

# Resonance Raman study of the cytochrome P-450 LM2-halothane intermediate complex

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Resonance Raman (RR) and absorption spectroscopic studies of purified rabbit liver cytochromes P-450 show that the form 2 isomer (LM2) but not the form 4 isomer (LM4) forms a long-lived complex with halothane after dithionite reduction, absorbing light at 470 nm, in which ferric 6-coordinated heme iron in the low-spin configuration is liganded to 2-chloro-1,1-difluoroethylene. The RR data exclude the possibility that the  $\text{CF}_3\text{CHCl}^-$  carbanion is a ligand and are consistent with the involvement of an active-site pocket in the cytochrome P-450 polypeptide.

Cytochrome P-450; Biotransformation; Resonance Raman spectroscopy; Halothane; 2-Chlorodifluoroethylene

## 1. INTRODUCTION

Many chemical substances form catalysis-dependent complexes with cytochrome P-450 (reviews [1,2]) detectable by special absorbances between 440 and 480 nm. This diagnostic feature is in many cases the only structural parameter of these complexes that is known and is used to define their structure by comparison with porphyrin model complexes. More is known about the structure of such 'intermediate complexes' originating in the course of reductive dehalogenation of halocarbons [3,4], in particular of a halothane-derived complex which exhibits unique stability in comparison with other intermediate complexes of cytochrome P-450 with halocarbons (e.g.  $\text{CCl}_4$ )

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*Abbreviations:* RR, resonance Raman; LM2, cytochrome P-450 form 2; LM4, cytochrome P-450 form 4; (P-450)<sub>red</sub>, reduced cytochrome P-450; (P-450-Hal)<sub>ox</sub>, oxidized cytochrome P-450-halothane complex; (P-450-Hal)<sub>red</sub>, reduced cytochrome P-450-halothane complex

[5], and which has been regarded as a complex between ferric 6-coordinated heme iron in the low-spin configuration and the  $\text{CF}_3\text{CHCl}^-$  carbanion [6]. It was assumed that this complex degrades via  $\beta$ -elimination to form 2-chloro-1,1-difluoroethylene, a well known product of halothane in vivo and in vitro [6-8].

In this study on the structure of the halothane complex, we have used resonance Raman (RR) spectroscopy which can provide unambiguous information on the oxidation, spin and ligation state of the heme iron [9]. This technique and the use of purified isoenzymes 2 (LM2) and 4 (LM4) of cytochrome P-450 contribute to the elucidation of the enzymatic process of the reductive branch of halothane biotransformation. Previous studies have shown that the halothane complex forms readily with the major phenobarbital-inducible isoenzyme of cytochrome P-450 LM2 [5,10] which is consistent with the fact that this isozymic form also generates the greatest amount of 2-chloro-1,1-difluoroethylene when incubated with halothane [11]. The present results exclude the possibility of a carbanion complex and suggest that 2-chloro-1,1-difluoroethylene is the complexing ligand.

## 2. MATERIALS AND METHODS

P-450 isoenzymes LM2 (13.5 nmol/mg) and LM4 (15.2 nmol/mg) were purified from liver microsomes of phenobarbital-treated rabbits [12] and solubilized in Emulgen 913 (Kao Chemicals, Tokyo). All samples contained 20% (v/v) glycerol, 0.2% Emulgen 913 and 150 mM potassium phosphate buffer (pH 7.3). The P-450-halothane complex (P-450-Hal) was prepared by adding a halothane-saturated glycerol-buffer solution (0.4 ml) to a suspension of P-450 (0.4 ml,  $10^{-5}$  M). Reduction of P-450-Hal was achieved by adding a small amount of dithionite (Merck, Darmstadt) to the solution. Halothane was bought from Ayerst Labs (New York).

Absorption spectra were recorded with a Shimadzu UV240 spectrometer. RR spectra were excited with the 406 and 476 nm lines of a  $Kr^+$  and  $Ar^+$  laser, respectively, with a power of 30 mW or less at the sample. The RR signals were accumulated by repetitive scanning. Details of the equipment are described in [13]. The spectral band width was  $4\text{ cm}^{-1}$ . The samples were deposited in a rotating cuvette in order to minimize thermal heating or photodegradation.

Before and after RR experiments the integrity of the sample was checked by the absorption spectra.

## 3. RESULTS

Excitation of the RR spectrum at 406 nm in the Soret transition of cytochrome P-450 LM2 (see fig.1A) predominantly enhances the totally symmetric stretching vibrations of the porphyrin macrocycle [9]. The strongest RR band in the frequency range displayed in fig.2A is  $\nu_4$  at  $1371\text{ cm}^{-1}$  which is known to be sensitive to the oxidation state of the heme iron. The observed frequency is typical for a ferric heme while upon reduction this band shifts down significantly. The frequencies of most of the bands above  $1400\text{ cm}^{-1}$  have been correlated with the core size of the porphyrin, i.e. the center-to-pyrrole-nitrogen distance  $d(C_1-N)$ . These are the bands at  $1500\text{ cm}^{-1}$  ( $\nu_3$ ),  $1553\text{ cm}^{-1}$  ( $\nu_{38}$ ),  $1562\text{ cm}^{-1}$  ( $\nu_{11}$ ),  $1580\text{ cm}^{-1}$  ( $\nu_2$ ),  $1602\text{ cm}^{-1}$  ( $\nu_{37}$ ), and  $1635\text{ cm}^{-1}$  ( $\nu_{10}$ ) [9,14].

Since  $d(C_1-N)$  is determined by the oxidation, spin and ligation state, the frequencies of these bands can be used to identify the electronic configuration of the central metal ion (spin state marker bands). The comparison with the characteristic values for different spin and ligation states (table 1) clearly shows that cytochrome P-450 LM2 (oxidized) is in the six-coordinated low-spin (6cLS) configuration. There is no evidence of even a small contribution from a high-spin (HS) configuration which should give rise to bands at down-shifted frequencies (see table 1).

Binding of halothane to cytochrome P-450 LM2

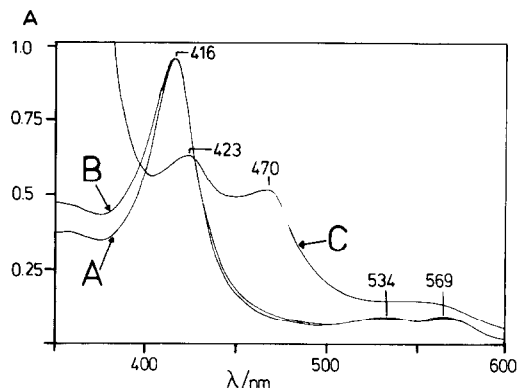


Fig.1. Absolute absorption spectra of cytochrome P-450<sub>ox</sub> LM2 (A), after binding of halothane (B) and after reduction of the cytochrome P-450 LM2-halothane complex with dithionite (C).

(P-450<sub>ox</sub>-Hal) induces a slight blue shift in the Soret absorption band (fig.1) as is observed for type-I substrate binding [15]. This is due to the partial population of the five-coordinated high-spin (5cHS) state. The RR spectrum of this complex (fig.3A) does not reveal significant differences from that of the substrate-free enzyme (fig.2A). However, minor spectral changes can be visualized by a difference spectrum (spectrum in fig.3A - spectrum in fig.2A) which is displayed in fig.2B. Here it can be seen that upon halothane binding, bands near  $1491$ ,  $1574$ ,  $1597$  and  $1628\text{ cm}^{-1}$  have appeared at the expense of those near  $1501$ ,  $1582$ ,  $1606$  and  $1638\text{ cm}^{-1}$ , respectively. The positive peaks in the difference spectrum correspond to the marker bands  $\nu_3$ ,  $\nu_2$ ,  $\nu_{37}$  and  $\nu_{10}$  of the 5cHS state (table 1), reflecting a small change of the spin state equilibrium [16].

Dithionite reduction of the halothane-bound cytochrome P-450 LM2 produces significant changes in the absorption spectrum (fig.1C). The Soret band has split into two components at 423 and 470 nm of nearly equal oscillator strength. The RR spectrum of this complex (P-450-Hal)<sub>red</sub> compared to P-450<sub>ox</sub>-Hal excited at 406 nm (fig.3B) exhibits most conspicuously a general decrease in absolute RR intensity and a particular lowering of the relative intensity of  $\nu_4$  ( $1371\text{ cm}^{-1}$ ) with respect to the other bands (fig.3A). This can easily be understood, since at Soret band excitation the RR intensity of the totally symmetric modes, in particular of  $\nu_4$ , is determined by the oscillator strength [17] which is much lower for the 423 nm

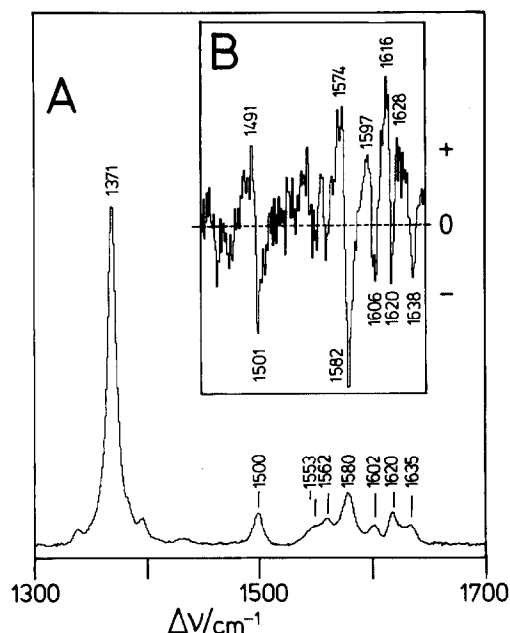


Fig.2. RR spectrum of cytochrome P-450<sub>ox</sub> LM2 (19  $\mu$ M) excited at 406 nm (A). The inset (B) displays the difference between the RR spectrum of cytochrome P-450<sub>ox</sub> LM2 after and before halothane binding (fig.3A-panel A, this figure).

band of (P-450-Hal)<sub>red</sub> than for the Soret band of P-450<sub>ox</sub>-Hal (see fig.1). The frequency of  $\nu_4$  (1372  $\text{cm}^{-1}$ ) is not very different from that of the oxidized P-450-Hal complex, indicating that the ox-

idation state of the heme iron ( $\text{Fe}^{3+}$ ) is preserved in this complex. This interpretation is substantiated by a comparison with the values of  $\nu_4$  of reduced protoporphyrin complexes (1358  $\text{cm}^{-1}$ ) [14] or of the reduced, substrate-free, cytochrome P-450 LM2 (1341  $\text{cm}^{-1}$ ), which are shifted to much lower frequencies (table 1). Also, the spin state marker bands  $\nu_3$  (1501  $\text{cm}^{-1}$ ),  $\nu_{11}$  (1564  $\text{cm}^{-1}$ ),  $\nu_2$  (1579  $\text{cm}^{-1}$ ),  $\nu_{37}$  (1604  $\text{cm}^{-1}$ ), and  $\nu_{10}$  (1635  $\text{cm}^{-1}$ ) appear at positions which unambiguously point to a ferric 6cLS configuration. It should be pointed out that most of the frequencies are higher than in P-450<sub>ox</sub>, indicating replacement of the original axial ligand in the 6th position.

During the RR experiment a slow growing-in of a band near 1360  $\text{cm}^{-1}$  was observed which was accompanied by an intensity decrease of the 1372  $\text{cm}^{-1}$  band. Based on the absorption spectra (CO complex) the intensity of the 1360  $\text{cm}^{-1}$  band could be correlated with the amount of P-450<sub>red</sub> which was formed during the RR experiment. Based on comparative studies with different laser powers, excitation wavelengths and dithionite concentrations, it was concluded that conversion to P-420 was induced by primary or secondary products of the photodecomposed dithionite.

Excitation of the RR spectrum of (P-450-Hal)<sub>red</sub> in the low-energy component of the Soret band at 476 nm gives a similar spectrum (not shown). However, due to the strong background, it was on-

Table 1

Frequencies ( $\text{cm}^{-1}$ ) of the oxidation and spin state marker bands of various states of P-450 and iron protoporphyrin model compounds<sup>a</sup>

Mode	P-450 <sub>ox</sub>	$\text{Fe}^{3+}\text{PP}(\text{ImH})_2^b$ (6cLS)	P-450 <sub>ox</sub> -Hal <sup>c</sup>	$\text{Fe}^{3+}\text{PPCl}^b$ (5cHS)	(P-450-Hal) <sub>red</sub>	(P-450-O <sub>2</sub> ) <sub>red</sub> <sup>d</sup>	P-450 <sub>red</sub> <sup>e</sup>
$\nu_4$	1371	1373	1370	1373	1372	1376	1341
$\nu_3$	1500	1502	1491	1491	1501	1503	—
$\nu_{38}$	1553	1554	1555	1533	1549	1553	—
$\nu_{11}$	1562	1562	1564	1553	1564	1565	1559
$\nu_2$	1580	1579	1574	1570	1579	1588	1586
$\nu_{37}$	1602	1602	1597	1591	1604	—	—
$\nu_{10}$	1635	1640	1628	1626	1635	1639	1606

<sup>a</sup> The subscripts ox and red refer to the oxidation state of the heme iron ( $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ ); 6cLS, six-coordinated low-spin; 5cHS, five-coordinated high-spin

<sup>b</sup> Data for the bis(imidazole) complex ( $\text{Fe}^{3+}\text{PPIMH}_2$ ) and the  $\mu$ -oxo dimer ( $\text{Fe}^{3+}\text{PPCl}$ ) of iron protoporphyrin IX are adopted from [13]

<sup>c</sup> Bands of the high-spin component of the oxidized cytochrome P-450 LM-halothane complex (P-450<sub>ox</sub>-Hal) as derived from the difference spectrum in fig. 2A

<sup>d</sup> Data for the reduced oxo complex of cytochrome P-450-CAM adopted from [21].

<sup>e</sup> From [19]

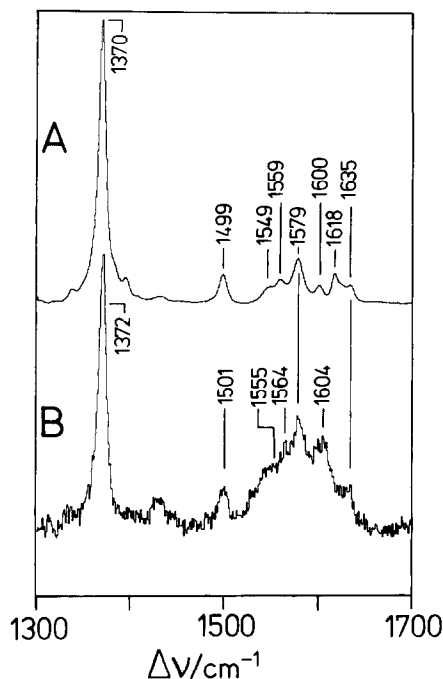


Fig.3. RR spectra of the cytochrome P-450 LM2-haloethane complex before (A) and after dithionite reduction (B) excited at 406 nm.

ly possible to identify the strongest band at 1372  $\text{cm}^{-1}$  ( $\nu_4$ ).

The haloethane-derived complex or a type-I-binding spectrum of cytochrome P-450 LM4 could not be detected in another series of absorption measurements.

#### 4. DISCUSSION

Both absorption as well as RR spectroscopic measurements demonstrate type I binding of haloethane to oxidized cytochrome P-450 LM2. As in the case of benzphetamine, the classic type I substrate, the concomitant shift of the spin state equilibrium from the 6cLS to 5cHS configuration is very small for LM2 solubilized in Emulgen 913 [16].

The complex formed from haloethane upon dithionite as well as enzymic reduction was formerly assumed, on the basis of comparative EPR and absorption spectroscopic studies of model complexes and liver microsomes, to be a 6cLS complex of heme ferric iron with the carbanion  $\text{CF}_3\text{CHCl}^-$  and thiolate as opposing axial ligands [6]. Thereby,

the previous suggestions [4] of an  $\text{Fe}^{2+}\text{-CHCF}_3$  (carbene) complex could be ruled out.

Our RR data confirm that the heme iron is in the 6cLS configuration, however, they can be reconciled with neither the carbene nor the carbanion complex. The frequency of the RR band  $\nu_4$ , whose main contributions are the  $\text{C}_a\text{-N}$  stretching vibrations, is highly sensitive to delocalization of the electrons in the porphyrin macrocycle [18]. The  $\text{C}_a\text{-N}$  stretching force constants become weaker and thus the frequency of  $\nu_4$  decreases by increasing the electron density in the antibonding  $\pi$ -orbitals of the porphyrin via back-donation from the  $d_\pi$ -orbitals of the central metal ion. Since back-donation is stronger in  $\text{Fe}^{2+}$  than in  $\text{Fe}^{3+}$ ,  $\nu_4$  shifts down upon reduction by about 10–15  $\text{cm}^{-1}$ . For example,  $\nu_4$  is at 1373  $\text{cm}^{-1}$  in methemoglobin ( $\text{Fe}^{3+}$ ) but at 1358  $\text{cm}^{-1}$  in deoxyhemoglobin ( $\text{Fe}^{2+}$ ). This effect can be significantly enhanced when one axial ligand exhibits high basicity like thiolate [18,19] which in  $(\text{P-450})_{\text{red}}$  lowers  $\nu_4$  to 1341  $\text{cm}^{-1}$ . In  $(\text{P-450-Hal})_{\text{red}}$ , however,  $\nu_4$  is at 1373  $\text{cm}^{-1}$  which is even higher than in  $(\text{P-450})_{\text{ox}}$ . Thus, the possibility of a ferrous carbene can be unambiguously ruled out. Also, the assignment to the ferric carbanion complex [20] is highly unlikely. The coordination of the heme iron by two strong electron donors (thiolate, carbanion) should lead to a significant decrease in the  $\nu_4$  frequency. Thus, one must conclude that the second axial ligand in  $(\text{P-450-Hal})_{\text{red}}$  is an electron acceptor. This conclusion is in line with the RR data on the carbon monoxide [19] or dioxygen [20] complexes of the reduced P-450, which both reveal a frequency upshift of  $\nu_4$  by 25–35  $\text{cm}^{-1}$  compared to  $(\text{P-450})_{\text{red}}$ . Both ligands possess empty  $\pi^*$ -orbitals which can accept electron density competing with the  $\pi^*$ -orbitals of the porphyrin. Thus, the respective frequencies of  $\nu_4$  are found to be as high as 1366 and 1376  $\text{cm}^{-1}$ . It is important to point out that a split Soret band, which had been taken as the main evidence for thiolate-carbanion ligation, is also found in the absorption spectrum of the  $(\text{P-450-Hal})_{\text{red}}$  as well as the CO complex of  $(\text{P-450})_{\text{red}}$  [22]. This implies that the electronic spectrum of the  $(\text{P-450-Hal})_{\text{red}}$  complex is also in line with a coordination configuration including a strong electron donor (thiolate) and electron acceptor.

At present, we are unable to identify unambiguously the second axial ligand. However,

among the intermediates which are possibly formed during reduction of halothane, there is no candidate which might serve as an electron acceptor except the final product,  $\text{CF}_2=\text{CHCl}$ , itself. Therefore, we tentatively assign the 2nd axial ligand to this olefin (fig.4). As in the case of  $\text{O}_2$  or  $\text{CO}$ , electron density may be transferred into the empty  $\pi^*$ -orbital of this molecule so that  $\nu_4$  increases compared to  $(\text{P-450-Hal})_{\text{ox}}$ . Ligation of chlorodifluoroethylene instead of a carbanion provides a better explanation for the stability of the complex and the failure to detect  $\text{CH}_3\text{ClHD}$  during reductive dehalogenation of halothane in the presence of  $\text{D}_2\text{O}$  [23] (see fig.4). This may imply that defluorination, favored by hyperconjugation of the  $\text{C-F}\sigma$  bond [24], takes place during transfer of a second electron. As further evidence that reduction is complete, it has been reported that, once formed, the complex is sufficiently stable to allow the removal of all substrate and reductant. Following this, if the complex is disrupted, 2-chloro-1,1-difluoroethylene is obtained as the major product [5].

Formation of the  $(\text{P-450-Hal})_{\text{red}}$  complex is a unique property of LM2. Attempts to detect this complex in LM4 failed, indicating that only the

specific protein environment of the heme in LM2 satisfies the structural requirements for the formation and stability of this complex. This may be achieved due to nestling of the  $\text{CF}_2$  group in the  $\text{O}_2$ -binding site, which may form a shape in cytochrome P-450 LM2 similar to the pocket observed in cytochrome P-450-CAM by X-ray crystallography [25]. In this way,  $\text{CF}_2=\text{CHCl}$  may be fixed in a specific orientation with respect to the porphyrin leading to an electronic interaction with the heme iron. It may be difficult to synthesize model compounds which feature this structural specificity.

It was not possible to detect the  $(\text{P-450-Hal})_{\text{red}}$  complex upon direct addition of  $\text{CF}_2=\text{CHCl}$  to  $(\text{P-450})_{\text{red}}$ . This may be explained as follows: only  $\text{CF}_2=\text{CHCl}$  originating in situ would be able to induce and use this favorable protein conformation near the heme iron and not external  $\text{CF}_2=\text{CHCl}$  intruding through the cleft amenable to substrate access.

While there has been some speculation regarding the possibility that a radical and a carbanion may be formed as cytotoxic intermediates in the reductive metabolism of halothane, these studies of the halothane complex offer a more complete picture;

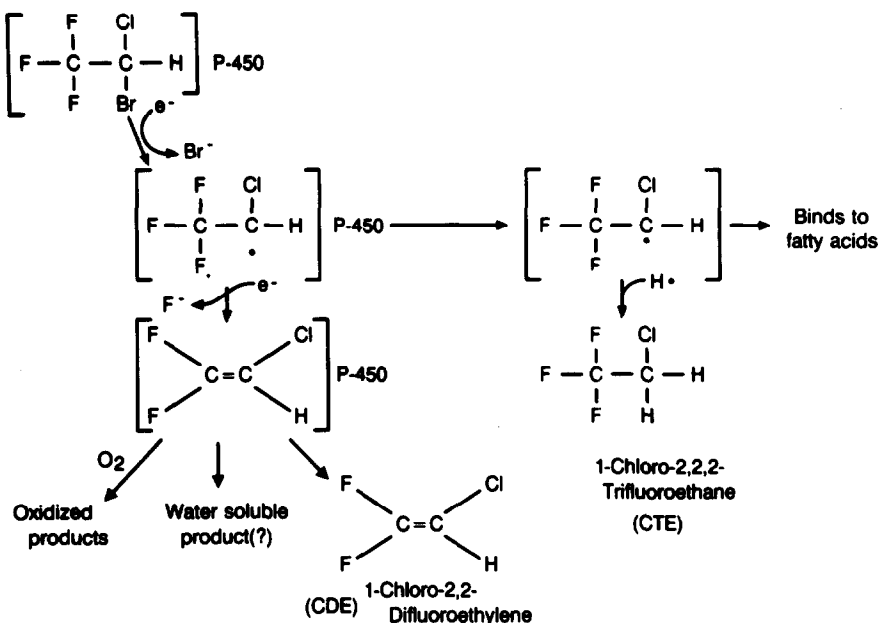


Fig.4. Formation and breakdown of the postulated cytochrome P-450 LM2-halothane intermediate complex.

they indicate that the production of the carbanion as a toxic intermediate is highly unlikely (see fig.4). Furthermore, as the formation of the complex appears to be a unique feature of phenobarbital-induced forms of the cytochrome corresponding to cytochrome P-450-LM2, it is tempting to suppose an involvement of this complex in the liver-toxic effects of halothane seen only in the phenobarbital-induced male hypoxic rat.

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