BINDING BEHAVIOUR OF SUBSTRATE ANALOGOUS SPIN-LABELLED *n*-ALKYLAMINES IN LIVER MICROSOMAL CYTOCHROME *P*-450

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1. Introduction

The binding affinity of alkylamines (type II substrates) to cytochrome *P*-450 from liver microsomes increases with elongation of the alkyl side chain [1]. These investigations revealed that hydrophobic interactions determine the binding properties not only of type I substrates but also of type II substrates. By spin labelling of such a homologous series it is possible to obtain detailed structural information about the local environment of the bound substrates. Whereas in [1] only the optically active binding of the substrates was analysed, it is possible by the use of spin labelled substrates, moreover, to determine the bound as well as the free substrate molecules in titration experiments. Although type II substrate analogous spin labels as SL-metyrapone bound to bacterial [2] as well as to liver microsomal cytochrome *P*-450 [3] were studied, the number of binding sites was not determined.

In this study a homologous series of SL-*n*-alkylamines with varying length of the alkyl side chain (m = 1, 2, 7, 10) [4] (see fig.1) was investigated with the aim to get local structural data of bound substrates of different chain length and to determine the number of binding sites in microsomal cytochrome *P*-450.

The following pertinent findings were shown: The SL-*n*-alkylamine with m = 10 was rigidly bound to *P*-450 indicating a well accessible large binding area.

Abbreviations: SL, spin labelled; *P*-450, partially purified solubilized cytochrome *P*-450 from liver microsomes; LM 2, electrophoretically homogeneous cytochrome *P*-450 from liver microsomes; EPR, electron paramagnetic resonance

With SL-*n*-decylamine the number of substrate binding sites n = 6 was determined for the first time in the solubilized cytochrome *P*-450 system. Depending on the length of bound substrates new low spin ferric signals appear and specific broadening of the spin label spectra occurs caused by the neighbourhood of the paramagnetic Fe^{3+}. The binding affinity of SL-*n*-alkylamines (m = 2 and 10) to LM 2 is less by one order of magnitude as in the case of solubilized *P*-450.

2. Materials and methods

Partially purified rabbit cytochrome *P*-450 was prepared according to the method of Lu et al. [5]. The cytochrome solutions usually containing 66 μM cytochrome *P*-450, 5.5 μM cytochrome *P*-420 and 2.5 μM cytochrome b_5 at a protein concentration of about 11 mg/ml were concentrated by ultrafiltration under nitrogen using Amicon XM-50 membranes. Electrophoretically homogeneous cytochrome *P*-450 was prepared from liver microsomes of phenobarbital-pretreated male rabbits. The purification procedure was a slight modification [6] of the method of Coon [7,8]. Protein was determined by the method of Lowry et al. [9] with ovalbumin as standard. The concentration of cytochrome *P*-450 and cytochrome *P*-420 were calculated by the method of Omura and Sato [10] from CO difference spectra. The cytochrome b_5 content was assayed from the difference spectra of NADH-reduced and oxidized forms [11].

Optical and EPR measurements were carried out with cytochrome *P*-450 in 0.1 M phosphate buffer, pH 7.45, containing 20% glycerol.

Optical absorption spectra were recorded with a Beckman Acta CV spectrophotometer at 25 ± 0.2°C in 1.0 cm cuvettes. The spectral dissociation constants (K_S) were calculated from Eadie plots.

EPR experiments were performed using a Varian E 3 spectrometer with a flat aqueous solution cell at 25 ± 0.1°C. The double integration and the subtractions of EPR signals were carried out by means of a computer (KRS 4200, VEB Robotron, Dresden, GDR) connected on-line with the EPR spectrometer [12,13]. The effect of substrate on the low spin EPR signal of cytochrome *P*-450 was measured at −190°C. The dissociation constants and the number of binding sites from the EPR data were calculated according to Scatchard [14]. Rotational correlation time of bound spin label was calculated by sucrose extrapolation [15] from the shift of outer extrema [16].

Scatchard and Eadie plots were evaluated using a computer BESM-6 (USSR). The program was based on the assumption of two classes of binding constants.

3. Results

By means of optical difference spectroscopy the complexes of *P*-450 with SL-*n*-alkylamines were found to exhibit the same absorption characteristics (maxima; minima) in the Soret region and also two apparent binding constants as the unlabelled *n*-alkylamines [1]. The differences in the K_S values at varying chain length of the SL-*n*-alkylamines, however, are less compared with the unlabelled derivatives [1]. The shortest SL-derivative (m = 1) shows the highest affinity. In order to analyse the nature of the second K_S value also the electrophoretically homogeneous LM-2 fraction was used and the binding constants for SL-*n*-alkylamines (m = 2; 10) were determined. In this case only one binding constant was obtained with an affinity one order of magnitude less than with the solubilized *P*-450 (table 1).

In the presence of *P*-450 the SL-*n*-alkylamines show EPR spectra characteristic for mean binding affinity which consist of overlapping spectra of both bound (ES) and free (S) substrate molecules being in equilibrium with each other (fig.1). For the calculation of the K_S values from the EPR spectra *P*-450 was titrated by adding increasing amounts of substrate. The line shape of the spectrum of the bound SL-substrate at different stages of titration remains constant. At different length of the alkyl side chain, however, the shape of the EPR spectrum is changed. For m = 1 the bound part in the EPR spectrum is strongly

Table 1
Spectral data for complexes of cytochrome *P*-450 with spin labelled *n*-alkylamines (see fig.1)

Substrate (m)		λ_{max} (nm)	λ_{min} (nm)	K_{S1} (mM)	K_{S2} (mM)
1	A	432	411	0.02	1.6
2	A	433	411	0.11	3.9
	B	434	413	0.96	–
7	A	432	411	1.0	7.9
10	A	432	411	0.09	1.6
	B	433	413	0.99	–

(A) partially purified cytochrome *P*-450 ($4 \cdot 10^{-6}$ M per haem)
(B) electrophoretically homogeneous fraction LM 2 ($3.4 \cdot 10^{-6}$ M.per haem)

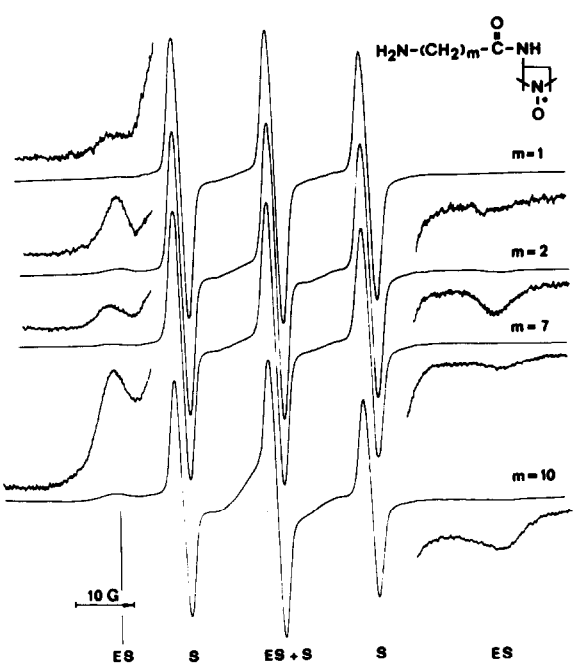

Fig.1. EPR spectra of spin labelled *n*-alkylamines after binding to *P*-450. ([E] $4.3 \cdot 10^{-4}$ M per haem; [S_0] $1 \cdot 10^{-4}$ M; microwave power, 20 mW; modulation, 4 G; T, 25°C.)

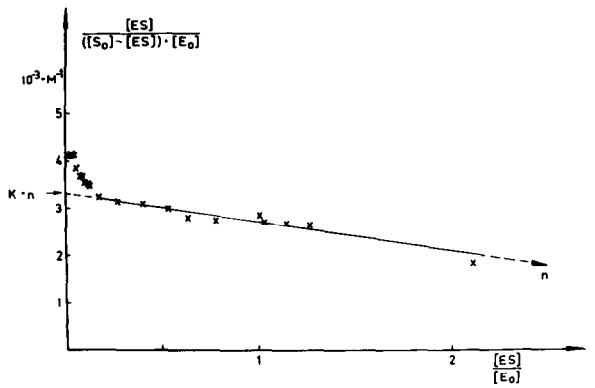

Fig.2. Scatchard plot of EPR data of spin labelled *n*-decylamine bound to *P*-450.

broadened which can be explained by dipole–dipole interactions between the iron and the NO-group (fig.1). From the Scatchard plot of EPR titration data two different binding affinities were obtained for the substrate $m = 10$. A K_{S2} value of 1.85 mM was calculated from the flat part of the curve to which $n_2 = 5.8 \pm 1.0$ binding sites per haem correspond (fig.2). The other K_S value was also calculated ($K_{S1} = 0.015$ mM, $n_1 =$ 0.02); however, these data are less accurate because of the very low concentrations of bound substrate.

The rotational correlation time of the bound label was determined for compound $m = 10$. The shift of the outer extrema of the spectrum extrapolated to infinite viscosity [15,16] is very small (0.5 G) indicating that $\tau_r > 300$ ns. Assuming a globular *P*-450 and a rigid attaching of the label an approx. wt 500 000 can be estimated.

In order to prove the influence of the substrate binding on the haem iron complex EPR spectra of *P*-450 were measured in the presence of substrates at low temperature. Depending on chain length of the SL-*n*-alkylamines new types of haem iron low spin signals occur which can be titrated by addition of substrates. New signals appear for $m = 1$ at $g = 2.047$ and 2.101 but for $m = 2$ and 10 at $g = 1.895$ and 2.521 (fig.3).

4. Discussion

Using SL-substrates EPR was applied to follow directly the binding of substrates to *P*-450 thus differentiating between free and bound molecules.

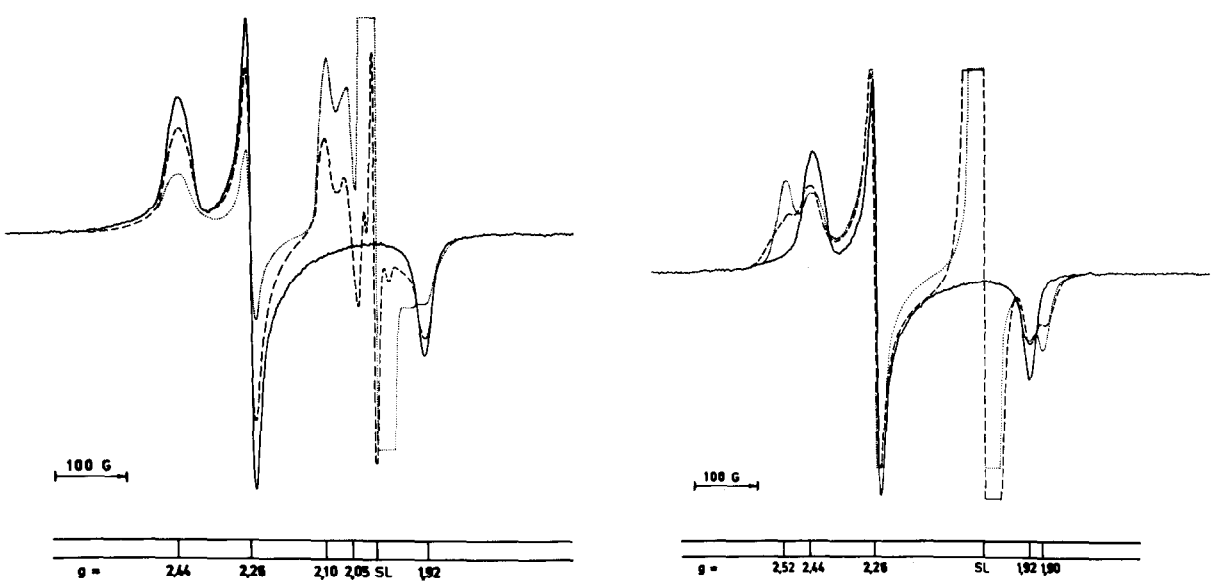

Fig.3. EPR spectra of ferric low spin *P*-450 in presence of spin labelled *n*-alkylamines. (Microwave power, 20 mW; modulation, 12.5 G; *T*, −190°C.) (A) – (————) without substrate; (– – – –) $m = 1$, $[S_0]$ $2.5 \cdot 10^{-4}$ M; (· · · · · · · ·) $m = 1$, $[S_0]$ $5 \cdot 10^{-3}$ M. (B) – (- - - - - - - -) $m = 2$, $[S_0]$ $5 \cdot 10^{-3}$ M; (· · · · · · · ·) $m = 10$, $[S_0]$ $5 \cdot 10^{-3}$ M.

From these data the binding constant and the number of binding sites were evaluated for *P*-450.

The existence of more than one binding site per haem iron gives rise to the question about the nature of these binding sites. From the appearance of new haem iron low spin signals in the presence of substrate it can be assumed that at least some of the six bound substrates are localized near the haem distorting the symmetry of the octahedral complex by conformational change in the *P*-450 molecule or by an exchange of one of the axial ligands. Moreover, in the case of SL-substrate $m = 1$ the observable magnetic interaction means that the binding site is indeed near to the haem iron*. The increasing binding affinity with elongation of unlabelled *n*-alkylamines is caused by hydrophobic interactions of the alkyl chain with the protein [1]. The smaller variation of the affinity of different SL-*n*-alkylamines as well as the low mobility of bound substrates at all chain length, however, indicates that the pyrrolidine group plays the main role for the non-covalent binding of SL-*n*-alkylamines at *P*-450.

In carbonic anhydrase using spin labelled sulfonamide inhibitors with different chain length a sharp increase of mobility occurs, if a distinct length of chain is exceeded indicating a cleft-like binding site [17]. In *P*-450, however, a rather different behaviour arises. The rigid binding also in the case of long chain SL-substrates suggests a well accessible large binding area, in which the bulky pyrrolidine group is stereochemically similar to large highly affine substrates [2,3] and therefore well adapted to the binding site at *P*-450. The assumption of a well accessible binding site at *P*-450 is further supported by earlier results [18].

The K_s values derived from the EPR spectra using the Scatchard plot (protein concentration: $4.3 \cdot 10^{-4}$ M) and from the optical spectra by use of the Eadie plot (protein concentration: $4 \cdot 10^{-6}$ M) are comparable (table 1). The accordance of binding constants determined by EPR and optical spectroscopy, respectively, suggests that six binding sites may exist at lower protein concentrations, too.

The comparison of the K_s values (table 1) binding affinity in partially purified *P*-450 and in the LM 2-fraction allows an insight into the role of lipids for the binding affinity. The lowered binding affinity may be caused by the removal of phospholipids in the LM 2-fraction. The disappearance of the second K_s value in the LM 2-fraction is possibly connected with the removal of isoenzymes.

*An estimation of distance between the haem iron and the NO-group in dependence on the chain length will be given in a following paper.

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