Thermodynamic studies of substrate binding and spin transitions in human cytochrome *P*-450 3A4 expressed in yeast microsomes

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An approach to the quantitative spectral analysis of substrate binding and inactivation of cytochrome *P*-450 in microsomes is described. The method is based on the application of the principal component analysis technique on the Soret-region spectra measured at different temperatures at various concentrations of substrate. This approach allowed us to study the thermodynamic parameters of substrate binding and spin transitions in human cytochrome *P*-450 3A4 expressed in yeast (*Saccharomyces cerevisiae*) microsomes. These parameters are discussed in comparison with the values reported earlier by Ristau et al. [(1979) Acta Biol. Med. Ger. **38**, 177–185] for rabbit liver cytochrome *P*-450 2B4 in solution with benzphetamine as a substrate. Our analysis shows the substrate-free states of 2B4 and 3A4 to be very similar. However, substrate binding seems to perturb haem–protein interactions in 3A4 in contrast with 2B4, where the effect of substrate binding on the thermodynamic parameters of spin transitions was insignificant. The implication of the results for the mechanism of substrate-induced spin shift is discussed.

INTRODUCTION

It is well known that cytochromes *P*-450 in the ferric state exist in a temperature-dependent equilibrium between the forms having haem iron in the low-spin and the high-spin states [1–5]. Most cytochromes *P*-450 are predominantly low-spin in the substrate-free state. Binding of the substrates usually shifts the equilibrium towards the high-spin form [1,3,6,7]. Spin equilibrium is also modulated by incorporation of the haemoprotein into membranes [6,8], changes in the membrane lipid composition [8], interactions of the haemoprotein with cytochrome b_5, NADPH-cytochrome *P*-450 reductase [9,10], and oligomerization of the haemoprotein [11,12].

The equilibrium between the high-spin and low-spin forms of cytochrome *P*-450 was shown to be extremely important for the function of both soluble bacterial and membrane-bound eukaryotic mono-oxygenases [13–15]. Modulation of the spin equilibrium of cytochrome *P*-450 seems to be one of the principal control mechanisms for the activity of microsomal mono-oxygenases. Thus detailed studies of the thermodynamic and kinetic parameters of the spin transition in the native environment (in the microsomal membrane) are very important for the understanding of the mechanisms of action and regulation of the system.

In the past 20 years many investigations have been undertaken to study the effects of various factors on the spin state of cytochromes *P*-450 in relation to the functional properties of the microsomal mono-oxygenase. To characterize the spin state of the haemoprotein the difference in Soret absorption spectra between the high-spin (λ_{max} 388–396 nm; *P*-450_{hs}) and low-spin (λ_{max} 416–418 nm; *P*-450_{ls}) haemoprotein is routinely used [4,5,16–20]. However, microsomal cytochromes *P*-450 undergo rather fast spontaneous inactivation to form the *P*-420 (inactive) state of the haemoprotein. Thus the quantitative analysis of spin transitions in microsomal cytochromes *P*-450 is complicated by the overlapping of the band of *P*-450_{ls} with the band of the *P*-420 form of the haemoprotein, which is located at 420–425 nm [21,22], and by the lack of spectral standards for the pure *P*-450_{ns}, *P*-450_{ls} and *P*-420 forms of microsomal cytochromes. In microsomes, such analysis is especially intricate because of the turbidity of the system and the overlapping of the *P*-450 absorbance bands with that of cytochrome b_5 and flavoprotein. In addition, many *P*-450-related transitions in microsomes are usually associated with some changes in turbidity. Under these conditions the classical methods of analysis of spectral changes are almost inapplicable.

We have introduced a functional model system of microsomal mono-oxygenase based on yeast microsomes containing expressed human cytochrome *P*-450 3A4 and human cytochrome b_5 with co-expressed NADPH-cytochrome *P*-450 reductase and cytochrome b_5 [23–25]. This system seems to be a very convenient tool for the physico-chemical and mechanistic studies of the function and regulation of microsomal mono-oxygenases. However, the above-mentioned difficulties seem to limit the study of the spin transition and substrate-binding processes here.

The present study was undertaken to develop an appropriate method for monitoring the cytochrome *P*-450 spin equilibrium in membranous systems. The approach we describe here is based on the principal component analysis (PCA) technique. This method, also known as bilinear factor analysis [26] or singular value decomposition (SVD) analysis [27], was first introduced in chemistry by Malinowski and Howery [28]. It is widely used nowadays to interpret absorbance and fluorescence spectral changes in complex biochemical systems [26–32]. Using this approach we have deduced the set of standard absorbance spectra of the high-spin, low-spin and *P*-420 states of pure ferric

Abbreviations used: DTT, dithiothreitol; *P*-420, inactive form of cytochrome *P*-450; *P*-450, active form of cytochrome *P*-450; *P*-450_{ls} and *P*-450_{hs}, low-spin and high-spin *P*-450 states of cytochrome *P*-450 respectively; PCA, principal component analysis.

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microsomal cytochrome *P*-450 2B4 from rabbit liver. Then, using PCA and the set of newly generated spectral standards, we have evaluated the thermodynamic parameters of substrate (bromokriptine) binding and spin transitions in yeast microsomes containing human cytochrome *P*-450 3A4. The study was undertaken in part to serve as a methodological basis for further studies of pressure-induced changes in substrate binding and spin equilibrium in cytochrome *P*-450 3A4-containing yeast microsomes. The hydrostatic pressure perturbation approach in combination with the PCA technique has been already used to study substrate binding and spin transitions in rabbit cytochrome *P*-450 2B4 in solution [12].

MATERIALS AND METHODS

Experimental

Materials

Cytochrome *P*-450 2B4 from rabbit liver was purified to electrophoretic homogeneity [33] and stored in 0.1 M Na/Hepes buffer containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and 20 % (v/v) glycerol at −70 °C.

Hepes, EDTA, D,L-DTT, benzphetamine and bromokriptine were obtained from Sigma Chemicals. Triton N-101 was the product of Fluka Chemicals.

Experimental procedure

All experiments were performed in 0.1 M Na/Hepes buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT at 25 °C unless otherwise indicated. Absorbance spectra were recorded with a computer-controlled Cary-219 (Varian) or Uvikon-940 (Kontron) spectrophotometers.

Methods of spectra processing and data fitting

PCA technique for separation of particular processes in a multicomponent system

To resolve the spin shift, the *P*-450 → *P*-420 transitions and the fluctuations in the turbidity from overall spectral changes, we used the method of PCA [26–28], of which the ultimate goal is to determine the minimum number of particular processes reflected in the overall spectral changes and to separate these changes into so-called principal components used to understand the nature of each particular process. PCA generates two series of quantities for each principal component. These quantities are the basis spectra and the vectors of loading factors. These two sets of parameters can respectively be thought of as differential spectra and the dynamics of apparent particular processes. The number of statistically significant principal components is an estimate of the minimum number of individual processes taking place in the system. These components do not necessarily correspond, however, to real transitions. They represent only one of the possible ways of decomposition and should be considered as a linear combination of the real transitions. To delineate such transitions some additional information is required, such as the extinction spectra of the pure components of the system and some suggestion as to the nature of the transitions. This information is then used to transform the initial results of PCA (to redistribute the spectral changes between the components) to get the exact stoichiometry and follow the course of the real processes.

The PCA algorithm was implemented in the SPAN module of our SpectraLab data acquisition and fitting software, as previously described [12]. In the present work we usually applied this method to the series of absorbance spectra in the range 340–600 nm digitized in 2 nm steps.

Curve fitting was performed by direct optimization with the nonlinear least-squares algorithms of Marquardt [34] and Nelder and Mead [35], implemented in the SpectraLab software package [12].

RESULTS AND DISCUSSION

Determination of the extinction standards of the *P*-450$_{hs}$, *P*-450$_{ls}$ and *P*-420 states of ferric cytochrome 2B4

Cytochromes *P*-450 are usually represented by a mixture of three spectral forms: *P*-450 high-spin (*P*-450$_{hs}$, Soret band at 388–392 nm), *P*-450 low-spin (*P*-450$_{ls}$, Soret band at 416–420 nm) and *P*-420 (Soret band approx. 425 nm) [21,22]. In the absence of substrate, most bacterial cytochromes *P*-450, such as *P*-450cam from *Pseudomonas putida* and *P*-450lin from *Pseudomonas incognita*, are predominantly low-spin. They can be completely converted into the high-spin form by the addition of an appropriate substrate [1,2,6]. High pressures were reported to convert *P*-450cam totally to the *P*420 state [21]. In contrast, the pure *P*-450$_{ls}$, *P*-450$_{hs}$ and *P*-420 forms of microsomal cytochromes *P*-450 are unavailable [5,13,16,36]. It is a common practice to use the extinction coefficients of the bacterial haemoproteins in an analysis of the spin equilibrium of microsomal cytochromes *P*-450 [4,16]. However, these coefficients, as well as the positions of the maxima, could well be different in the bacterial and the microsomal haemoproteins. Moreover, microsomal cytochromes *P*-450 exhibit easy spontaneous conversion to the *P*-420 state, which must be taken into account in a quantitative analysis of the *P*-450 spin transitions. For such an analysis it was necessary to obtain the set of spectral standards of *P*-450$_{ls}$, *P*-450$_{hs}$ and *P*-420 for the microsomal haemoprotein. Because of considerable similarity of amino acid sequences and similar functional properties of microsomal cytochromes *P*-450 2B4 and 3A4, we have assumed that the spectral standards of *P*-450 2B4 could also be used for *P*-450 3A4.

To produce the set of spectral standards of *P*-450 2B4 we used the spectra of bacterial cytochromes as initial estimates for further refinement. We have chosen the spectra of the substrate-free *P*-450lin and camphor-bound *P*-450cam as initial estimates for *P*-450$_{ls}$ and *P*-450$_{hs}$ respectively. The spectrum of the pressure-generated *P*-420 form of *P*-450cam [21] was used as the initial spectral standard of *P*-420. To refine these standards for *P*-450 2B4, the series of 2B4 spectra recorded as a function of temperature and the results of spectrophotometric titration of 2B4 by benzphetamine were used. These series were subjected to the PCA procedure. In both cases the first principal component was enough to fit approx. 96 % of the changes. As expected, the first basis spectrum was close to the differential spectrum of the low-to-high spin transition (Figure 1b). The second minor component (approx. 3 % of the changes) was apparently due to the *P*-450 → *P*-420 transition. For the first cycle of refinement a series of benzphetamine titrations was used (Figure 1). The estimates of the spectral standards were approximated by the linear combination of the first two basis spectra and the average spectrum of the series (Figures 1c to 1d). Then the initial estimates of the standards were replaced with newly generated ones and the process was repeated with the series of spectra recorded at different temperatures (results not shown). This iterative procedure was repeated until the changes in the estimates of spectral standards became negligible. Then, to refine the spectral standard of *P*-420, the same approach was applied to the series of spectra recorded at different times during the *P*-450 → *P*-420 transition initiated by the addition of 6 M urea or 1.6 M sodium iodide to a solution of 2B4 [36] (results not shown). The extinction coefficients of *P*-450$_{hs}$, *P*-450$_{ls}$ and *P*-420 were adjusted for the

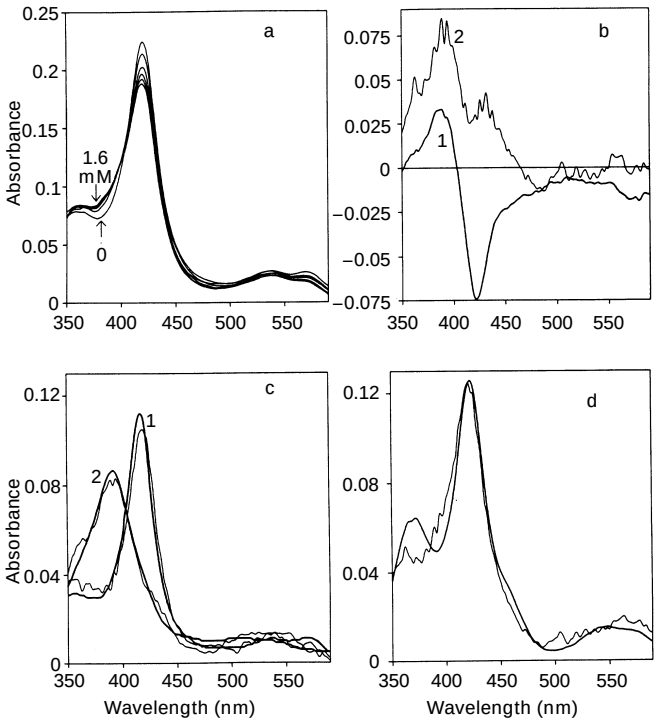


Figure 1 Refinement of the spectral standards of the pure *P*-450$_{ls}$, *P*-450$_{hs}$ and *P*-420 states of *P*-450 2B4

(**a**) A series of spectra of cytochrome *P*-450 2B4 recorded at various concentrations of benzphetamine (0, 0.25, 0.5, 0.75, 1 and 1.6 μM). Conditions: 2.2 μM 2B4 in 0.1 M Na/Hepes buffer (pH 7.4)/1 mM EDTA/1 mM dithioerythritol. (**b**) The first (1) and second (2) basis spectra obtained by application of PCA to this series. (**c**) Spectral standards of the *P*-450$_{ls}$ state of *P*-450lin (trace 1) and the *P*-450$_{hs}$ state of *P*-450cam (trace 2) (bold lines) along with their approximations by 2B4 spectra shown in (**a**) and (**b**). (**d**) The spectrum of the pressure-generated *P*-420 state of *P*-450cam (bold line) along with its approximation by the spectra shown in (**a**) and (**b**).

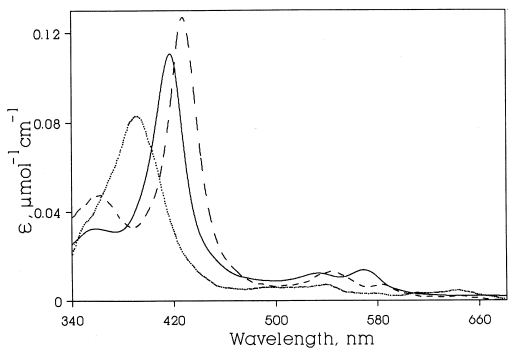


Figure 2 Standard extinction spectra of the pure species of cytochrome *P*-450 2B4: *P*-450$_{ls}$ (solid line), *P*-450$_{hs}$ (dotted line) and *P*-420 (broken line)

best conformity with the concentrations of *P*-450 and *P*-420 measured by the method of Imai and Sato [36].

The set of extinction standards obtained by this procedure is shown in Figure 2. The standards of 2B4 high-spin and low-spin forms correspond well to those known [5] for bacterial haemoproteins. The spectrum of the oxidized *P*-420 state of 2B4 is characterized by two maxima at 365 and 426 nm, in good agreement with those reported for the *P*-420 state of cytochrome *P*-450cam, where these maxima were found near 360–370 and 420–425 nm [21,22]. The extinction coefficient for the *P*-420 state of 2B4 at 426 nm was found to be 0.128 $\mu M^{-1} \cdot cm^{-1}$.

The concentrations of *P*-420 and total *P*-450 (both high-spin and low-spin states) given by least-squares fitting with the newly generated standards show no more then 5% difference from those determined by the method of Imai and Sato [36]. Our evaluations of the percentage of *P*-450$_{hs}$ were compared with the values determined by the temperature-difference method of Ristau et al. [16]. These values were also in good agreement with the results obtained by our method.

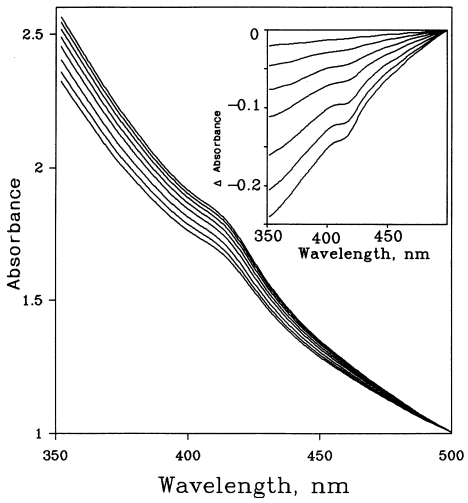


Figure 3 Temperature-induced spectral changes in NF25 microsomes in the presence of 1 μM bromokriptine

The conditions were: NF25 yeast microsomes containing 1.6 μM cytochrome *P*-450 3A4 in 100 mM Na/Hepes buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT and 1 μM bromokriptine. The optical pathlength was 5 mm. Spectra were measured at 4 °C (uppermost spectrum), 8, 12, 16, 20, 25, 30 and 35 °C. Inset: the same spectra shown as a difference from the first spectrum of the series (at 4 °C).

Analysis of temperature-induced spectral changes

To study the thermodynamic parameters of substrate binding and spin equilibrium of 3A4 we have measured the series of spectra of absorbance of microsomes at different temperatures at various concentrations of bromokriptine.

A series measured at 10 μM bromokriptine is shown in Figure 3. The most prominent changes in the absorption spectra are caused by temperature-dependent turbidity drift. To resolve this drift from the spectral transitions of cytochrome *P*-450 we applied PCA. A single principal component was enough to cover 99.9% of the spectral changes here (Figure 4). The contribution of turbidity drift was so high that it could not be resolved from the spectral changes of cytochrome *P*-450 by PCA at this stage. The first basis spectrum always reflected both the drift in turbidity and the changes in *P*-450 spectra. Because of the very low significance of the other principal components, their basis spectra were very noisy and could not be used for further analysis. However, all spectral transitions of interest were expected to be a combination of the pure spectra of the haemoprotein substates. Correspondingly, the second derivative of the first basis spectrum fits well the derivative of the differential spectrum of the high-to-low spin transition calculated from the 2B4 spectral standards (Figure 4a, inset). Thus the first basis spectrum can be thought of

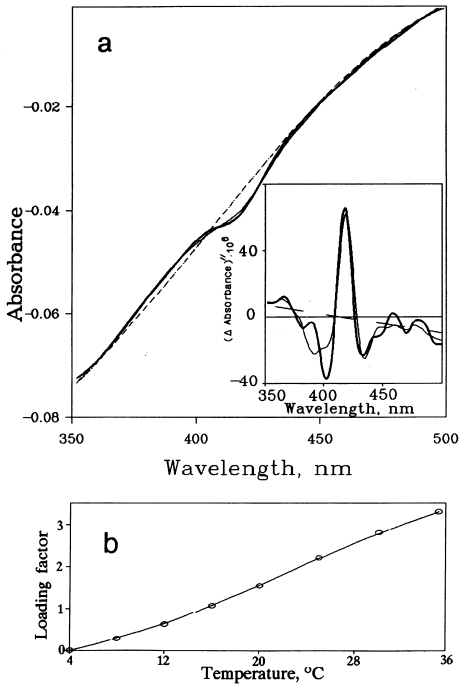


Figure 4 Application of PCA to the data set shown in Figure 3

(**a**) First basis spectrum (bold line). The thin solid line shows the result of its being fitted by a combination of the differential spectrum of transition the transition from low spin to high spin calculated from 2B4 spectral standards and the approximation of the turbidity component by a third-order polynomial (broken line). Inset: second derivatives of the same basis spectrum (bold line), fitting curve (thin solid line) and polynomial (broken line). (**b**) Temperature dependence of the loading factor for the first principal component.

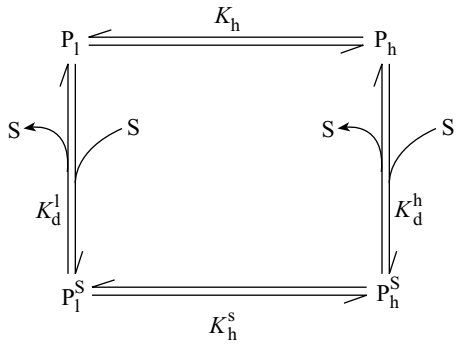
as a linear combination of the spectral standards of *P*-450 with the turbidity component. The contribution of turbidity could be approximated by a low-order polynomial. The square correlation coefficient for the fitting of the basis spectrum by a combination of a third-order polynomial and the standard differential spectrum of the high-to-low spin transition (Figure 4a, thin solid line) was higher than 0.999.

The polynomial component of the fitting accounts for the approximation of the 'spectrum' of turbidity drift. To compensate for the drift this polynomial was multiplied by the corresponding loading factors and subtracted from the spectra. To compensate for the invariant turbidity contribution in the absorbance, the averaged spectrum of the series was fitted by a linear combination of the spectral standards of 2B4 *P*-450_{ls}, *P*-450_{hs} and *P*-420, the standard extinction spectra of cytochrome b_5 and a third-order polynomial. The polynomial part of the fitting was subtracted from the series.

The results of this correction are shown in Figure 5a. Application of PCA to the series of corrected spectra yields two principal components, which fit more than 99 % of the residual spectral changes. The first principal component applies mainly to the low-to-high spin shift, the second to the *P*-450 → *P*-420 transition taking place at high temperatures (Figures 5b to 5d). The basis spectra of the first two principal components, obtained by PCA of the series of spectra measured at various concentrations of bromokriptine (0–100 μM), were identical (square coefficient of cross-correlation more than 0.98). The changes in the concentrations of *P*-450 species calculated from the results of the factor analysis are shown in Figure 5(c).

Thermodynamic analysis of the substrate binding and spin equilibria of 3A4

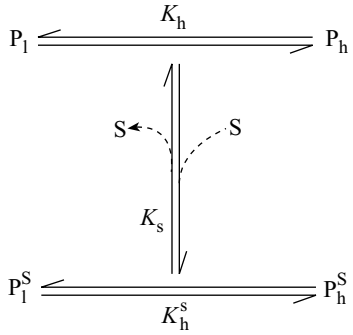
The scheme for the substrate binding and spin transitions in cytochrome *P*-450 can be drawn as follows:



$$K_h = [P_h]/[P_l];\ K_h^s = [P_h^s]/[P_l^s];$$
$$K_d^l = [P_l][S]/[P_l^s];\ K_d^h = [P_h][S]/[P_s^h] \quad (1)$$

where P and PS correspond to cytochrome *P*-450 and its complex with the substrate respectively, and indices l and h designate respectively the low-spin and high-spin states of the haemoprotein.

To simplify the case, the two independent substrate binding equilibria of *P*-450_{hs} and *P*-450_{ls} can be effectively replaced by a single equilibrium characterized by apparent dissociation constant, K_s [16]:



$$K_s = \frac{([P_l]+[P_h])[S]}{[P_l^s]+[P_h^s]};$$

$$K_s = \frac{(1+K_h)K^l{}_d}{1+K^s{}_h} = \frac{(1+K_h)K^l{}_dK^h{}_d}{K^h{}_d+K^l{}_dK_h} \quad (2)$$

Analysis of this model yields the following equation for the apparent equilibrium constant of the overall spin equilibrium:

$$F_h = \frac{[P_h^s]+[P_h]}{[P]_0} = \frac{K_s(K_h+1)+[S](K^s{}_h+1)}{([S]+K_s)(K^s{}_h+1)(K_h+1)} \quad (3)$$

where $[P]_0 = [P_h^s]+[P_h]+[P_l^s]+[P_l]$.

It should be noted that, although the changes in the concentrations of *P*-450 species found by PCA seem to be precise and well reproducible, the values of the absolute concentrations and therefore the evaluations of F_h are less accurate. To obtain the concentrations of *P*-450_{ls}, *P*-450_{hs} and *P*-420 at each temperature, the estimates of concentrations at the initial (lowest) temperature

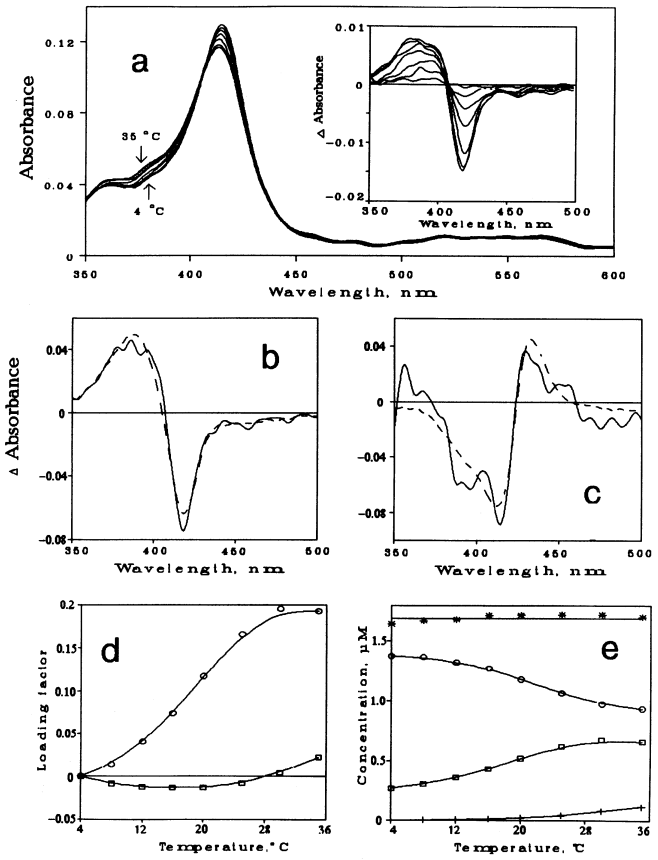


Figure 5 Resolution and quantitative analysis of the temperature-induced spectral changes in cytochrome *P*-450 3A4 by PCA

The same series of spectra are shown as in Figure 3 after correction for the turbidity changes: the polynomial component of the first basis spectrum (Figure 4a, broken line) was multiplied by the values of the loading factor (Figure 4b) for the corresponding temperature and subtracted from the original spectra. The invariant turbidity contribution was corrected for by the fitting of the first spectrum of the series by a combination of spectral standards of cytochromes *P*-450 2B4 and b_5 with a fourth-order polynomial. (**a**) Absolute spectra. Inset: the same spectra shown as the differences from the first spectrum of the series. (**b**, **c**) The results of PCA application here: first (**b**) and second (**c**) basis spectra with the results of their fitting by spectral standards of cytochrome *P*-450 2B4 (broken lines). (**d**) Temperature dependences of the loading factors for the first (○) and second (□) principal components. (**e**) Temperature dependences of the concentrations of *P*-450$_{ls}$ (○), *P*-450$_{hs}$ (□), *P*-420 (+) and total 3A4 concentration (∗), calculated from the PCA results.

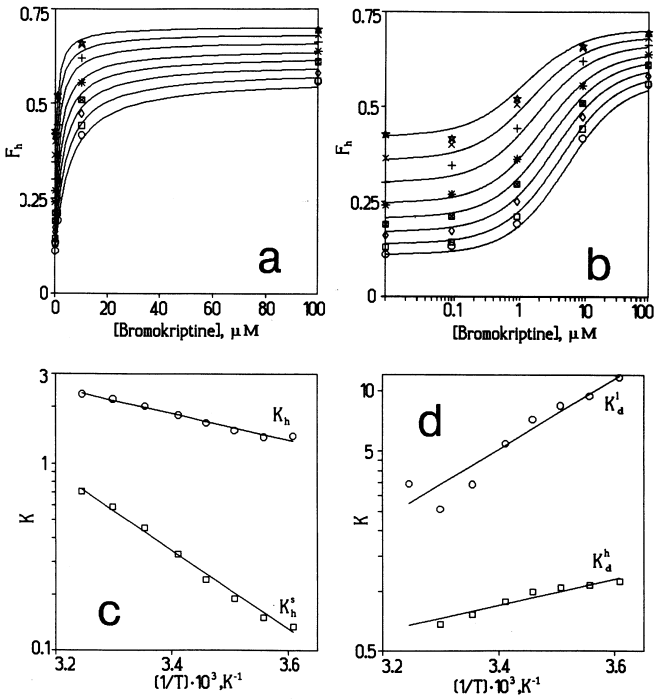


Figure 6 Determination of the thermodynamic parameters of bromokriptine binding and spin transitions of cytochrome *P*-450 3A4 in NF25 microsomes

(**a**, **b**) Dependences of the fraction of cytochrome *P*-450$_{hs}$ on bromokriptine concentration at various temperatures: 4 (○), 8 (□), 12 (◇), 16 (⊠), 20 (∗), 25 (+), 30 (×) and 35 (☆) °C. Solid lines show the global fitting of data by eqn. (3) to the set of constants calculated from the thermodynamic parameters given in Table 1. (**c**, **d**) van't Hoff plots for the equilibrium constants of substrate binding (**d**) and spin transitions (**c**).

were used to offset the changes quantified by PCA. This was done by a straightforward fitting of the first spectrum in the series by a combination of spectral standards of cytochromes *P*-450 and b_5 and the polynomial approximation of the turbidity component. Because there is considerable turbidity as well as overlapping of the cytochrome b_5 and *P*-450 bands, the results of the fitting are only estimates of the concentrations of the 2B4 species.

The fitting of the dependence of apparent F_h on [S] at each temperature was used to improve the estimates of F_h by iterative backward correction. This was based on the proposition that the estimates of the changes in the concentrations of *P*-450$_{hs}$, *P*-450$_{ls}$ and *P*-420 with temperature are accurate enough but the absolute concentrations and therefore the F_h values for the same [S] might contain a constant deviation. Thus for each particular temperature the dependences of F_h on [S] were fitted to eqn. (1) (Figures 6a and 6b). The deviations in fit were averaged over the temperature for each particular [S] [i.e. over each column of points in Figures 6(a) and 6(b)]. These values of mean deviation for each [S] were used as a constant displacement to correct the whole data set corresponding to this particular concentration of the substrate [i.e. to shift a column of points in Figures 6(a) and 6(b)]. It should be noted that the deviations were not more than 20% relative to F_h and had no systematic dependence on [S]. The procedure was recycled until F_h displacements became negligible (less than 5% of the value). For the present data set only two iteration cycles were necessary to reach this condition. The coefficient of correlation in the first iteration (for the uncorrected data set) was 0.978. The final fit had $\rho^2 = 0.998$. The absolute changes in F_h values resulting from this data correction procedure did not exceed 0.15. This fitting gave us the values of K_h, K^S_h and K_s for each temperature. Then K^h_d and K^l_d were calculated from eqn. (2). Temperature dependences of K_h, K^S_h, K^h_d and K^l_d obeyed the van't Hoff equation with $\rho^2 \geqslant 0.965$ (Figures 5c and 5d).

The thermodynamic parameters of the spin transitions and the substrate binding are given in Table 1. Compared with the parameters determined by Ristau and co-authors [16] for purified rabbit liver cytochrome *P*-450 2B4 in solution, our values reveal the same general regularities. ΔH and ΔS for the spin transition of the substrate-free form and for the substrate binding to the low-spin state are very similar for these two cytochromes. However, the spin transitions of the haemoprotein–substrate complexes as well as the substrate binding to the high-spin haemoproteins exhibit considerable differences. The spin tran-

Table 1 Thermodynamic parameters of spin equilibrium and bromokriptine binding for cytochrome *P*-450 3A4 in yeast microsomes compared with the values reported for cytochrome *P*-450 2B4 in solution with benzphetamine as a substrate

Data for rabbit liver cytochrome *P*-450 2B4 with benzphetamine as a substrate are taken from [16]. The values of the equilibrium constants and ΔG were recalculated for 25 °C from ΔH and ΔS given in the original article. 1 e.u. = 1 cal · mol^{-1} · K^{-1}.

	K_h		K^S_h		K^I_d		K^h_d	
	3A4	2B4	3A4	2B4	3A4	2B4	3A4	2B4
Equilibrium constant at 25 °C	0.48	0.10	1.9	0.47	4.5 μM	350 μM	0.80 μM	44 μM
ΔH (kJ · mol^{-1})	40.6	44.4	13.4	38.0	−33.0	−31.4	−12.6	−26.4
ΔS (J · mol^{-1} · K^{-1})	130	130	50.2	121	−213	−171	−159	−172
ΔS (e.u.)	31	31	12	29	−51	−41	−38	−41
ΔG at 25 °C (kJ · mol^{-1})	1.84	5.64	−1.57	1.90	30.5	19.7	34.8	24.9

sition of the substrate complex of 3A4 in microsomes has ΔH and ΔS values that are both lower than the corresponding parameters found by Ristau et al. for 2B4 in solution (Table 1). Our recent results on the thermodynamics of 2B4 spin transitions in microsomes and proteoliposomes show that this difference reflects some important distinction between these two cytochromes as it is retained even on incorporation of 2B4 into microsomal or proteoliposomal membrane (E. V. Apletalina and D. R. Darydov, unpublished work). Substrate binding seems to perturb haem–protein interactions in 3A4, in contrast with 2B4, where the effect of substrate binding on the thermodynamic parameters of spin transitions was insignificant.

According to the theory used by Ristau et al. the excess of the entropy change over the expected entropy of the spin shift itself [approx. 2 e.u. (cal · mol^{-1} · K^{-1}; 1 cal ≈ 4.18 J)] results from the decrease in the number of the haem protein contacts. Each broken contact gives a contribution to the overall entropy change of approx. 2–3 e.u. [16]. That gives 10–15 contacts for substrate-free 3A4 as well as for both substrate-free and benzphetamine-bound 2B4. However, following the same speculation, the transition from low spin to high spin in the 3A4–bromokriptine complex is associated with the disappearance of only five haem protein contacts. Thus, in contrast with benzphetamine binding to 2B4, binding of the substrate to 3A4 results in profound changes in protein–haem interactions, substantially favouring dissociation of the fifth haem ligand and the transition from low spin to high spin. This is reflected in the fact that the binding of bromokriptine to 3A4 induces a very prominent increase in the high-spin fraction of the haemoprotein (from 12 % in the substrate-free to approx. 70 % in the bromokriptine-saturated state at 20 °C). This effect is much higher than that found for benzphetamine binding to 2B4 (from 7 % to approx. 30 % at 20 °C).

In the low-spin state both cytochromes have similar values of both ΔH and ΔS of interactions with substrate. In contrast, in the high-spin state of the haemoproteins, the value of ΔH for the interaction of 3A4 with bromokriptine is less than half that for benzphetamine binding to 2B4 in solution. However, this difference disappears when 2B4 is incorporated into proteoliposomes containing cytochrome b_5 (E. V. Apletalina and D. R. Davydov, unpublished work). Thus it reflects the effect of the membrane and/or cytochrome b_5, rather than any dissimilarity of the haemoproteins.

In summary, we consider the proposed strategy to be very useful in the analysis of the content of the high-spin, low-spin and *P*-420 states of cytochromes *P*-450. It allowed us to resolve spin shift and inactivation transitions into two independent components. The method is also applicable to turbid systems (microsomes and proteoliposomes) containing at least 1 μM of *P*-450 even in the presence of other haemoproteins (such as cytochrome b_5). The strategy allowed us to obtain a set of spectral standards of the pure *P*-450$_{hs}$, *P*-450$_{ls}$ and *P*-420 states of the rabbit cytochrome *P*-450 2B4 (LM2); these were applicable to human cytochrome *P*-450 3A4. These results could serve as a basis for further detailed physico-chemical studies of *P*-450 spin transitions, substrate binding and protein–protein interactions in yeast microsomes containing human cytochrome 3A4.

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