# SPIN-LABELLED ISOCYANIDES AS STEREOCHEMICAL PROBES FOR THE ACTIVE CENTER OF CYTOCHROME *P*-450

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Received 11 December 1978
Revised version received 12 March 1979

## 1. Introduction

Spin-labelled substrate analogues have been widely used to obtain a deeper insight into stereochemical properties of the active sites of enzymes [1,2]. In this way the spin-labelled inhibitor metyrapone [3,4] as well as spin-labelled alkylamines [5] have been used to study cytochrome *P*-450.

Both types of compounds are so-called type II substrates of *P*-450 which, it has been suggested, are bound to the heme iron as well as to hydrophobic binding sites [3,6]. For the isocyanides, being type II substrates, heme iron binding is also to be assumed. A spin-labelled isocyanide (4-isocyanide-1-oxyl-2,2,6,6-tetramethylpiperidine (I in fig.1)), has been studied [7,8] but with controversial results. The significant broadening of the ESR signal from this spin label bound to *P*-450 led to the suggestion of a very strong magnetic interaction between it and the ferric heme iron [8]. However, an unbroadened triplet signal and the absence of a spectral part with low mobility with the same label was observed [7] from which it was concluded that the binding affinity is rather low and that the spin label binds to a region more distant from the heme iron. In this study, therefore, two spin-labelled isocyanides with different chain lengths between the isocyanide and the spin-label group (I, II in fig.1) were used in order to decide between the controversial results and to get more information about the active site of *P*-450.

The results indicate that both isocyanides bind with high affinity to the active site of *P*-450. But only the label with the shorter distance between the isocyanide and the spin-label group shows a strong magnetic interaction of the spin-label group with the paramagnetic heme iron. This behaviour is reflected in the ESR spectrum of the label as well as in that of the heme iron which was also recorded.

In earlier investigations of the interaction of spin-labelled isocyanides with *P*-450, liver microsomes were used [7,8]. But comparable investigations of solubilized, partially purified *P*-450 and an electrophoretically homogeneous *P*-450, LM 2 [10] show that only LM 2 binds isocyanide II with a 1 : 1 stoichiometry as determined from a Scatchard plot.

*Abbreviations:* *P*-450, cytochrome *P*-450; solubilized *P*-450, solubilized, partially purified *P*-450 according to [9]; LM 2, electrophoretically homogeneous fraction of *P*-450 from liver microsomes of phenobarbital-induced male rabbits; ESR, electron spin resonance

## 2. Materials and methods

Partially purified *P*-450 from liver microsomes (phenobarbital induced male rabbits) was prepared by the methods in [9]. The initial *P*-450 solutions (40–60 μM *P*-450 at ~7–10 mg protein/ml) contained ~10–15% cytochrome *P*-420 and ~5–10% cytochrome $b_5$. The solutions were concentrated at 4°C by ultrafiltration under nitrogen using Amicon XM-50 membranes up to 0.3–0.5 mM *P*-450 final conc. For details, see figure and table legends.

Electrophoretically homogeneous LM 2 was prepared from phenobarbital-induced rabbit liver microsomes, with slight modifications according to [10,11]. Protein was determined by the Lowry method [12] with ovalbumin as standard.

The concentration of *P*-450 and cytochrome *P*-420 were calculated from CO-difference spectra by the method in [13]. The cytochrome $b_5$ content was assayed from the NADH-reduced and oxidized forms [14].

The isocyanide groups of the spin-labels were synthesized by a general method from the respective amines, chloroform and sodium hydroxide. They were purified on a silica gel column using benzene as the eluent.

Optical and ESR measurements were carried out with *P*-450 in 0.1 M phosphate buffer (pH 7.45), containing 20% glycerol (v/v).

Optical absorption spectra were recorded using a Beckman Acta CV spectrophotometer at 298 ± 0.2 K in 1.0 cm cuvettes. The app. $K_s$-values were calculated from Eadie plots.

ESR experiments were performed using a Varian E3 spectrometer with a flat, aqueous-solution cell at 298 ± 0.1 K. The double integration and the subtractions of ESR signals were carried out using a computer (KRS 4200, GDR) connected on-line with the ESR spectrometer [15,16]. The effect of substrate on the low spin ESR signals of *P*-450 was measured at 77 K. The dissociation constants and the number of binding sites were calculated from the ESR data according to Scatchard [17]. Scatchard and Eadie plots were evaluated using the computer BESM-6 (USSR). The program used to calculate the nonlinear regression assumed two classes of binding constants.

## 3. Results

Binding of isocyanides I and II to solubilized *P*-450 results in optical difference spectra of type II with maxima and minima at 433 nm and 411 nm, respectively. The $K_s$-values for both are in the μM range indicating tight binding (see table 1).

The binding of both I and II to solubilized *P*-450 is characterized by two $K_s$-values differing one from another by a factor of 100 and 200, respectively. Compared with the solubilized *P*-450 the binding of each isocyanide to LM 2 gives one straight line in the Eadie plot indicating only one dissociation constant. These values are 4-fold higher than the $K_{s1}$-values for solubilized *P*-450 indicating a weaker binding of LM 2 towards these isocyanides. This difference in the $K_{s1}$-values may be explained by the heterogeneity of solubilized *P*-450 [3] as well as by the almost complete absence of phospholipids from LM 2, the presence of which lowers the $K_s$-value [18].

The relatively high values of $K_{s2}$ for both I and II and solubilized *P*-450 together with their disappearance for LM 2 means that additional unspecific binding sites are characterized by the $K_{s2}$-values.

The ESR spectra of both isocyanides in the presence of *P*-450 are shown in fig.1. The upper one represents the spectrum of I without a slow motion signal of the spin label. In the presence of *P*-450, however, the total spin concentration measured by double-integrated ESR spectra of I is lowered as compared to that in the absence of *P*-450, indicating that a part of isocyanide I is bound to the enzyme (fig.2). The signal of this part is broadened by the strong magnetic interaction of the spin-label group with the

Table 1
Optically determined apparent dissociation constants for complexes of *P*-450 with spin-labelled isocyanides ($E_o = 2 \times 10^{-6}$ M/heme)

| Isocyanide (fig.1) | *P*-450 | $\lambda_{max}$ | $\lambda_{min}$ | $K_{s1}$ (μM) | $K_{s2}$ (μM) | Maximal distance[a] > N–O · · · · –N=C (Å) |
|---|---|---|---|---|---|---|
| I | Solubilized | 433 | 411 | 1.8 | 150 | 6 |
| I | LM 2 | 433 | 412 | 7.8 | – | |
| II | Solubilized | 433 | 411 | 4.3 | 830 | 10 |
| II | LM 2 | 433 | 412 | 16 | – | |

[a] From Dreiding models

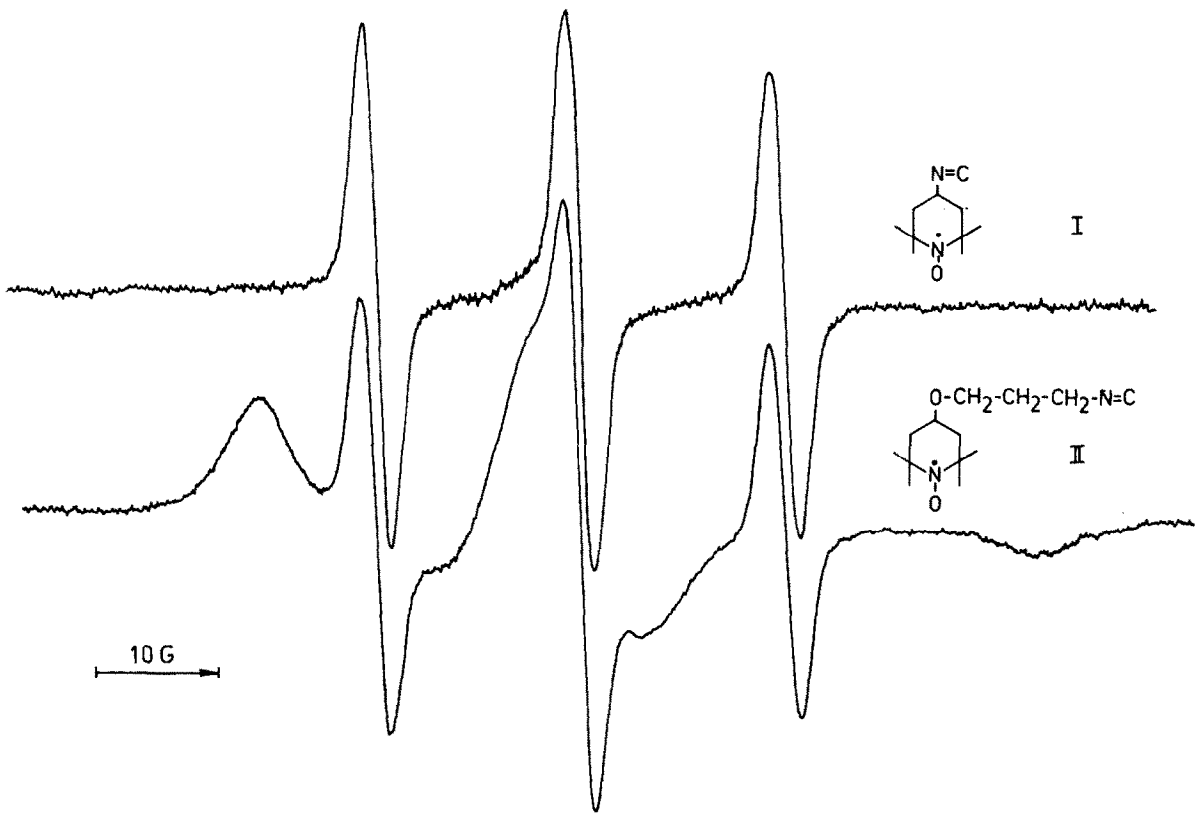


Fig.1. ESR spectra of spin-labelled isocyanides in the presence of solubilized *P*-450; $E_0 = 2 \times 10^{-4}$ M/heme; $S_0 = 5 \times 10^{-5}$ M; microwave power 18 mW; modulation 4 G; $T = 25°C$; gain $1.5 \times 10^5$ (I), $1 \times 10^5$ (II).

paramagnetic ferric heme iron. The amount of bound I was calculated from the difference between the signal for the unbound part in the presence of *P*-450 and the control value for I in buffer.

In contrast to I, the ESR spectrum of isocyanide II in the presence of *P*-450 exhibits a part of the spectrum due to immobilized spin label with distinct low and high field peaks (fig.1). From this spectrum the bound part of II can be determined directly by computer subtraction [15,16] showing that the total spin concentration (sum of bound and unbound II in the presence of *P*-450) is identical with the control value (i.e., in buffer solution) (fig.2).

From a series of titration experiments the spin concentrations of mobile and immobilized I and II were determined by means of computer separation of the two respective spectral parts.

On the supposition that the mobile part of the spectrum corresponds to the unbound, and the immobile part to the bound spin-labelled isocyanide the dissociation constant of the spin-labelled isocyanide–*P*-450 complex together with the number of binding sites were evaluated using a Scatchard plot. Analogous to the two classes of dissociation constants ($K_s$) determined by optical spectroscopy and also by ESR titration of solubilized *P*-450, two classes were obtained differing one from another by a factor of ~50 (I) and ~400 (II), respectively (table 2). The ESR-determined $K_s$ values roughly agree with the optically determined constants, indicating that the dissociation of the spin-labelled isocyanide–*P*-450 complex is nearly independent of the enzyme concentration (*P*-450 concentrations used: optical, $2.0 \times 10^{-6}$ M; ESR, $2.0 \times 10^{-4}$ M). The Scatchard plot revealed 0.35 binding sites with

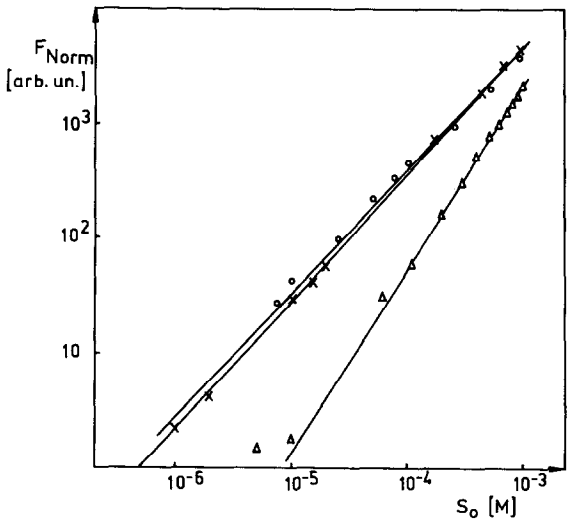


Fig.2. Total intensity (double integral) ($F_{Norm}$) of ESR spectra against added concentration of spin-labelled isocyanides ($S_0$): (×–×–×) I or II in buffer solution; (△–△–△) I with solubilized *P*-450 ($E_0$ = 2 × $10^{-4}$ M/heme); (○–○–○) II with solubilized *P*-450 ($E_0$ = 2 × $10^{-4}$ M/heme).

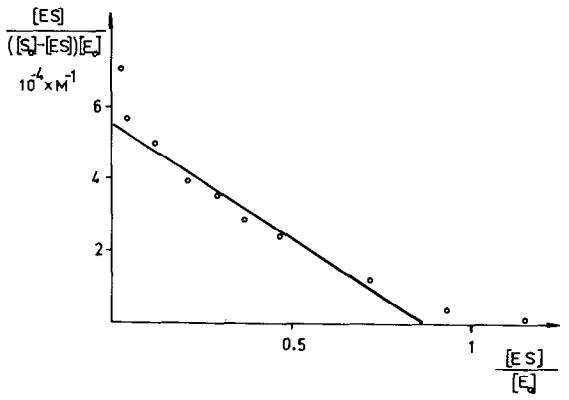


Fig.3. Scatchard plot from ESR data of isocyanide spin-label II bound to *P*-450 LM 2.

higher affinity for both I and II and values of 3 and 4 for I and II, respectively, for the binding site with lower affinity.

In order to elucidate the origin of the number of binding sites as well as of the two binding classes in the solubilized *P*-450 an ESR titration of LM 2 with isocyanide II was carried out. In accord with the optical results, the Scatchard plot (fig.3) of the ESR titration data of LM 2 revealed only one dissociation constant identical with the optically determined one, supporting the assumption that the heterogeneity of solubilized *P*-450 may be one reason for the observation of a binding class with less affinity. Moreover, the number of binding sites is shifted from an inconsistent value of 0.35 to nearly 1 (0.85 ∓ 0.13) (table 2) indicating a 1 : 1 stoichiometry of the binding of II to LM 2.

In order to prove the influence of spin-labelled isocyanide binding on the heme complex of *P*-450, the ESR spectra of the ferric heme iron of *P*-450 were measured at 77 K. In the presence of I a new low spin ferric heme iron spectrum with the following *g*-values appears: $g_1 = 2.19; g_2 = 2.08; g_3 = 1.97$. In the presence of II, however, only a small shift of the low spin *g*-values of the heme iron is observed: $g_1$ from 2.44–2.45; $g_2$ from 2.26–2.30; $g_3$ from 1.92–1.91 (fig.4).

Table 2
ESR determined apparent dissociation constants and binding sites for complexes of *P*-450 with spin-labelled isocyanides ($E_0$ = 2 × $10^{-4}$ M/heme, solubilized cytochrome *P*-450; $E_0$ = 2.2 × $10^{-4}$ M/heme, LM 2)

| Isocyanide (fig.1) | *P*-450 | $K_{s1}$ (μM) | $n_1$ | $K_{s2}$ (μM) | $n_2$ |
|---|---|---|---|---|---|
| I | Solubilized | 4.6 | 0.35 | 220 | 2.8 |
| II | Solubilized | 2.1 | 0.34 | 780 | 3.9 |
| II | LM 2 | 16 | 0.85 | – | – |

## 4. Discussion

Usually substrate binding to *P*-450 is analysed by means of substrate-induced spectral changes of the heme chromophore [6]. From this procedure, however, no conclusions can be drawn about the number of binding sites of the enzyme. On the other hand, the binding of spin-labelled substrates can be directly measured using the ESR method leading to the number of bound substrate molecules and to a better understanding of the stereochemical properties of the binding site. Utilizing the methodological advantages of com-

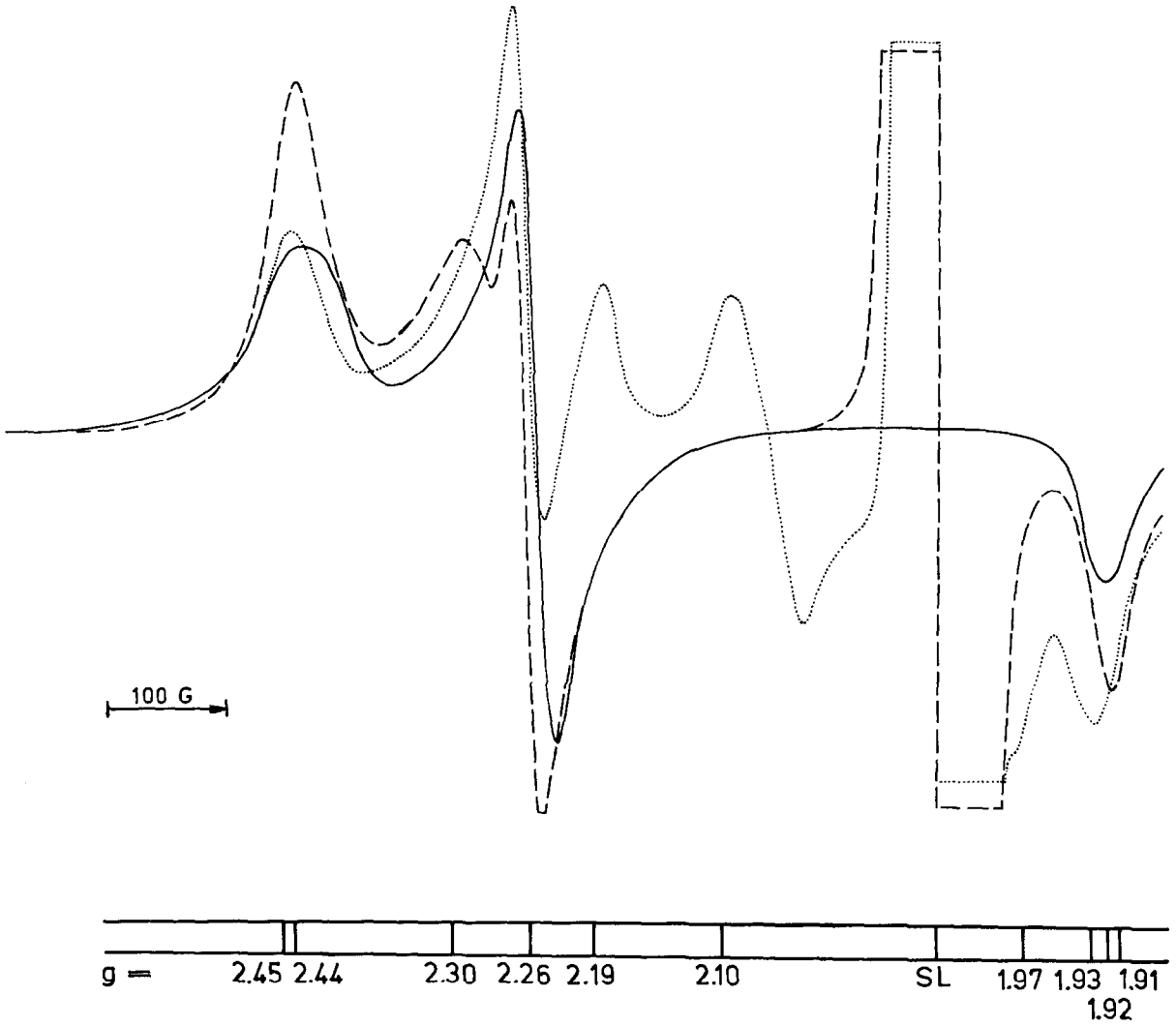


Fig.4. ESR spectra of the ferric heme iron of solubilized *P*-450 in the presence of spin-labelled isocyanides: (——) *without* substrate; (· · ·) I, 5.4 × $10^{-4}$ M; (– – –) II, 6.8 × $10^{-4}$ M; microwave power 18 mW; modulation 12.5 G; $T$ = −190°C; $E_o$ = 2 × $10^{-4}$ M/heme.

puterized evaluation of the spectra, differentiation between bound and unbound spin-labelled substrate is possible. This allows the use of a Scatchard plot from which 1 : 1 stoichiometry between LM 2 and isocyanide II was obtained. The structural similarity between both investigated isocyanides suggests they have the same binding site.

It is well known [19] that isocyanide derivatives are bound to ferrous iron in different hemoproteins. Moreover, isocyanide derivatives in the exceptional case of *P*-450 are bound also to ferric iron [20]. The reason for this may be seen in the better $\pi$-electron donor properties of the heme iron of *P*-450 caused by the axial mercaptide group occupying the 5th coordination site of the heme iron [21]. From these considerations it is suggested that the isocyanide group of both I and II are bound to the heme iron. Because of different distances between the spin-label

and the isocyanide group of both compounds (6 Å in I, 10 Å in II) the binding of the isocyanide group leads necessarily to different distances between the NO-group and the heme iron. Therefore, I can form strong dipolar interactions between the paramagnetic heme iron and the spin-label group resulting in an almost completely broadened signal of the bound part of I. On the other hand, the larger distance between the spin-label group and the heme iron of II does not permit such strong interactions thus leading to a well-pronounced signal of bound isocyanide II.

Assuming a binding of the isocyanide group to the ferric heme iron the change of the ESR spectra of the iron on the binding of the spin-labelled isocyanide is also explicable. The formation of a new spectrum of the ferric heme iron on the binding of I may be explained by intensive interactions between the spin-label group and the paramagnetic heme iron considering that the unpaired electron of the heme iron is delocalized into the porphyrin orbitals (in the myoglobin–cyanide complex this delocalization was determined to be ~25% [22]). Moreover, it is possible that the unpaired electron of the spin-label group of I may interact with the conjugated $\pi$-electron system of the porphyrin forming a $\pi$-complex (stacking). Further experiments to clarify the origin of the new low spin signals of the ferric heme iron in the presence of I are now under way.

On the other hand, the larger distance between the spin-label group and the heme iron of bound II does not induce such profound changes in the ESR spectrum of the heme iron of *P*-450. The only small changes of the *g*-values are explicable by the binding of the isocyanide group to the heme iron by which one of the axial ligands originating from the protein is displaced from the 6-coordinated heme iron. This obviously is not the mercaptide sulfur because the characteristic ligand field of the heme iron formed by this ligand [23] is maintained.

## Acknowledgements

The authors are indebted to Dr D. Schwarz and Dr O. Ristau from the Central Institute of Molecular Biology for the use of their computer programs for spin-label processing and nonlinear regression with two classes of binding constants and for valuable discussions.

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