Formation of a Ferric Carbanion Complex from Halothane and Cytochrome P-450: Electron Spin Resonance, Electronic Spectra, and Model Complexes†

Hans Heinrich Ruf,* Hansjuergen Ahr, Wolfgang Nastainczyk, Volker Ullrich,‡ Daniel Mansuy, Jean-Paul Battioni, Regino Montiel-Montoya,§ and Alfred Trautwein§

ABSTRACT: The spectral intermediate complex formed upon reduction of halothane, $CF_3CHClBr$, by microsomal cytochrome P-450 with a Soret band at 470 nm exhibited a characteristic electron spin resonance (ESR) spectrum at g = 2.71, 2.27, and 1.80, indicating a low-spin ferric heme complex. Iron(III) porphyrin model complexes, $[Fe(TPP)(CF_3CHCl)(RS)]^-$, having both the carbanion CF_3CHCl^- and thiolate RS^- as axial ligands, showed hyperporphyrin spectra as well as ESR signals and ligand field parameters similar to those of the halothane-derived complex of cytochrome P-450. Hence, the spectral intermediate was assigned to a complex of ferric cytochrome P-450 with the 2,2,2-trifluoro-1-chloroethane carbanion, CF_3CHCl^-, derived from halothane by reductive debromination. The stability of such a complex with an iron–carbon bond at cytochrome P-450 is discussed.

Liver microsomal cytochrome P-450 can catalyze the reductive metabolism of many halogenated aliphatic hydrocarbons (Diaz Gomez et al., 1973; Uehleke et al., 1973a; Mansuy et al., 1974; Cox et al., 1976; Wolf et al., 1977; Ahr et al., 1982; Nastainczyk et al., 1982). During these reactions, spectral intermediates of cytochrome P-450 with red-shifted Soret bands between 450 and 475 nm are formed (Reiner & Uehleke, 1971; Uehleke et al., 1973b), which have been explained as complexes of this hemoprotein with an iron–carbon bond (Mansuy et al., 1974; Wolf et al., 1977). These complexes have been assigned either to ferrous carbene complexes, for instance, $[(P\text{-}450)Fe^{II}(CCl_2)]$ derived from CCl_4 (Ahr et al., 1980), or, tentatively, to a ferric carbanion complex $[(P\text{-}450)Fe^{III}(CHClCF_3)]$ derived from halothane, $CF_3CHClBr$ (Ahr et al., 1982). Usually, these assignments are based on the identification of metabolites and on the comparison of optical spectra with those of heme model complexes with known structure. The use of additional spectroscopic methods would make such assignments more conclusive. Furthermore, such complexes at cytochrome P-450 may shed light on its catalytic mechanism since oxene as well as superoxide anion complexes have been proposed as intermediates in the monooxygenation cycle of cytochrome P-450 (Ullrich et al., 1982; White & Coon, 1980). In addition, the reductive metabolism of polyhalogenated compounds seems to be responsible for the observed hepatotoxicity of these chemicals (Recknagel & Glende, 1973; McLain et al., 1979;), and carbene or carbanion intermediates may play a potentiating or inhibitory role in this process (Ullrich et al., 1978; Ahr et al., 1980).

Carbon tetrachloride, the most widely studied hepatotoxin, produces a band at 460 nm under reducing conditions in liver microsomes (Reiner & Uehleke, 1971). This intermediate complex slowly releases CO, which is explained by hydrolysis of the carbene complex $[(P\text{-}450)Fe^{II}(CCl_2)]$ (Wolf et al., 1977; Ahr et al., 1980). The preparation of well-characterized model complexes of ferrous iron porphyrins with the dichlorocarbene as a ligand substantiates this assignment (Mansuy et al., 1977, 1978). These complexes are diamagnetic and therefore do not show ESR[1] signals.

The widely used inhalation anaesthetic halothane, $CF_3CHClBr$, forms a band at 470 nm in liver microsomes under reducing conditions (Uehleke et al., 1973b). In analogy to CCl_4 and by the use of the carbene precursor 1,1,1-trifluoro-2-diazoethane, CF_3CHN_2, which produces a similar band at 468 nm, the spectral intermediate from halothane was tentatively assigned to a ferrous cytochrome P-450 carbene complex (Mansuy et al., 1974). Doubt was cast on this assignment when the study of the products of reductive halothane metabolism revealed that the intermediate complex with the band at 470 nm releases the olefin CF_2CHCl, which is best explained as the product of a β-elimination of a ferric carbanion complex, $[(P\text{-}450)Fe^{III}(CHClCF_3)]$ (Ahr et al., 1982). A model complex, formed with Fe(TPP) and halothane under reducing conditions, can be isolated and shows properties different from the carbene model complex $[Fe^{II}(TPP)(CCl_2)]$. By elementary analysis, mass spectrometry, and ^1H NMR, this complex, derived from halothane, has been identified as the ferric carbanion complex $[Fe^{III}(TPP)(CHClCF_3)]$ (Mansuy & Battioni, 1982).

The reduction of benzyl halides by microsomal cytochrome P-450 produces the corresponding toluenes and exhibits spectral intermediates absorbing at 478 nm, which have also been assigned to ferric carbanion complexes $[(P\text{-}450)Fe^{III}(CH_2Ar)]$ (Mansuy & Fontecave, 1983). Similar complexes of heme proteins with a single iron(III)–carbon bond have been recently reported to be formed upon in situ oxidation of monosubstituted hydrazines. Iron(III)–phenyl complexes were formed upon oxidation of phenylhydrazine by hemoglobin or myoglobin (Kunze & Ortiz de Montellano, 1983), catalase (Ortiz de Montellano & Kerr, 1983), and cytochrome P-450

† From the Departments of Physiological Chemistry (H.H.R., H.A., W.N., and V.U.) and Applied Physics (R.M.-M. and A.T.), University of Saarland, D-6650 Homburg, Federal Republic of Germany, and the Laboratoire de Chimie de l'Ecole Normale Superieure (D.M. and J.P.B.), F-75321 Paris Cedex 05, France. *Received April 6, 1984.* This work was supported by the Deutsche Forschungsgemeinschaft, SFB 38. A preliminary report has been presented (Ullrich et al., 1982).

‡ Present address: Faculty of Biology, University of Constance, D-7750 Constance, FRG.

§ Present address: Institute of Physics, Medical School Luebeck, D-2400 Luebeck 1, FRG.

[1] Abbreviations: ESR, electron spin resonance; TPP, *meso*-tetraphenylporphyrin; PPIXDME, protoporphyrin IX dimethyl ester; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; DMF, *N*,*N*-dimethylformamide; halothane, 2-chloro-2-bromo-1,1,1-trifluoroethane; Mb, myoglobin; EDTA, ethylenediaminetetraacetic acid.

0006-2960/84/0423-5300$01.50/0

(Battioni et al., 1983a; Jonen et al., 1982). Analogous iron(III)–methyl complexes are formed upon oxidation of methylhydrazine by hemoglobin or myoglobin (Mansuy et al., 1982) and cytochrome P-450 (Battioni et al., 1983a). Most recently, the existence of an iron–carbon bond has been proven by the X-ray structure of the complex $[(Mb)Fe^{III}(C_6H_5)]$ formed by the interaction of phenylhydrazine with crystals of myoglobin (Ringe et al., 1984). Alkyliron porphyrins, as model complexes, have been obtained by the reaction of monosubstituted hydrazines with iron porphyrins (Battioni et al., 1983b).

Here we report further proofs on the ferric carbanion nature of the intermediate complex of cytochrome P-450 with halothane. We used ESR as a magnetic resonance method that can clearly differentiate between the valence states of heme iron, in particular between ferrous carbene and ferric carbanion complexes of cytochrome P-450 in spite of similar optical spectra, both of the hyperporphyrin type. We were able to detect an ESR signal in microsomes concomitant to the band at 470 nm and, by use of ferric porphyrin model complexes with thiolate and the carbanion CF_3CHCl^- as axial ligands, to assign the ESR signal to a cytochrome P-450 Fe(III)–$CHClCF_3$ complex. We further discuss factors that could stabilize this structure.

Materials and Methods

Preparation of Microsomes. Male Sprague-Dawley rats (100–150 g) were used after treatment with sodium phenobarbitone (80 mg/kg of body wt., ip, daily for 3 days) or Aroclor 1254 (500 mg/kg of body wt., ip, a single dose 4 days prior to use). Liver microsomes were prepared by differential centrifugation (Frommer et al., 1970). The protein content was determined by the biuret method (Gornall et al., 1949) and cytochrome P-450 by difference spectra (Omura & Sato, 1964).

Chemicals. Halothane (Fluothane) was kindly provided by ICI-Pharma, Plankstadt, FRG. The Fe(TPP) model complex of halothane was prepared as described (Mansuy & Battioni, 1982). Thiolates were prepared as described as sodium salts with dibenzo-18-crown-6 in DMF (Ruf et al., 1979).

Spectroscopic Measurements. Optical (electronic) differences spectra of microsomes were recorded with an Aminco-DW 2 dual-wavelength photometer. We used cuvettes with an optical path length of 1 cm. Electronic spectra of the heme model complexes were measured in ESR sample tubes. This procedure permitted recording of both electronic and ESR spectra of the same sample, which was important for the characterization of transient species (Ruf et al., 1979). For electronic spectra, a glass rod was inserted into the ESR sample tube above the volume active for ESR, yielding an effective optical path length of 0.1 mm. The sample beam of the Aminco-DW 2 was focused by a $f = 10$ cm glass lens on an aperture of 4×1 mm^2 that was just in front of the ESR tube in the height of the glass spacer. The reference beam was attenuated by a suitable grid. Sample tubes with the aperture were inserted into a coldfinger dewar with liquid nitrogen, which was placed in the focus of the sample beam of the Aminco-DW 2.

ESR was measured in the same sample tubes below the glass insert. We used a Varian E-9 ESR spectrometer operating at about 9.16-GHz microwave frequency and a power of 40 mW. The samples were placed in a dewar insert in the Varian E-231 rectangular cavity where a flow of cold nitrogen gas maintained a sample temperature of 90 ± 1 K. The spectrometer was connected to a digital computer (Data General Nova 820), which was used for signal averaging as well as scaling, superposition, and double intergration of the stored ESR spectra. Spin concentrations were determined from the double integrals with 1 mM $Cu^{II}EDTA$ as a standard and by correcting for different g values (Aasa & Vänngard, 1975). g values were calculated from the magnetic fields as given by the Fieldial with DPPH (diphenylpicrylhydrazyl) as a g marker. Solvent blanks were subtracted from all displayed ESR spectra. Ligand field parameters were computed by programs in FORTRAN IV (Bohan, 1977; Herrick, 1976).

Mössbauer spectra have been recorded in constant acceleration mode with a 30-mCi $^{57}Co(Rh)$ source. Samples were sealed vacuum tight in a copper block, and aluminized mylar windows were used for the transmission of the 14.4-keV γ-rays. Low-temperature measurements have been performed in a continuous-flow cryostate. Experimental data were analyzed by a least-squares procedure using Lorentzians. Isomer shifts are given with respect to α-Fe at room temperature.

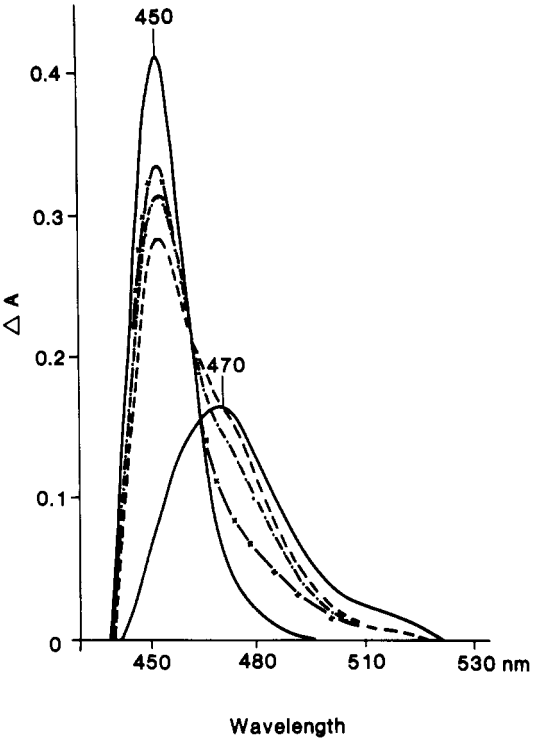


FIGURE 1: Difference spectra of the interaction of halothane with microsomal cytochrome P-450. Halothane (1 mM final concentration) and sodium dithionite (5 mM) were added to microsomes (2 mg of protein/mL) from phenobarbitone-pretreated rats. The spectrum (solid line) with the band at 470 nm was recorded after 5 min. Then CO was bubbled through the cuvette, and the spectra [(– –), (–·–), (–×–)] were recorded in 5-min intervals. Finally, $K_3Fe(CN)_6$ (10 mM) was added, and the sample was again reduced with an excess of dithionite (solid line with the band at 450 nm). The temperature was 30 °C.

Results

Formation of the Spectral Intermediate in Microsomes. During reductive metabolism of halothane by liver microsomes, a spectral intermediate with a band at 470 nm was formed (Mansuy et al., 1974; Ahr et al., 1982), indicating a cytochrome P-450 metabolite (Figure 1). After the addition of CO, the intermediate decomposed slowly as can be seen by the slow increase of the band at 450 nm, representing the ferrous carbonyl complex. Oxidation with $K_3Fe(CN)_6$ destroyed the intermediate complex immediately, releasing the product 2-chloro-1,1-difluoroethylene (Ahr et al., 1982). After a second addition of excess $Na_2S_2O_4$ and CO, the remaining cytochrome P-450 could be determined. From the difference

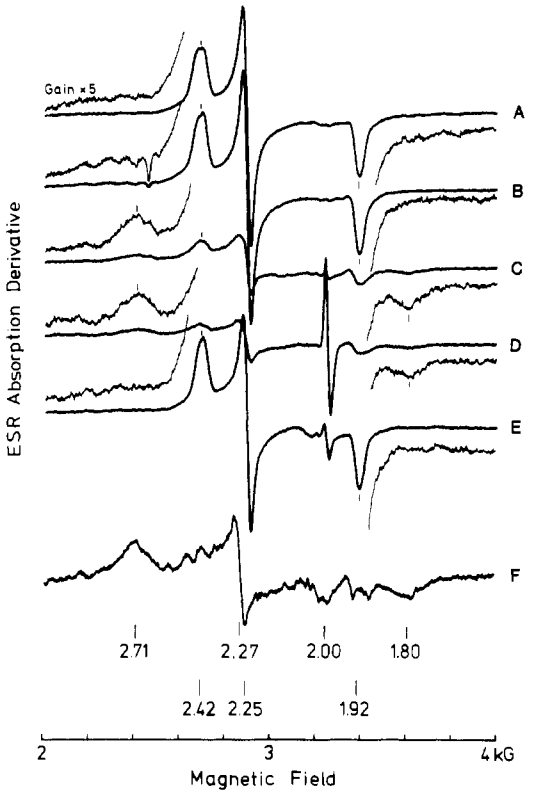


FIGURE 2: ESR spectra of the interaction of halothane with microsomal cytochrome P-450. The spectra were (A) microsomes induced with Arochlor 1254 (51 mg of protein/mL, 200 μM cytochrome P-450) and with subsequent additions of (B) 10 mM halothane, (C) 3 mM dithionite (incubated for 2.5 min at 20 °C), (D) CO (bubbled for 20 s and frozen within another 20 s), and (E) 10 mM $K_3Fe(CN)_6$. The spectra were recorded at a temperature of 90 K with 25-G modulation amplitude and a gain of 500. The inserted wings of the spectra are displayed with 5-fold gain. Trace F was computed from trace C from which 13% of trace A was subtracted to cancel the residual signal of cytochrome P-450 (displayed with 4-fold gain).

of the band at 450 nm under CO between the first and second addition of dithionite, the amount of the 470 nm absorbing complex was estimated.

Both amount and stability of this complex in microsomes reduced with dithionite depended on the pretreatment of the rats. After phenobarbital or Aroclor 1254, up to 50% of cytochrome P-450 formed this complex.

ESR Signal of the Spectral Intermediate in Microsomes. Further characterization of this complex was attempted by ESR, which could give information on valence state and axial ligands of heme iron. A broad ESR signal at $g = 2.71$ and 1.80 was observed in microsomes after the addition of halothane and dithionite, under conditions where the band at 470 nm was formed. This broad signal with its corresponding low amplitude was not found in the earlier study (Mansuy et al., 1974) and could only be detected when the modulation amplitude was increased to 25 G (Figure 2).

It is emphasized that the signal at $g = 2.71$ and 1.80 reflected the behavior of the intermediate absorbing at 470 nm in every respect: namely, (i) the signal was formed only after the addition of both halothane and dithionite, (ii) the signal so formed was decreased only very slowly after the addition of CO, (iii) the signal was destroyed immediately after oxidation with $K_3Fe(CN)_6$, and (iv) the signal was not observed when the sample was bubbled with CO before the addition of halothane and reductant. The recovery of the low-spin signal of native cytochrome P-450 after oxidation with $K_3Fe(CN)_6$ indicated that the signal corresponded to an intermediate complex of cytochrome P-450 and not to a denatured form of the cytochrome (Figure 2).

After subtracting the residual signal of native, nonreduced cytochrome P-450, the signal was identified as a rhombic ESR signal with g values at 2.71, 2.27, and 1.80 (Figure 2, trace F). Its double integral corresponded to about 40% of total cytochrome P-450, which agreed well with the estimate obtained from the optical spectra. The low amplitude of this signal, compared to native cytochrome P-450, was explained by the larger width of this signal. Since spin concentrations are calculated by double integration of ESR absorption derivative signals, the result of this procedure is proportional not only to the amplitude but also to the square of the width of the signal. This explains why visual inspection often underestimates the spin concentrations of low but broad signals. With anaerobic microsomes and NADPH as reductant, the same ESR signal was observed although at slightly lower amplitude.

We examined the ESR spectra of reduced microsomes in the presence of other polyhalogenated compounds for which spectral complexes between 450 and 470 nm have been reported (Wolf et al., 1977). Remarkably, no ESR signal was observed with CCl_4 under conditions where CCl_4 formed a spectral intermediate with a band at 460 nm (Ahr et al., 1980). Likewise, CF_3CHN_2 with dithionite or NADPH, which has been shown to produce a band at 468 nm and has been assumed to be a precursor for a cytochrome P-450–carbene complex, [(P-450)$Fe^{II}CHCF_3$] (Mansuy et al., 1974), did not produce a comparable ESR signal.

From these findings, the ESR signal was assigned to the intermediate complex absorbing at 470 nm. The ESR signal clearly indicated a low-spin ferric heme complex. Remarkably, the ESR signal showed the largest g tensor anisotropy of all cytochrome P-450–ligand complexes reported so far (Dawson et al., 1982; Ruf et al., 1979).

Spectroscopic Characterization of Fe(TPP)–$CHClCF_3$ Model Complexes. The preparation and characterization of the ferric carbanion complex [Fe^{III}(TPP)($CHClCF_3$)] have been described by Mansuy & Battioni (1982). Its structure has been clearly assigned on the basis of elementary analysis, mass spectrometry, and ^1H NMR. We extended its characterization by ESR and Mössbauer spectroscopy and attempted to prepare such a complex with a thiolate as the second axial ligand in order to get a more suitable model for the corresponding cytochrome P-450 complex. As already mentioned, from its reversible formation and the hyperporphyrin nature of the band at 470 nm, we concluded that cysteinate sulfur, the native fifth ligand in cytochrome P-450, was still bound as a ligand in the intermediate. The complex [Fe^{III}(TPP)-($CHClCF_3$)], prepared according to Mansuy & Battioni (1982) and dissolved at −78 °C under strictly anaerobic conditions in CH_2Cl_2, showed optical absorption bands at 414, 518, and 615 nm at 77 K (Figure 3, trace C), which favorably compare to 410 and 521 nm, the previously reported bands at room temperature. The observed ESR signal at $g = 3.0$, 2.3, and 1.6 (spectrum not shown) identified this complex as a low-spin ferric heme complex. A small signal at $g = 6$ (less than 15% of total heme) increased after aeration and warming of the sample. Thus the high-spin ferric heme corresponding to the $g = 6$ signal was more likely an oxidation product of the O_2-sensitive [Fe^{III}(TPP)($CHClCF_3$)] complex than part of a spin equilibrium of the carbanion complex itself.

To further probe the structure of the [Fe^{III}(TPP)($CHClCF_3$)] complex, we recorded Mössbauer spectra of a powder sample handled anaerobically in a glovebox. The spectra were taken in the temperature range between 4.2 and 255 K

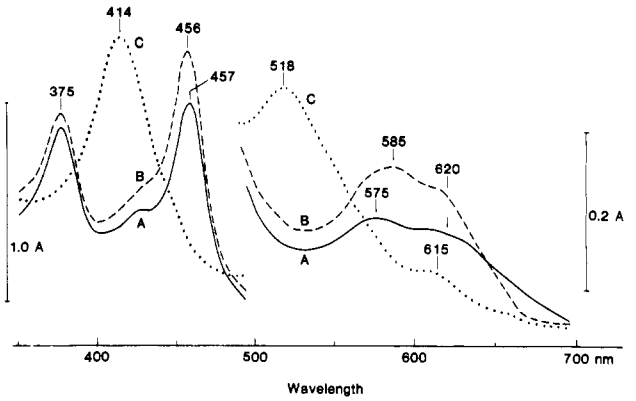
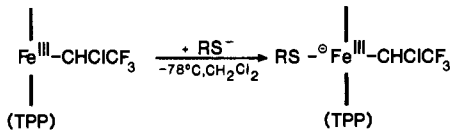


FIGURE 3: Electronic spectra of Fe(TPP) model complexes at 77 K. (A) $[Fe^{III}(TPP)(CHClCF_3)]$ complex with added thiolate $C_6HF_4S^-$. About 400 μL of the carbanion complex $[Fe^{III}(TPP)(CHClCF_3)]$ (about 0.5 mM in CH_2Cl_2) was mixed with 20 μL of thiolate (0.4 M C_6HF_4SNa and 0.2 M dibenzo-18-crown-6 in DMF) in an ESR tube below −60 °C under strictly anaerobic conditions. After the glass spacer was adjusted, the sample was frozen in liquid N_2. (B) $[Fe^{III}(TPP)(RS)_2]^-$ complex. A total of 10 μL of thiolate was mixed with 300 μL of $[Fe^{III}(TPP)(Cl)]$ (1 mM in CH_2Cl_2) at −60 °C and frozen in liquid nitrogen. (C) $[Fe^{III}(TPP)(CHClCF_3)]$ complex. Solid $[Fe^{III}(TPP)(CHCl,CF_3)]$ was dissolved in CH_2Cl_2 under anaerobic conditions. The about 1 mM solution was frozen in liquid nitrogen. The effective light path was 0.1 mm.

Scheme I



(without applied magnetic field) and at 4.2 K with applied magnetic field of 0.1 T perpendicular to the γ-beam. The main feature of the obtained spectra accounted for about 88% of the total absorption area (at 4.2 K). The parameters of this dominating subspectrum were isomer shift $\delta = 0.28$ mm s^{-1} and quadrupole splitting $\Delta E_Q = 2.54$ mm s^{-1} at 4.2 K and $\delta = 0.22$ mm s^{-1} and $\Delta E_Q = 2.33$ mm s^{-1} at 255 K. Applying a magnetic field of 0.1 T perpendicular to the γ-beam did not change the Mössbauer pattern; this indicated that the electronic spin relaxation stayed in the fast relaxation mode even under the application of moderate fields. These data indicated that we were concerned with a low-spin ferric heme complex, corroborating the ESR results. The Mössbauer parameters of the remaining subspectrum (12% of the total absorption area) indicated the presence of a high-spin ferric heme complex.

In order to bind a thiolate ligand trans to the carbanion ligand, we added an excess of the (2,3,5,6-tetrafluorobenzo)thiolate $C_6HF_4S^-$ to the $[Fe^{III}(TPP)(CHClCF_3)]$ complex in CH_2Cl_2, at −78 °C under strictly anaerobic conditions. The drastic changes in the optical (Figure 3) and ESR spectra (Figure 4) represented the binding of the thiolate (Scheme I). Comparable results have been obtained with the more negative 1-butylthiolate (spectra not shown).

The optical spectrum changed to a hyperporphyrin spectrum with a split Soret band at 375 and 457 nm and a broad band in the visible at 575 nm (Figure 3, trace A). The ESR spectrum consisted of two low-spin ferric heme species (Figure 4, trace A), both with decreased anisotropy of the g tensor, indicating binding of thiolate sulfur as ligand to heme iron (Ruf et al., 1979). The spectrum at $g = 2.38$, 2.28, and 1.93 was almost identical with the spectrum of the $[Fe^{III}(TPP)(RS)_2]^-$ complex Figure 4, trace B), which was prepared at −78 °C by adding an excess of thiolate to $[Fe^{III}(TPP)Cl]$ in

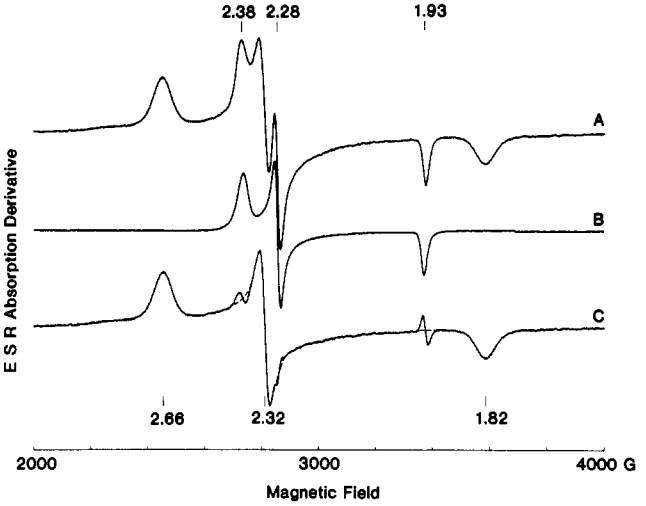


FIGURE 4: ESR spectra of the $[Fe^{III}(TPP)(CHClCF_3)(RS)]^-$ and $[Fe^{III}(TPP)(RS)_2]^-$ model complexes: (A) $[Fe^{III}(TPP)(CHClCF_3)]$ complex with added thiolate $C_6HF_4S^-$ (the same sample as in Figure 3A); (B) $[Fe^{III}(TPP)(RS)_2]^-$ complex (the same sample as in Figure 3B); (C) computed spectrum of the thiolate carbanion complex (25% of spectrum B was subtracted from spectrum A to cancel the bisthiolate complex). The spectra were recorded at a temperature of 90 K with a modulation amplitude of 10 G and a gain of 100.

Table I: Comparison of Hyperporphyrin Spectra of Cytochrome P-450 and Ferric Porphyrin Model Complexes with Thiolates or Carbanions[a]

complex	Soret bands (nm)
$[(P\text{-}450)^-SC_6H_{17}]$	378, 471[b,c]
$[(P\text{-}450)^-CHClCF_3]$	470[c]
$[Fe^{III}(TPP)(SC_4H_9)_2]^-$	382, 465
$[Fe^{III}(TPP)(SC_4H_9)(CHClCF_3)]^-$	383, 466
$[Fe^{III}(TPP)(SC_6HF_4)_2]^-$	375, 456
$[Fe^{III}(TPP)(SC_6HF_4)(CHClCF_3)]^-$	375, 457

[a] For details, see Figure 4. [b] Nastainczyk et al. (1976). [c] Difference spectrum.

CH_2Cl_2. Subtraction of this spectrum from the composite spectrum yielded a residual spectrum at $g = 2.66$, 2.32, and 1.82, corresponding to 80% of total spin concentration (Figure 4, trace C). This latter species was assigned to a $[Fe^{III}(TPP)(RS)(CHClCF_3)]^-$ complex from its g values and ligand field parameters (see below).

Hence, the optical spectrum (Figure 3, trace A) was also composite: 20% of the $[Fe^{III}(TPP)(RS)_2]^-$ complex with a very similar hyperporphyrin spectrum (Figure 3, trace B) and 80% of the complex $[Fe^{III}(TPP)(CHClCF_3)(RS)]^-$. The pure spectrum of the latter complex could be obtained by subtraction. But since spectra A and B were qualitatively very similar and the subtraction of 20% of spectrum B would be a minor correction, the pure spectrum will be very similar to spectrum A. Thus, the optical spectrum of the $[Fe^{III}(TPP)(CHClCF_3)(RS)]^-$ complex was identified as a hyperporphyrin spectrum with bands at 375, 457, and 575 nm. Remarkably, very similar hyperporphyrin Soret bands were observed for the complexes with either two thiolates or one thiolate and the CF_3CHCl^- carbanion as axial ligands both for the models and cytochrome P-450 (Table I).

The $[Fe^{III}(TPP)(CHClCF_3)(RS)]^-$ complexes appeared to be fairly unstable intermediates. Even under carefully maintained anaerobic conditions and low temperature during dissolving and mixing, their yield does not exceed 80% with $C_6HF_4S^-$ or 75% with $C_4H_9S^-$. This was explained both by the inherent instability of the complex and by the presence of the hemin impurity with the $g = 6$ signal in the starting carbanion complex (less than 15%), which could directly form

Table II: ESR Parameters of Ligand and Intermediate Complexes of Cytochrome P-450

no.[a]	complex	g_1	g_2	g_3	k
1	RLM_{PB}[b]	2.41	2.25	1.92	1.13
1	RLM_{ARO}[c]	2.43			1.17
		2.40	2.25	1.92	1.11
2	RLM_{PB} + halothane + $Na_2S_2O_4$	2.705	2.27	1.80	1.14
2	RLM_{ARO} + halothane + $Na_2S_2O_4$	2.71	2.26	1.80	1.13
3	RLM_{PB} + *N*-methylimidazole	2.536	2.255	1.877	1.12
4	RLM_{PB} + *n*-octylamine	2.494	2.248	1.898	1.14
5	RLM_{PB} + metyrapone	2.48	2.257	1.892	1.10
6	RLM_{PB} + diethylphenylphosphine	2.49	2.28	1.88	1.10
1	cytochrome $P\text{-}450_{CAM}$[d]	2.45	2.26	1.91	1.15
3	$P\text{-}450_{CAM}$ + *N*-methylimidazole	2.535	2.259	1.873	1.11
4	$P\text{-}450_{CAM}$ + *n*-octylamine	2.494	2.255	1.894	1.13
5	$P\text{-}450_{CAM}$ + metyrapone	2.48	2.26	1.88	1.05
6	$P\text{-}450_{CAM}$ + diethylphenylphosphine	2.50	2.28	1.88	1.11
7	$P\text{-}450_{CAM}$ + C_4H_9SH	2.37	2.25	1.94	1.21

[a] Numbers correspond to Figure 5. [b] RLM_{PB}, rat liver microsomes from rats pretreated with phenobarbitone. [c] RLM_{ARO}, rat liver microsomes from rats pretreated with Aroclor 1254. [d] Cytochrome P-450 from *Pseudomonas putida* grown on *d*-camphor; data from Ruf et al. (1979).

Table III: ESR Parameters of Hemin Thiolate Model Complexes[a]

no.[b]	complex	g_1	g_2	g_3	k
□[b]	$[Fe^{III}(TPP)(SC_6F_4H)]$				
2	$+^-CHClCF_3$[c]	2.678	2.321	1.807	1.17
3	+*N*-methylimidazole	2.470	2.291	1.885	1.10
4	+*n*-octylamine	2.547	2.286	1.862	1.12
5	+pyridine	2.493	2.314	1.871	1.11
6	+diethylphenylphosphine	2.544	2.329	1.826	1.06
7	$+C_6F_4HS^-$	2.382	2.280	1.932	1.22
×[b]	$[Fe^{III}(PPIXDME)(SC_4H_9)]$				
3	+*N*-methylimidazole	2.360	2.235	1.940	1.16
4	+*n*-octylamine	2.388	2.232	1.934	1.16
5	+pyridine	2.362	2.241	1.939	1.17
6	+diethylphenylphosphine	2.385	2.257	1.927	1.14
7	$+C_4H_9S^-$	2.309	2.229	1.958	1.25
◇[b]	$[Fe^{III}(TPP)(SC_4H_9)]$				
2	$+^-CHClCF_3$[c]	2.510	2.246	1.902	1.19
3	+*N*-methylimidazole	2.355	2.218	1.947	1.19
4	+*n*-octylamine	2.378	2.215	1.943	1.19
5	+pyridine	2.345	2.225	1.950	1.22
6	+diethylphenylphosphine	2.357	2.230	1.947	1.22
7	$+C_4H_9S^-$	2.332	2.208	1.964	1.37

[a] Prepared according to this work or Ruf et al. (1979). [b] Numbers and symbols correspond to Figure 5. [c] For preparation, see text.

the bisthiolate complex after the addition of an excess of thiolate. The $[Fe^{III}(TPP)(CHClCF_3)(RS)]^-$ complexes were sensitive against traces of air or higher temperature than −60 °C. They decomposed under such conditions via various species, showing ESR signals between the original thiolate carbanion complexes and the final bisthiolate complexes. These species with Soret bands around 435 nm were not further investigated. The instability of the complex made the recording of Mössbauer spectra infeasible.

Ligand Field Parameters. Although the g values of low-spin ferric hemes depend on the axial ligands, direct comparisons of g values of complexes with different porphyrins and different thiolates as ligands may be greatly misleading. The meaningful parameters for such comparisons are the ligand field parameters that describe the tetragonal (μ) and rhombic (R) distortion of an octahedral ligand field. Plots of "rhombicity" R/μ vs. "tetragonality" μ/λ (λ is spin–orbit coupling constant) were introduced (Blumberg, 1967; Peisach et al., 1974) to identify the axial ligands of heme. Tetragonality is a measure of the electronegativity of the axial ligands compared to the four pyrrol nitrogens of the porphyrin. Rhombicity depicts covalent bonding of the axial ligands, which disturbs the tetragonal axis. When we attempted ligand field analyses of the g values, we encountered the problem of "ordering the g values". This is the problem of assigning the experimental g values g_1, g_2, and g_3 from the powder spectra to the signed components of the g tensor g_x, g_y, and g_z in a molecular coordinate system. This has been discussed in detail (Blumberg, 1967; Peisach et al., 1974; Bohan 1977; Taylor 1977) and can be visualized as the choice of a proper coordinate system. For hemes without a thiolate ligand, the tetragonal axis has been found to be normal to the heme plane whereas, for cytochrome P-450 and hemes with thiolate ligands, this axis is nearly in the heme plane (Devaney, 1980). Since we intended to maintain consistency with published parameters (Peisach et al., 1974; Ullrich et al., 1979), we ordered the g values as $g_x \sim -2.4$, $g_y \sim 2.2$, and $g_z \sim -1.9$ according to Bohan's convention III and kept this ordering consistently for all spectra although the cytochrome P-450 carbanion complex with $R/\mu = 0.75$ violated $R/\mu = {}^2/_3$, a criterion for proper ordering.

A first approximation of the ligand field parameters μ and R was computed with the orbital reduction factor $k = 1$ (Bohan, 1977) from the g values summarized in Tables II and III. The parameters were refined by an iteration program (Herrick 1976), where k was allowed to vary. The orbital reduction factor k for consistent solutions was found between $k = 1.05$ and 1.37 (Tables II and III).

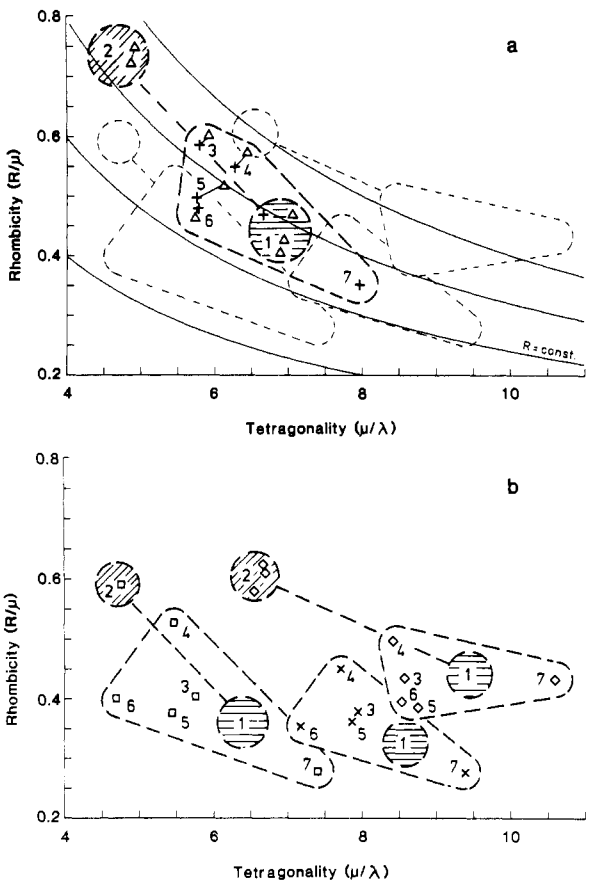
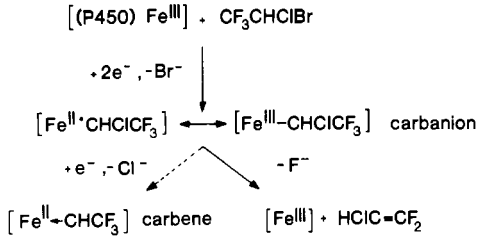


FIGURE 5: Comparison of ligand field parameters of cytochrome P-450 complexes (a) and Fe(III)–porphyrin–thiolate model complexes (b), both with various sixth ligands. The complexes corresponding to the symbols are (Δ) $P\text{-}450_{RLM}$, (+) $P\text{-}450_{CAM}$, (□) $[Fe^{III}(TPP)(SC_6HF_4)]$, (◇) $[Fe^{III}(TPP)(SC_4H_9)]$, and (×) $[Fe^{III}(PPIXDME)(SC_4H_9)]$. The various sixth ligands corresponding to the numbers are (1) native cytochrome, (2) CF_3CHCl^-, (3) imidazole, (4) alkylamine, (5) pyridine, (6) phosphine, and (7) thiolate (for details, see Table II and III).

The calculated ligand field parameters were plotted in Figure 5 for four different groups of complexes, (i) cytochrome

Scheme II



P-450, (ii) [Fe^{III}(PPIXDME)(SC_4H_9)], with various sixth ligands, including the CF_3CHCl^- carbanion. Although the values of the ligand field parameters varied considerably for the four compounds with the same sixth ligand, due to the different electronic properties of the porphyrins and thiolates, the patterns for the different compounds with various ligands were remarkably similar. In particular, the similar relative positions of the halothane-derived complex of cytochrome P-450 and its Fe(TPP) model complexes indicated that in all these cases the nature of the sixth ligand was identical, namely, the CF_3CHCl^- carbanion.

Discussion

During reductive metabolism of halothane, rat liver microsomes produce a band at 470 nm that was assigned to a cytochrome P-450–metabolite complex. We could detect an ESR signal at $g = 2.71$, 2.27, and 1.81 concomitant in every respect with the appearance of the band at 470 nm. This ESR signal unequivocally proved the low-spin ferric heme nature of the metabolite complex. Any proposed structure for this complex has to explain two peculiarities of the ESR signal, namely, the large anisotropy of the g tensor and the sensitivity to oxidation. The finding that the intermediate quantitatively releases the product 2-chloro-1,1-difluoroethylene has led to the hypothesis that the CF_3CHCl^- carbanion complex of cytochrome P-450 is the precursor of the product (Ahr et al., 1982) according to Scheme II.

The study of model complexes where a thiolate RS^- was added as the second axial ligand to the carbanion complex with the known structure [Fe^{III}(TPP)($CHClCF_3$)] clearly resulted in electronic and ESR spectra analogous to those of the cytochrome P-450 complex. Hence, these data provide strong evidence for the carbanion structure [(P-450)$Fe^{III}CHClCF_3$] for the complex absorbing at 470 nm.

The earlier assignment as a ferrous heme carbene complex (Mansuy et al., 1974) was derived from the observation that the addition of the carbene precursor 1,1,'-trifluorodiazoethane to reduced microsomal cytochrome P-450 produced a band at 468 nm, almost identical with the spectral intermediate with halothane at 470 nm. Since in our experiments the addition of this diazo compound to microsomes did not produce an ESR signal characteristic of the ferric carbanion complex, the diazo compound apparently formed a different complex with a band at 468 nm, possibly a ferrous carbene complex [(P-450)-Fe^{II}($CHCF_3$)]. It is remarkable that similar absorption bands for corresponding carbanion and carbene complexes have been reported for the model complexes [Fe^{III}(TPP)($CHClCF_3$)] and [Fe^{II}(TPP)($CClCF_3$)], which both exhibit Soret bands at 410 nm, although with different intensities (Mansuy & Battioni, 1982). The CCl_4-derived metabolite complex of cytochrome P-450 showing a band at 460 nm has been assigned to a low-spin ferrous dichlorocarbene complex, [(P-450)Fe^{II}(CCl_2)] (Wolf et al., 1977), which was substantiated by the preparation and characterization of porphyrin model complexes, e.g., [Fe^{II}(TPP)(CCl_2)] (Mansuy et al., 1977, 1978). In agreement with this assignment, we did not observe a corresponding ESR signal with microsomal cytochrome P-450 under conditions where the band at 460 nm was formed with CCl_4 and dithionite.

Scheme II predicts that carbanion complexes, produced by reduction with two electrons, were at least unstable intermediates in the formation of carbene complexes. It is tempting to speculate on the factors that stabilize the carbanion with respect to the carbene in the case of halothane. The main factor may be the electron-withdrawing effect of the trifluoromethyl group, which stabilizes the carbon–chlorine bond and decreases the rate of carbene formation. If the β-elimination of fluoride, forming the olefin, also is a comparably slow process, the intermediate carbanion complex can accumulate to a measurable amount.

Other possible stabilizing factors in the enzyme, such as steric hindrance, accessibility, or polarity effects, are unknown at present, but it might be speculated that these factors may be effective differently in the different forms of cytochrome P-450, the amount of which depends on the pretreatment of the animals. Thiolate as trans ligand as in cytochrome P-450 should increase the electron density at the α-carbon, which would facilitate the α-elimination of the chloride and destabilize the carbanion complex. In fact, the addition of thiolate to the carbanion model complex [Fe^{III}(TPP)($CHClCF_3$)] apparently destabilized the carbanion complex.

The spectral properties of the carbanion complexes shed some light on the nature of the iron carbon, which may be described as Fe^{II}·C ↔ Fe^{III}–C ↔ Fe^{III}–C. The ESR signals demonstrated that we were concerned with ferric carbanion complexes and not ferrous carbon radical complexes. The low spin state of the 5-coordinate [Fe^{III}(TPP)($CHClCF_3$)] complex indicated that this Fe–C bond is highly covalent, providing the strong ligand field yielding a low-spin heme complex with a single axial ligand.

With thiolate as a second axial ligand, the CF_3CHCl^- carbanion complex showed electronic spectra very similar to the spectra of the bisthiolate complexes, both of the hyperporphyrin type (Table I). This indicated that in both coordinations [$-S^-Fe^{III}S^-$–] and [$-S^-Fe^{III}C^-$–] the ligands exert similar charge-transfer interactions on the porphyrin electrons by which hyperporphyrin spectra have been explained (Hanson et al., 1976; Loew et al., 1981). On the other hand, the ligand field parameters derived from ESR indicate different ligand fields for the iron 3d electrons for both ligand arrangements. The larger tetragonality of the bisthiolate complexes can be explained by the higher electronegativity of RS^- compared to CF_3CHCl^-. The higher rhombicity with the carbanion ligand points to a strong covalency of the Fe–C bond with low axial symmetry, presumably due to nonaxial π-back-bonding. Therefore, the Fe–C bond is probably neither purely ionic nor a pure σ-bond, but strong covalency, additional π-bonding, and charge-transfer interaction are suggested from the spectral data.

Acknowledgments

We are greatly indebted to G. Herrmann and V. Eichinger for their skillful technical assistance.

References

Aasa, R., & Vänngard, T. (1975) *J. Magn. Reson. 19*, 308–315.

Ahr, H. J., King, L. J., Nastainczyk, W., & Ullrich, V. (1980) *Biochem. Pharmacol. 29*, 2855–2861.

Ahr, H. J., King, L. J., Nastainczyk, W., & Ullrich, V. (1982) *Biochem. Pharmacol. 31*, 383–390.

Battioni, P., Mahy, J. P., Delaforge, M., & Mansuy, D. (1983a) *Eur. J. Biochem. 134*, 241–248.

Battioni, P., Mahy, J. P., Gillet, G., & Mansuy, D. (1983b) *J. Am. Chem. Soc. 105*, 1399–1401.

Blumberg, W. E. (1967) in *Magnetic Resonance in Biological Systems* (Ehrenberg, A., Malmstrom, B. G., & Vanngard, T., Eds.) pp 119, Pergamon Press, New York.

Bohan, T. L. (1977) *J. Magn. Reson. 26*, 109–118.

Cox, P. J., King, L. J., & Parke, D. V. (1976) *Xenobiotica 6*, 365–375.

Dawson, J. H., Andersson, L. A., & Sono, M. (1982) *J. Biol. Chem.* 257, 3606–3617.

Devaney, P. W. (1980) Ph.D. Thesis, University of Illinois, Urbana, IL.

Diaz Gomez, M. I., Castro, J. A., de Ferreyra, E. C., d'Acorta, N., & de Castro, C. R. (1973) *Toxicol. Appl. Pharmacol. 25*, 534–541.

Frommer, U., Ullrich, V., & Staudinger, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 903–912.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–767.

Hanson, L. K., Eaton, W. A., Sligar, S. G., Gunsalus, I. C., Gouterman, M., & Connel, C. R. (1976) *J. Am. Chem. Soc. 98*, 2672–2674.

Herrick, R. C. (1976) Ph.D. Thesis, University of Illinois, Urbana, IL.

Jonen, H. G., Werringloer, J., Prough, R. A., & Estabrook, R. W. (1982) *J. Biol. Chem. 257*, 4404–4411.

Kunze, K. L., & Ortiz de Montellano, P. R. (1983) *J. Am. Chem. Soc. 105*, 1380–1381.

Loew, G. H., Herman, Z. S., Rohmer, M.-M., Goldblum, A., & Pudzianowski, A. (1981) *Ann. N.Y. Acad. Sci. 367*, 192–218.

Mansuy, D., & Battioni, J. P. (1982) *J. Chem. Soc., Chem. Commun.*, 638–639.

Mansuy, D., & Fontecave, M. (1983) *Biochem. Pharmacol. 32*, 1871–1879.

Mansuy, D., Nastainczyk, W., & Ullrich, V. (1974) *Naunyn-Schmiedeberg's Arch. Pharmacol. 285*, 315–324.

Mansuy, D., Lange, M., Chottard, J. C., Guerin, P., Morliere, P., Brault, D., & Roufee, M. (1977) *J. Chem. Soc., Chem. Commun.*, 648–649.

Mansuy, D., Lange, M., Chottard, J. C., Bartoli, J. F., Chevrier, B., & Weiss, R. (1978) *Angew. Chem. 90*, 828–829.

Mansuy, D., Battioni, P., Mahy, J. P., & Gillet, G. (1982) *Biochem. Biophys. Res. Commun. 106*, 30–36.

McLain, G. E., Sipes, I. G., & Brown, B. R. (1979) *Anesthesiology 51*, 321–326.

Nastainczyk, W., Ruf, H. H., & Ullrich, V. (1976) *Chem.-Biol. Interact. 14*, 251–263.

Nastainczyk, W., Ahr, H. J., & Ullrich, V. (1982) *Biochem. Pharmacol. 31*, 391–396.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.

Ortiz de Montellano, P. R., & Kerr, D. (1983) *J. Biol. Chem. 258*, 10558.

Peisach, J., Blumberg, N. E., & Adler, A. (1973) *Ann. N.Y. Acad. Sci. 206*, 310–327.

Recknagel, R. O., & Glende, E. A. (1973) *CRC Crit. Rev. Toxicol. 2*, 263–296.

Reiner, O., & Uehleke, M. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1048–1052.

Ringe, D., Petsko, G. A., Kerr, D. E., & Ortiz de Montellano, P. R. (1984) *Biochemistry 23*, 2–4.

Ruf, H. H., Wende, P., & Ullrich, V. (1979) *J. Inorg. Biochem. 11*, 189–204.

Taylor, C. P. S. (1977) *Biochim. Biophys. Acta 491*, 137–149.

Uehleke, H., Hellmer, K. M., & Tabarelli, S. (1973a) *Xenobiotica 3*, 1–11.

Uehleke, H., Hellmer, K. M., & Tabarelli-Poplawski, S. (1973b) *Naunyn-Schmiedeberg's Arch. Pharmacol. 279*, 39–52.

Ullrich, V., King, L. J., Wolf, C. R., & Nastainczyk, W. (1978) in *Advances in Pharmacology and Therapeutics* (Cohen, Y., Ed.) Vol. 9, pp 131–138, Pergamon Press, Oxford.

Ullrich, V., Sakurai, H., & Ruf, H. H. (1979) *Acta Biol. Med. Germ. 38*, 287–297.

Ullrich, V., Ahr, H. J., Castle, L., Kuthan, H., Nastainczyk, W., & Ruf, H. H. (1982) in *The Biological Chemistry of Iron* (Dunford, H. B., Dolphin, D., Raymond, K. N., & Sieker, L., Eds.) pp 413–425, D. Reidel, Dordrecht, Boston, and London.

van Dyke, R. A., & Wood, C. L. (1973) *Anesthesiology 38*, 328–332.

White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.

Wolf, C. R., Mansuy, D., Nastainczyk, W., Deutschmann, G., & Ullrich, V. (1977) *Mol. Pharmacol. 13*, 698–705.