^{1}H NMR T_1 relaxation rate study on substrate orientation of fluoromethylanilines in the active sites of microsomal and purified cytochromes P_{450} 1A1 and 2B1

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Abstract The present study describes ^{1}H NMR T_1 relaxation rate studies on fluoromethylanilines bound to the active sites of microsomal and purified cytochromes P_{450} 1A1 and 2B1. From the data obtained, insights into the average orientation of the substrates with respect to the paramagnetic Fe^{3+} centre in the cytochromes P_{450} could be derived. Particular attention was paid to a possible extra relaxation pathway for methyl protons compared to the aromatic protons, due to the rotational motion of the CH_3 around the σ-C-CH_3 bond. However, this effect appeared to be minimal and to result in at most a few percent underestimation of the actual distance of the methyl protons to the Fe^{3+} centre. Furthermore, the data obtained demonstrate that all aromatic protons are at about the same average distance from the paramagnetic centre. The results also demonstrate that the fluoromethylanilines are bound to the active sites of cytochromes P_{450} 1A1 and 2B1 in a similar way. A time-averaged orientation of the substrate with the Fe^{3+} above the aromatic ring, with the π-orbitals of the aromatic ring and those of the porphyrin rings in a parallel position, providing possibilities for energetically favourable π-π interaction defines the orientation which best fits the results of the present study. Possibilities for a flip-flop rotation around an axis in the plane of the aromatic ring can be included in this picture, as such rotations would still result in a similar average distance of all aromatic protons to the Fe^{3+} paramagnetic centre. The results obtained also indicate that possible differences in metabolite patterns resulting from conversion of the fluoromethylanilines by different cytochromes P_{450}, especially P_{450} 1A1 and 2B1, are unlikely to be caused by a specific orientation of the substrate imposed by the substrate binding site of the enzyme.

Key words: Cytochrome P_{450}; Fluoromethylaniline; ^{1}H NMR; T_1 relaxation measurement

1. Introduction

Several studies in the literature describe T_1 relaxation measurements investigating the manner of binding of substrates to the active sites of cytochromes P_{450} [1–5]. All of these studies report a stereoselective orientation of the substrate in the active site of the cytochrome P_{450}, or even a link between the observed specific way of binding of the substrate and the regioselectivity of its hydroxylation by different P_{450} isozymes. However, some investigations have reported conflicting results. For example, the stereoselective orientation of 4-hydroxyacetanilide with respect to the Fe^{3+} centre in cytochromes P_{450} 1A1 and 2B1 was indicated to be different, but the actual difference reported appeared to be opposite in independent studies [2,3]. Using ^{1}H NMR T_1 relaxation rate measurements, the acetamino group was reported to be closer to the Fe^{3+} in P_{450} 2B1 than in P_{450} 1A1 [2], whereas in a study using ^{15}N and ^{13}C T_1 relaxation rate measurements the opposite was concluded, and the nitrogen atom of the acetamido group was reported to be closer to the Fe^{3+} in cytochrome P_{450} 1A1 than in P_{450} 2B1 [3].

Furthermore, recent results with various halogenated benzene derivatives have provided evidence for the hypothesis that relatively small substrates become bound to the relatively aspecific and large active sites of the mammalian cytochromes P_{450} in such a way that the active site does not impose a stereoselective positioning of the substrate with respect to the (activated) metal centre. This conclusion follows from the observation that the regioselectivity of the aromatic hydroxylation of fluorobenzene derivatives and fluoroanilines did not vary significantly with change in the cytochrome P_{450} enzyme pattern catalysing the hydroxylation [6,7]. Instead, the regioselectivity of the aromatic hydroxylation of a series of fluorobenzenes and fluoroanilines could be explained and even predicted on the basis of the calculated reactivity of the various carbon centres for an electrophilic attack. Together, these results rather suggest an orientation of the aromatic substrate in the active site of the cytochromes P_{450} with all protons at equal avarage distances from the Fe^{3+} centre, providing no distance limitations to hydroxylations at specific positions on the basis of a protein-imposed stereoselective orientation of the substrate in the active site of the cytochromes P_{450}.

The objective of the present study was to gain greater insights into this question related to the presence or absence of a stereoselective positioning of relatively small substrates in the active sites of cytochromes P_{450}. Cytochromes P_{450} 1A1 and 2B1 were used as the P_{450} enzymes for the study. Fluoromethylanilines were chosen as the model substrates. These substrates were chosen for several reasons. First, insight into factors that determine the regioselectivity of the cytochrome P_{450} catalysed conversion of methylanilines is of importance because *N*-hydroxylation pathways lead to bioactivation and mutagenic metabolites, whereas aliphatic side chain methyl and aromatic ring hydroxylations lead to products that can be excreted upon phase II conjugation [8–12]. Second, the fluoromethylanilines are relatively small molecules. Third, the molecules contain an amino group that may be especially suitable for a possible specific orienting interaction with active site amino acids. Such an orienting interaction between a cytochrome P_{450} amino acid residue and an amino moiety of the substrate has for example been reported for cytochrome P_{450} debrisoquine 4-hydroxylase

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[13]. Fourth, the molecules contain a methyl substituent, which makes it possible to study whether the distances obtained for methyl protons, which have a possible additional relaxation pathway due to the rotation of the methyl group around the σ-C-CH_3 bond [14], can actually be compared to distances determined for aromatic protons which lack this additional relaxation pathway. In addition, preferential side-chain C-hydroxylation of the methyl moiety over aromatic ring hydroxylation has been reported for *p*-methylanilines and toluene [15–17], while for *o*-methylaniline the opposite is true [18]. Information on average proton distances for both the methyl and aromatic protons may provide an answer to the question of whether the preferential site for hydroxylation originates from a closer proximity of the respective hydrogens to the active Fe^{3+} centre of the cytochromes P_{450}. Finally, the substrates have a good water solubility making T_1 NMR relaxation measurements under saturating substrate condition possible.

2. Materials and methods

2.1. Chemicals

4-Fluoro-2-methylaniline, 3-fluoro-4-methylaniline and 3-fluoro-2-methylaniline were all obtained from Aldrich (Steinheim, Germany). 2-Fluoro-4-methylaniline was purchased from Acros Chimica (Geel, Belgium).

2.2. Protein preparations

Preparation of purified cytochromes P_{450} 1A1 and 2B1 as well as of microsomes was carried out essentially as previously described [19,20]. The amount of cytochrome P_{450} was measured according to the method of Omura and Sato [21]

2.3. ^1H NMR T_1 relaxation rate measurements

^1H NMR T_1 relaxation rate measurements were performed on a Bruker CXP 500 NMR spectrometer at 296.2 K. The longitudinal relaxation time (T_1) was determined by a 180°–90° inversion recovery method. Irradation of the water signal was employed to reduce the disturbance by this resonance. The delay time (τ) between the pulses for the samples containing purified or microsomal cytochromes P_{450} and for the samples containing only substrate was 0.1 and 0.2 s, respectively. About 12 τ values were applied and 32 scans for each τ value were recorded. Samples contained 0.1 M potassium phosphate pH 7.25 (containing 20% glycerol) or a solution of (microsomal) cytochrome P_{450} in this buffer (final concentration 1.26 μM cytochrome P_{450}), or 0.4 M potassium phosphate pH 7.4 (containing 20% glycerol) or a solution of purified cytochrome P_{450} in this buffer (final concentration 1.26 μM cytochrome P_{450}), 0.1 mM EDTA, 5 mM substrate added from a 0.25 M stock solution in dimethylsulfoxide and 1% D_2O for locking the magnetic field. The total sample volume was 0.511 ml. Samples were made oxygen free by four cycles of evacuation and filling with argon.

To correct for the diamagnetic contribution to the longitudinal relaxation time the T_1 of a blank containing CO-dithionite-reduced cytochrome P_{450} was determined.

The corrected T_1 value ($1/T_1$corr = $1/T_1$observed $-1/T_1$blank) was used to calculate the distance (r) of the respective proton to the para-

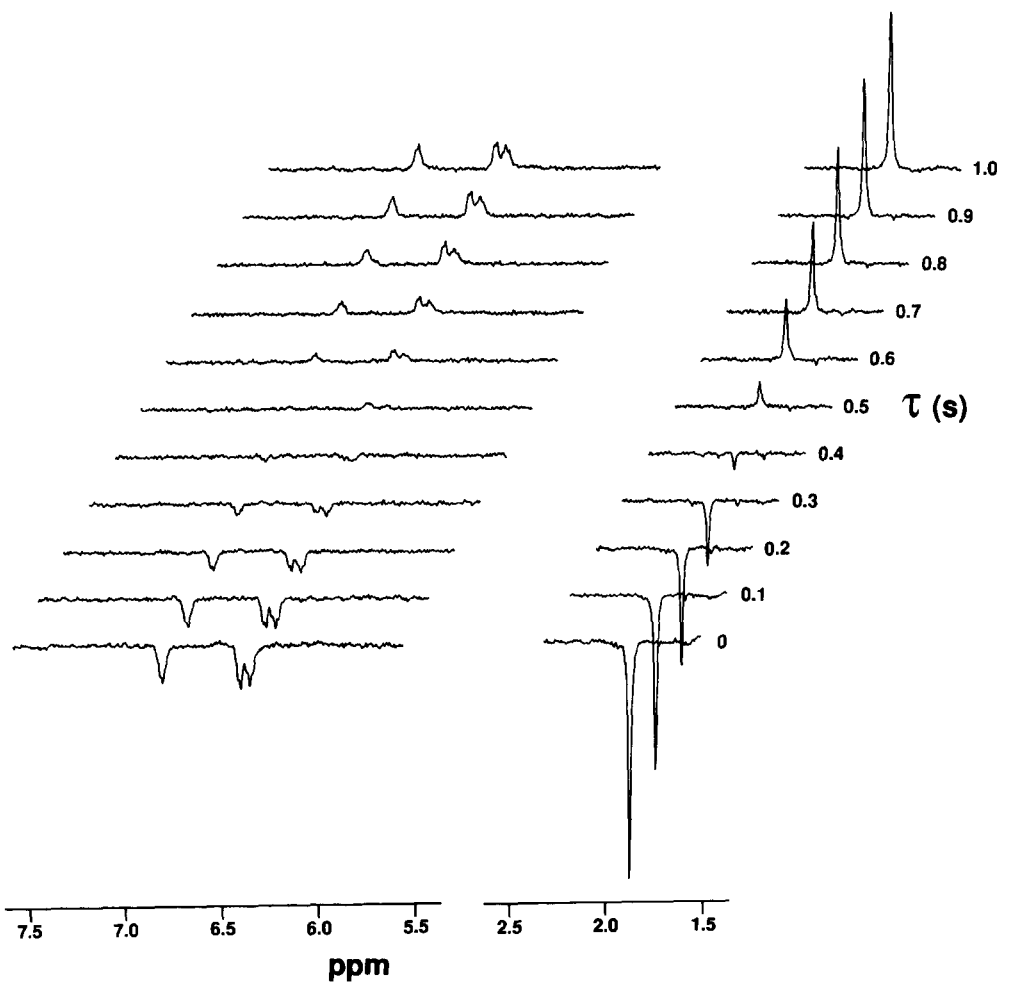

Fig. 1. ^1H NMR spectra showing the inversion recovery sequence (180°–τ–;90° pulse sequence) of 3-fluoro-2-methylaniline in the presence of microsomal cytochrome P_{450} derived from phenobarbital preteated rats. The water signal at 4.7 ppm has been omitted.

magnetic iron essentially as described by Novak and Vatsis [1], using the Solomon-Bloembergen equation, assuming the Fe^{3+} in both P_{450} 1A1 and 2B1 to be in the high spin conformation [1,22,23], and taking a τ_c value of 10^{-10}. This value for τ_c for cytochromes P_{450} may actually vary between 10^{-10} and 10^{-11} [24]. However, using a factor of 10^{-11} would result in values that are 68% of those obtained with a value of 10^{-10}. This changes the absolute values of the distances obtained by a constant factor, and does not influence relative differences obtained for protons in one substrate molecule bound to the active site of a specific cytochrome P_{450}.

2.4. Calculation of optimised fluoromethylaniline geometry

The optimised geometry for the various fluoromethylanilines was calculated on a Silicon Graphics Indigo^2 using Insight (Biosym, CA, USA). The semi-empirical molecular orbital method was used, applying the AM1 Hamiltonian from the AMPAC program.

3. Results

3.1. ^1H NMR T_1 relaxation measurements on fluoromethylanilines bound to β-naphthoflavone and phenobarbital induced microsomal cytochromes P_{450}

Previous ^1H NMR relaxation rate studies on the interaction of benzo(*a*)pyrene with microsomal cytochromes P_{450} clearly demonstrated that differences in the orientation of a substrate in the active sites of cytochromes P_{450} 1A1 and 2B1 can be detected in studies with β-naphthoflavone and phenobarbital induced microsomal preparations, respectively [5]. Thus, as a first approach, the binding of several fluoromethylanilines to cytochromes P_{450} was studied using β-naphthoflavone and phenobarbital induced microsomal preparations. The cytochrome P_{450} population in these microsomes contains 71 and 55% of cytochromes P_{450} 1A1 and 2B1, respectively [25].

Fig. 1 presents the results of a ^1H NMR T_1 relaxation measurement for 3-fluoro-2-methylaniline bound to phenobarbital induced microsomal cytochromes P_{450}. A plot of the natural logarithm of the peak at infinite delay time (M_∞) minus the peak area at the specific delay time τ (M_τ) against this delay time τ gives a straight line with a slope of $-1/T_1$, from which the T_1 relaxation time can be derived (Fig. 2). Tables 1 and 2 present the T_1 values thus obtained for the various substrate protons in the presence of microsomes from β-naphthoflavone and phenobarbital treated rats, respectively. T_1 values for the CO-reduced blank samples are also listed. From these T_1 and T_1 CO-blank values the distances of the respective protons to the Fe^{3+} centre in the active site of the microsomal cytochromes P_{450} were calculated, the results obtained also being presented in Tables 1 and 2. The distances of the various aromatic protons to the Fe^{3+} cytochrome P_{450} metal centre do not vary significantly for the aromatic protons of the various fluoromethylanilines. Based on these results as well as the calculated optimal geometry of the fluoromethylaniline molecules, the expected distance for the methyl protons to the Fe^{3+} centre can be calculated. This was carried out assuming the Fe^{3+} centre to be at the measured distance away from the aromatic protons and, thus, averaged in time above the centre of the aromatic ring.

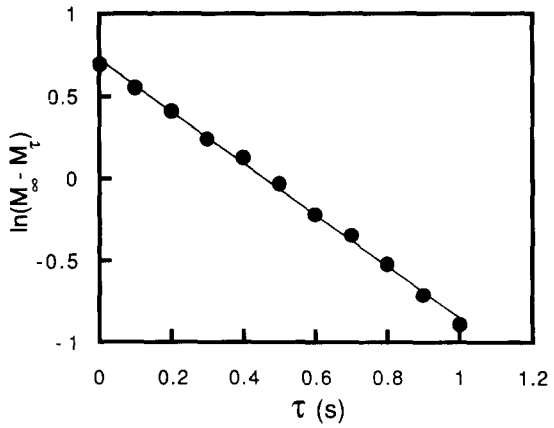

Fig. 2. Plot showing $\ln(M_\infty - M_\tau)$ plotted against τ for the H_4 proton of 3-fluoro-2-methylaniline in the presence of microsomal cytochrome P_{450} derived from phenobarbital preteated rats (Fig. 1). The slope of this curve equals $-1/T_1$.

Table 1
T_1 relaxation rates for protons of different fluoromethylanilines in a system containing microsomal cytochrome P_{450} from β-naphthoflavone pretreated rats, containing 71% P_{450} 1A1 [25] and in a CO-dithionite reduced P_{450} blank sample (T_1 CO-blank)

Substrate/proton	T_1 (s)	T_1 CO-blank (s)	r(Fe-H) (Å)	r(Fe-H_{methyl}) calculated (Å)
4-Fluoro-2-methylaniline				
H_3	0.834 ± 0.034	1.187 ± 0.062	6.27 ± 0.39	
H_5	0.824 ± 0.087	1.202 ± 0.018	6.20 ± 0.06	
H_6	0.759 ± 0.013	1.086 ± 0.012	6.16 ± 0.04	
H_{CH3}	0.704 ± 0.004	0.848 ± 0.126	6.69 ± 0.62	6.65 ± 0.06
3-Fluoro-2-methylaniline				
H_4	0.646 ± 0.065	0.908 ± 0.110	6.04 ± 0.08	
H_5	0.665 ± 0.120	0.927 ± 0.098	6.09 ± 0.70	
H_6	0.616 ± 0.074	0.846 ± 0.206	6.05 ± 0.07	
H_{CH3}	0.588 ± 0.019	7.727 ± 0.058	6.23 ± 0.21	6.51 ± 0.02
2-Fluoro-4-methylaniline				
H_3	0.689 ± 0.109	0.955 ± 0.038	6.14 ± 0.36	
$H_5 + H_6$	0.609 ± 0.053	0.830 ± 0.093	6.06 ± 0.15	
H_{CH3}	0.564 ± 0.047	0.704 ± 0.084	6.28 ± 0.19	6.55 ± 0.05
3-Fluoro-4-methylaniline				
H_2	0.482 ± 0.127	0.593 ± 0.067	6.18 ± 0.59	
$H_5 + H_6$	0.423 ± 0.001	0.561 ± 0.028	5.78 ± 0.08	
H_{CH3}	0.494 ± 0.033	0.597 ± 0.037	6.29 ± 0.07	6.44 ± 0.26

From these T_1 values the distances between the respective protons and the paramagnetic iron were calculated as described in section 2.

Table 2
T_1 relaxation rates for protons of different fluoromethylanilines in a system containing microsomal cytochrome P_{450} from phenobarbital pretreated rats, containing 55% P_{450} 2B1 [25] and in a CO-dithionite reduced P_{450} blank sample (T_1 CO-blank)

Substrate/proton	T_1 (s)	T_1 CO-blank (s)	r(Fe-H) (Å)	r(Fe-H_{methyl}) calculated (Å)
4-Fluoro-2-methylaniline				
H_3	0.766 ± 0.033	1.100 ± 0.118	6.16 ± 0.26	
H_5	0.718 ± 0.035	1.033 ± 0.093	6.09 ± 0.30	
H_6	0.638 ± 0.004	0.944 ± 0.060	5.91 ± 0.10	
H_{CH3}	0.651 ± 0.014	0.838 ± 0.070	6.31 ± 0.04	6.50 ± 0.12
3-Fluoro-2-methylaniline				
H_4	0.516 ± 0.106	0.958 ± 0.099	5.38 ± 0.25	
H_5	0.542 ± 0.164	0.893 ± 0.087	5.57 ± 0.55	
H_6	0.429 ± 0.064	0.794 ± 0.155	5.22 ± 0.19	
H_{CH3}	0.462 ± 0.096	0.743 ± 0.030	5.46 ± 0.34	5.89 ± 0.16
2-Fluoro-4-methylaniline				
H_3	0.706 ± 0.152	0.941 ± 0.061	6.28 ± 0.47	
$H_5 + H_6$	0.646 ± 0.114	0.849 ± 0.081	6.23 ± 0.44	
H_{CH3}	0.581 ± 0.025	0.706 ± 0.052	6.44 ± 0.07	6.69 ± 0.03
3-Fluoro-4-methylaniline				
H_2	0.398 ± 0.033	0.706 ± 0.035	5.20 ± 0.12	
$H_5 + H_6$	0.395 ± 0.007	0.691 ± 0.022	5.21 ± 0.06	
H_{CH3}	0.440 ± 0.064	0.664 ± 0.010	5.52 ± 0.31	5.72 ± 0.01

From these T_1 values the distances between the respective protons and the paramagnetic iron were calculated as described in section 2.

Comparison of the distances thus calculated and those actually observed demonstrates that the observed values are generally 96.5 ± 2.2% (n = 8) of those calculated. This implies that, although the T_1 relaxation rate of the methyl protons in a fluoromethylaniline is generally faster than that of the aromatic protons, this effect is also observed in the blank and is independent of the presence of a paramagnetic centre. Thus, the distances obtained for the methyl protons can be well compared to those determined for the aromatic protons.

Furthermore, comparison of the results presented in Tables 1 and 2 indicates that there are no significant differences between the distances observed with the two microsomal cytochrome P_{450} preparations, suggesting that the average way of binding and, more precisely, the average distance of the protons of a given substrate to the paramagnetic Fe^{3+} in the active site of the various cytochromes P_{450} are comparable.

3.2. *^{1}H NMR T_1 relaxation measurements on 4-fluoro-2-methylaniline and 2-fluoro-4-methylaniline bound to purified cytochromes P_{450} 1A1 and 2B1*

To further support the observations obtained with the β-naphthoflavone and phenobarbital induced microsomal cytochromes P_{450}, a ^{1}H NMR T_1 relaxation rate study was performed with purified cytochromes P_{450} 1A1 and 2B1 using two

Table 3
T_1 relaxation rates for 4-fluoro-2-methylaniline and 2-fluoro-4-methylaniline in a system containing purified cytochrome P_{450} 1A1 or 2B1 and in a CO-dithionite reduced P_{450} blank sample (T_1 CO-blank)

Substrate/proton	T_1 (s)	T_1 CO-blank (s)	r(Fe-H) (Å)	r(Fe-H_{methyl}) calculated (Å)
4-Fluoro-2-methylaniline/cytochrome P450 1A1				
H_3	1.638 ± 0.057	2.004 ± 0.034	7.61 ± 0.25	
H_5	1.649 ± 0.026	2.089 ± 0.078	7.44 ± 0.27	
H_6	1.424 ± 0.058	1.987 ± 0.389	6.91 ± 0.57	
H_{CH3}	1.084 ± 0.051	1.243 ± 0.007	7.54 ± 0.23	7.70 ± 0.35
4-Fluoro-2-methylaniline/cytochrome P450 2B1				
H_3	2.033 ± 0.155	2.858 ± 0.226	7.31 ± 0.81	
H_5	2.077 ± 0.176	2.926 ± 0.264	7.33 ± 0.74	
H_6	1.549 ± 0.436	2.694 ± 0.426	6.55 ± 0.47	
H_{CH3}	1.197 ± 0.137	1.465 ± 0.036	7.22 ± 0.40	7.45 ± 0.42
2-Fluoro-4-methylaniline/cytochrome P450 1A1				
H_3	1.191 ± 0.150	1.448 ± 0.090	7.25 ± 0.19	
$H_5 + H_6$	1.089 ± 0.133	1.305 ± 0.093	7.23 ± 0.23	
H_{CH3}	0.896 ± 0.103	1.008 ± 0.108	7.48 ± 0.42	7.62 ± 0.01
2-Fluoro-4-methylaniline/cytochrome P450 2B1				
H_3	1.718 ± 0.011	2.118 ± 0.030	7.63 ± 0.04	
$H_5 + H_6$	1.468 ± 0.039	1.814 ± 0.030	7.38 ± 0.18	
H_{CH3}	1.122 ± 0.038	1.220 ± 0.075	7.77 ± 0.39	7.87 ± 0.17

From these T_1 values the distances between the respective protons and the paramagnetic iron were calculated as described in section 2.

of the fluoromethylanilines as the substrate. The results obtained are presented in Table 3. Comparison of the results obtained with those listed in Tables 1 and 2 for the microsomal cytochromes P_{450} demonstrates that the average distances obtained with the purified enzymes are generally somewhat (17.7 ± 3.6%) longer than those observed with the microsomal cytochromes P_{450}, however, the overall effects observed are similar. No significant differences are observed in the distances between the aromatic protons and the Fe^{3+} in the active site of the two enzymes.

4. Discussion

The ^1H NMR T_1 relaxation rate studies on fluoromethylanilines bound to the active sites of microsomal and purified cytochromes P_{450} 1A1 and 2B1 were used to investigate the presence or absence of a preferential orientation of the substrates in the active site of the cytochromes P_{450}. Such a specific orientation would be reflected in different distances for the various protons in the substrates. An increased avarage distance of specific protons in the substrate molecule might result in an influence on the regioselectivity upon subsequent biotransformation to hydroxylated products. It should be mentioned that the actual orientation of the substrate bound to the Fe^{3+} resting state of the cytochrome P_{450} could be different from that of the substrate in the activated high-valent iron-oxo porphyrin form actually performing the hydroxylation step, as reported for thiocamphor bound to cytochrome P_{450cam} [26]. However, an interaction between an active site amino acid and, for example the amino moiety of the substrate, could very well persist upon conversion of the Fe^{3+} form to the high-valent iron-oxo form of the enzyme and, thus, influence the regioselectivity of the hydroxylation by an effect on the actual distance of the possible reaction sites from the active metal centre. Such an influence of substrate orientation on the regioselectivity of the reaction imposed by the active site of the cytochrome P_{450} has been reported, for example, for amino-containing substrates converted by cytochrome P_{450} debrisoquine 4-hydroxylase [13], but have also been suggested for the conversion of, e.g., 4-hydroxyacetanilide by cytochrome P_{450} 1A1 or 2B1 [2,3]. However, from the data obtained in the present study for the binding of fluoromethylanilines to the active site of microsomal and purified cytochromes P_{450} 1A1 and 2B1, it follows that all aromatic protons are at about the same average distance from the paramagnetic centre. The results also demonstrate that the fluoromethylanilines are bound to the active sites of cytochromes P_{450} 1A1 and 2B1 in a similar way. Thus, the binding characteristics of the relatively small fluoromethylanilines to the active sites of cytochrome P_{450} 1A1 and 2B1 provide no indications for a stereoselective orientation that may influence the regioselectivity of the oxidation.

Special attention was paid to the average distance obtained from the T_1 relaxation measurements for the methyl protons in the fluoromethylanilines. This was done for two reasons, first, the existence of an extra relaxation pathway for methyl protons compared to aromatic protons, due to a rotational motion of the CH_3 around the σ-C-CH_3 bond, might hamper the comparison of distances obtained for aromatic protons to those determined for methyl protons. Second, preferential side-chain C-hydroxylation of the methyl moiety over aromatic ring hydroxylation has been reported for *p*-methylanilines. Information on average proton distances for both the methyl and aromatic protons provides an answer to the question of whether this preferential side chain C-hydroxylation originates from a closer proximity of the methyl than of the aromatic protons to the active Fe^{3+} centre of the cytochromes P_{450}. Clearly, the results of the present study provide support for the methyl protons having higher T_1 relaxation rates than the aromatic protons, but the same effect is observed in the T_1 relaxation rates of the CO-reduced blanks, resulting in calculated average distances for the methyl protons that can be reliably compared to distances obtained for aromatic protons. The actual distances observed indicate that the methyl protons are not closer to the Fe^{3+} paramagnetic centre than the aromatic protons, providing no basis for the easier hydroxylation of the *p*-methylanilines at the methyl group than in the aromatic ring.

Taken together, the results obtained support a time-averaged orientation of the substrate in the active site in which the location of the Fe^{3+} is above the aromatic ring, with the π-orbitals of the aromatic ring and the π-orbitals of the porphyrin rings in a parallel position providing possibilities for energetically favourable π-π interaction. Possibilities for a flip-flop rotation around an axis in the plane of the aromatic ring can be included in this picture, as such rotations would still result in a similar average distance of all aromatic protons to the Fe^{3+} paramagnetic centre.

Furthermore, the results obtained in the present study support the conclusion that possible differences in metabolite patterns resulting from conversion of the fluoromethylanilines by different cytochromes P_{450}, especially P_{450} 1A1 and 2B1, and also the preferential sites for hydroxylation of the various sites in fluoromethylanilines, are unlikely to be defined by a specific orientation of the substrate, imposed by the substrate binding site of the cytochromes P_{450}. This conclusion supports previous conclusions from studies on regioselectivity of aromatic hydroxylation, from which it emerged that chemical reactivity instead of stereoselective binding imposed by the amino acids of the active site of the cytochromes P_{450} is the major determinant in setting the outcomes of the regioselectivity of the hydroxylation of relatively small benzene derivatives by cytochromes P_{450} [6,7].

References

[1] Novak, R.F. and Vatsis, K.P. (1982) Mol. Pharmacol. 21, 701–709.
[2] Van de Straat, R., De, Vries, J., De Boer, H.J.R., Vromans, R.M. and Vermeulen, N.P.E., (1987) Xenobiotica 17, 1–9.
[3] Myers, T.G., Thummel, K.E., Kalhorn, T.F. and Nelson, S.D. (1994) J. Med. Chem. 37, 860–867.
[4] Crull, G.B., Kennington, J.W., Garber, A.R., Ellis, P.D. and Dawson, J.H. (1989) J. Biol. Chem. 264, 2649–2655.
[5] Castro-Maderal, L. and Sullivan, P.D. (1992) FEBS Lett. 296, 249–253.
[6] Cnubben, N.H.P., Vervoort, J., Veeger, C. and Rietjens, I.M.C.M. (1992) Chem.-Biol. Interact. 85, 151–172.
[7] Rietjens, I.M.C.M., Soffers, A.E.M.F., Veeger, C. and Vervoort, J. (1993) Biochemistry 32, 4801–4812.
[8] Zimmer, D., Mazurek, J., Petzold, G. and Bhuyan, B.K. (1980) Mut. Res. 77, 317–326.
[9] Yoshimi, N., Sugie, S., Iwata, H., Niwa, K., Mori, H., Hashida, C. and Shimizu, H. (1988) Mut. Res. 206, 183–191.
[10] Arhenius, E. (1969/70) Chem.-Biol. Interact.1, 361–380.
[11] Kiese, M. (1963) Arch. Exp. Pathol. Pharmakol. 244, 387–404.
[12] Pai, V., Bloomfield, S.F., Jones, J. and Gorrod, J.W. (1978) in:

Biological Oxidation of Nitrogen (Gorrod, J.W., Ed.), Elsevier/North-Holland, Amsterdam.
[13] Wolff, T., Distlerath, L.M., Worthington, M.T., Groopman, J.D., Hammons, G.J., Kadbular, F.F., Prough, R.A., Martin, M.V. and Guengerich, F.P. (1985) Cancer Res. 45, 2116–2122.
[14] Kalk, A. and Berendsen, H.J.C. (1976) J. Magn. Reson. 24, 343–366.
[15] Boeren, S., Tyrakowska, B., Vervoort, J., Hoffman, E., De Teunis, K., Veldhuizen, A. and Rietjens, I.M.C.M. (1992) Xenobiotica 22, 1403–1423.
[16] Tyrakowska, B., Boeren, S., Geurtsen, B. and Rietjens, I.M.C.M. (1993) Drug Metab. Dispos. 21, 508–519.
[17] Hanzlik, R.P. and Ling, K.-H. J. (1990) J. Org. Chem. 55, 3992–3997.
[18] Son, O.S., Everett, D.W. and Fiala, E.S. (1980) Xenobiotica 10, 457–468.
[19] Rietjens, I.M.C.M. and Vervoort, J. (1989) Xenobiotica 19, 1297–1305.
[20] Balvers, W.G., Boersma, M.G., Veeger, C. and Rietjens, I.M.C.M. (1992) Biochim. Biophys. Acta 1117, 179–187.
[21] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378.
[22] Mildvan, A.S. and Gupta, R.K. (1978) Methods Enzymol. 49, 322–359.
[23] Wolf, C.R. and Oesch, F. (1983) Biochem. Biophys. Res. Commun. 111, 504–511.
[24] Bertini, I., Banci, L. and Luchinat, C. (1989) Methods Enzymol. 177, 246–263.
[25] Thomas, P.E., Reik, L.M., Ryan, D.E. and Levin, W. (1983) J. Biol. Chem. 258, 4590–4598.
[26] Paulsen, M.D. and Ornstein, R.L. (1993) Protein Eng. 6, 359–365.