in 0.1 M potassium phosphate buffer, pH 7.4, with 20% (v/v) glycerol and 0.1 mM dithiothreitol. The concentrations of *P*-450 and *P*-420 were determined according to Omura and Sato [11]. The cytochrome b_5 content was assayed from the difference spectra of the NADH-reduced and oxidized form [12].

Benzphetamine hydrochloride was prepared by

Abbreviations: $P\text{-}450_{cam}$ Cytochrome *P*-450 from *Pseudomonas putida*, $P\text{-}450_{LM}$ Cytochrome *P*-450 from liver microsomes

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extraction and crystallization in a pure form from Didrex^R (Upjohn Com., Katamazoo, Michigan, USA) and checked by mass spectroscopy. Sodium hexobarbital and methphenethamine as Spasman^R were obtained from VEB Arzneimittelwerk Dresden, GDR. Imidazole and cyanide were commercial products (analytical grade). Aniline was distilled twice before use.

All spectrophotometric measurements were carried out using a Beckman Acta CV spectrophotometer. Difference spectra were recorded by cooling the sample cuvette to 5°C and heating the reference cuvette to 30°C. The spectra were obtained with the same sample within one hour; in this time no denaturation of *P*-450 in the cuvette at 30°C could be observed. The temperature was measured within the cuvette holder by means of calibrated thermistors. Measurements were carried out under a stream of dry nitrogen.

3. Results

From the absorption spectra of $P\text{-}450_{LM}$ in fig.1 it can be seen that in the presence of type I substrates a small but significant blue-shift of the Soret absorption band occurs indicating that by substrate binding the high spin-state increases and moreover that an intermediate spin-value has been formed. Because intermediate spin-values represents mixtures of two spin-states they can be quantitatively analyzed by recording temperature difference spectra. Figure 2 shows such original spectra of $P\text{-}450_{LM}$ in the presence of benzphetamine in the Soret region.

A titration at a temperature-difference of a maximum of $\Delta T = 18°C$ is sufficient to record well resolved temperature-difference spectra which show maxima at 418 nm and minima at 386 nm indicating the low spin and the high spin band, respectively. In fig.3 it can be seen that also in the visible region the high spin bands at 495 nm and 641 nm and the low spin bands at 538 nm and 568 nm are well titratable by temperature dependence. From the sign of the high spin bands (maxima) and of the low spin bands (minima) respectively it can be established that with higher temperature the high spin-state increases and conversely that with lower temperature the low spin-state increases. This indicates that the ground-state of

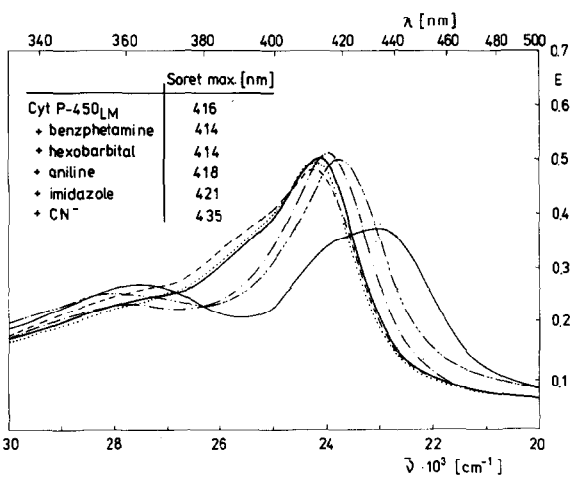

Fig.1. Optical absorption spectra in the Soret region of solubilized rabbit liver cytochrome *P*-450 in the presence of various substrates (the concentration values of substrates are given as final concentrations). (———) Without substrate. (– – – –) With benzphetamine 3.4 mM. (· · · · · · ·) With hexobarbital 20 mM. (· – · – · – · –) With aniline 18 mM. (– ·· – ·· – ·· –) With imidazole 3.2 mM. (———) With cyanide, added as solid KCN. Concentration of *P*-450 64.2 μM, *P*-420 6 μM, cytochrome b_5 3 μM, layer-thickness 0.2 cm.

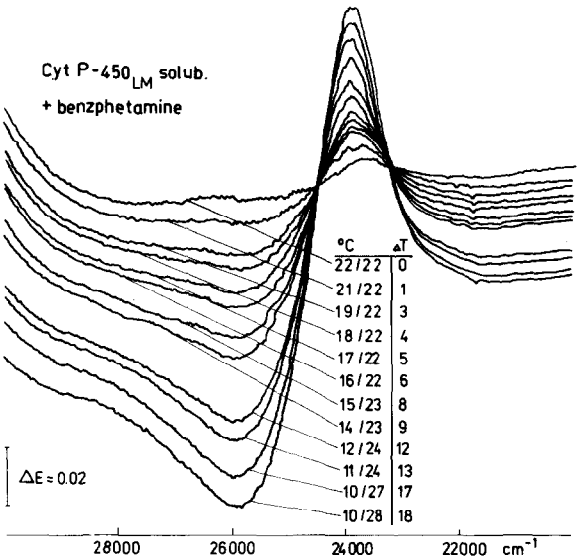

Fig.2. Temperature-difference titration in the Soret region of solubilized rabbit liver cytochrome *P*-450 in the presence of benzphetamine. The concentrations were the same as in fig.1, layer-thickness 0.2 cm.

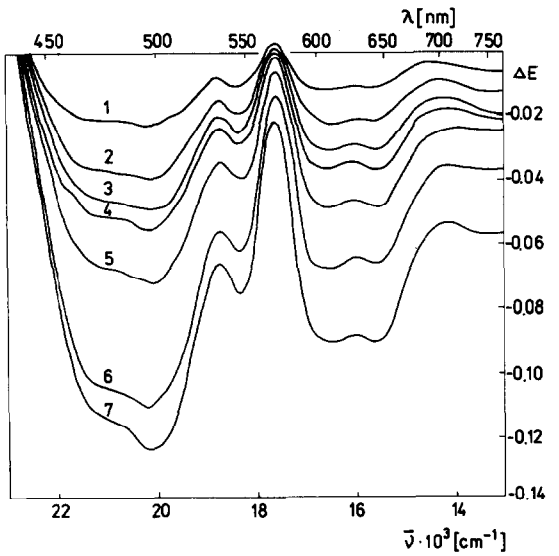

Fig.3. Temperature-difference titration in the visible region of solubilized rabbit liver cytochrome *P*-450 in the presence of benzphetamine. Temperature-differences 1 = 3°C, 2 = 6°C, 3 = 8°C, 4 = 10°C, 5 = 12°C, 6 = 18°C, 7 = 21°C. The concentrations were the same as in fig.1, layer-thickness 1.0 cm.

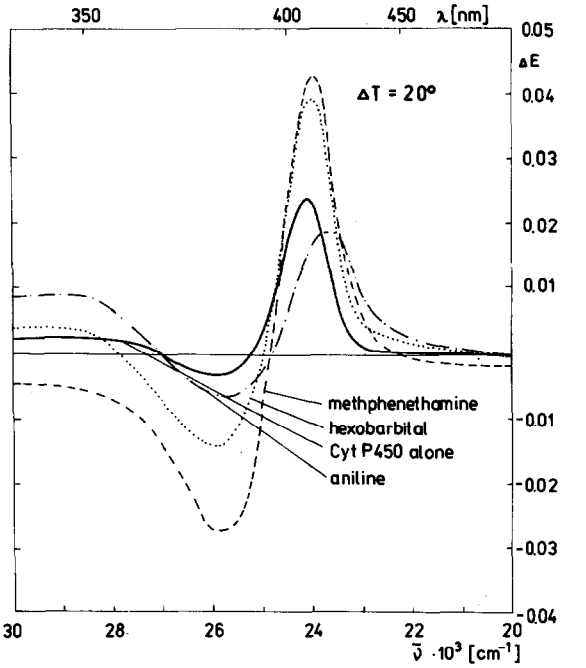

Fig.4. Temperature-difference spectra in the Soret region of solubilized rabbit liver cytochrome *P*-450 in the presence of various substrates (the concentration values of substrates are given as final concentrations). (————) Without substrate. (– – – – –) Met-phenethamine 4.5 mM. (· · · · · · · · ·) Hexobarbital 20 mM. (– · – · – · –) Aniline 18 mM. The concentration of the enzyme was the same as in fig.1, temperature-difference 20°C, layer-thickness 0.1 cm.

$P\text{-}450_{LM}$ in the presence of benzphetamine is low spin which is valid also for $P\text{-}450_{LM}$ in the presence of other substrates and without substrate. From this behaviour it is suggested that in the presence of type I substrates as well as in their absence the axial ligands of the heme iron are maintained.

From fig.4 it can be seen 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 individual substrate on the spin-state. In agreement with the blue-shift of the Soret band of $P\text{-}450_{LM}$ in the presence of type I substrates the spin equilibrium of $P\text{-}450_{LM}$ (predominantly low spin) is shifted to higher spin-values and therefore a large temperature-dependence is observed. Without substrate the temperature-dependence of $P\text{-}450_{LM}$ is small but significant at the relatively small temperature-difference of $\Delta T = 20°C$. In the presence of type II substrates such as aniline the temperature-dependence of the spectrum of $P\text{-}450_{LM}$ is likewise small. No significant temperature-difference spectra are observed in the presence of imidazole or cyanide.

For a quantitative estimation of the spin-state changes with temperature the temperature-difference spectra increasing with an increase in ΔT were simulated by measuring a met-hemoglobin solution with various amounts of azide therefore containing definite amounts of low spin and high spin proportions against the fully saturated complex. Taking the exact amounts of low spin and high spin portions in the met-hemoglobin complex as standard values, a spin-state change of about 5% was calculated for $P\text{-}450_{LM}$ in the presence of benzphetamine, at a temperature-difference of $\Delta T = 20°C$. Furthermore, taking into account the Soret maxima of $P\text{-}450_{cam}$ in absence of substrate (low spin) and in the presence of camphor (high spin) [1] according to [4] it can be calculated for $P\text{-}450_{LM}$ without substrate 8% high spin and in the presence of benzphetamine 16% high spin. From these data we calculate the thermodynamic parameter of the spin

equilibrium of $P\text{-}450_{LM}$ in the presence of benzphetamine ΔH = 3.4 kcal/M and ΔS = 14 cal/deg/M*.

4. Discussion

The binding of substrates to $P\text{-}450_{LM}$ is connected only with a small gain of energy [13]. On the other hand from the Tanabe-Sugano-diagram [14] it is known that in transition-metal complexes with ligand field-strengths near the intersection of spin-pairing energy, only a small amount of energy is necessary to shift the spin-state from one into the other. At d^5 complexes the low spin term t_{2g}^5 reacts at small changes of the ligand field-strengths and the new quality of spin-state (high spin or low spin) is produced. Therefore the small gain of energy produced by substrate-binding is sufficient to shift the spin-equilibrium which indeed in the presence of a substrate exhibits a small enthalpy-value.

A high spin complex is characterized by a weaker ligand field and longer binding distances between the metal and the ligands than in a low spin complex (iron out of the heme plane) [15]. Obviously in such a complex one of the axial heme-iron ligands of *P*-450 – necessary for the binding of oxygen – can be separated much more easily than in a low spin complex. The second step in the reaction cycle of *P*-450 showing an enhanced rate in the presence of substrates [16] is connected with the displacement of one of the axial ligands producing the 5-coordinated ferro–heme complex. The existence of such a complex being able to bind oxygen is proved by the spin-value $S = 2$ (found with $P\text{-}450_{cam}$) [17]. Because spin equilibria have short relaxation times [18] the fact that by substrate binding only a portion of *P*-450 is shifted to the high spin-state cannot be a limiting factor for the overall reaction rate of $P\text{-}450_{LM}$.

Summarizing the results it is suggested that the shift of the equilibrium to the high spin-state caused by substrate binding to the enzyme is the trigger for the energy consuming process of the heme-iron reduction.

*At this time further values of $P\text{-}450_{LM}$ in the presence of other substrates cannot be calculated because the small spin-state changes at substrate binding in $P\text{-}450_{LM}$ render a precise evaluation of the optical data more difficult, but by a Gaussian analysis of the temperature difference spectra, now under investigation, values with a higher degree of accuracy can be obtained.

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