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Theoretical Insights into the Reductive Metabolism of CCl_4 by Cytochrome P450 Enzymes and the CCl_4-dependent Suicidal Inactivation of P450

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The anaerobic metabolism of CCl_4 by P450 enzymes was investigated using quantum chemical calculations. It was found that under anaerobic conditions, the substrate CCl_4 might undergo one or two subsequent one-electron reductions to generate different reactive metabolites, trichloromethyl radical ($\cdot CCl_3$) and dichlorocarbene ($:CCl_2$) respectively. Meanwhile, it was the reduced ferrous haem complex rather than the unreduced ferric haem complex that could directly achieve such reductions. Based on the formation of the former reactive metabolite, a further one-electron reduction could take place with the assistance of a proton to yield the latter reactive species, *i.e.*, a further reductive dechloridation of $\cdot CCl_3$ could take place *via* a novel S_E3 mechanism. In addition, the $\cdot CCl_3$ species was capable of binding covalently to the *meso*-carbon atom of the prosthetic group, leading to the suicidal destruction of P450 enzymes. Whereas, the $:CCl_2$ species was involved in the CCl_4-dependent reversible P450 inhibition as its hydrolysis product, CO, but it was not significantly involved in the CCl_4-dependent irreversible P450 destruction. It is obvious that the reductive metabolism of CCl_4 to reactive intermediates by P450 enzymes is an essential prerequisite for its toxicity.

Introduction

Cytochrome P450 enzymes are ubiquitous in nature.[1] They essentially participate in the biosynthesis of endogenously active compounds to sustain the homeostasis and equilibrium, but also oxidative and reductive conversion of xenobiotics to potentially reactive products or less toxic metabolites.[2,3] Therein, the reactions of cytochrome P-450 with organic halides can be an important means of both activation and detoxification of such xenobiotics *in vivo*.[4-7]

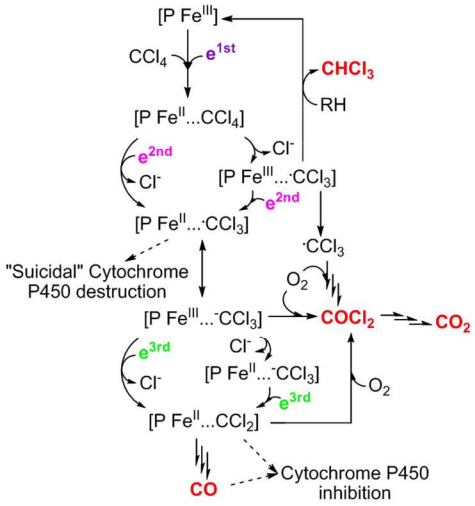
Carbon tetrachloride (CCl_4), as the simplest perhalogenated alkane, is of considerable industrial importance.[8-11] It has been widely used as a dry-cleaning fluid, as an extinguishant, in the manufacture of other halogenated compounds and for other uses. The metabolic activation of CCl_4 to reactive intermediates is an essential prerequisite for the hepatotoxicity produced both *in vivo* and *in vitro* by such toxic agent.[8,12-14] Experiments performed in microsomes and in reconstituted cytochrome P450-containing systems indicate that P450 enzyme is the sole site for such metabolic activation.[15-17] Furthermore, comparison of the metabolic reaction rate of different human P450 forms with CCl_4 indicates that CYP2E1 is the major one responsible for CCl_4 bioactivation.[18]

Since no hydrogen atom exists in CCl_4, direct hydroxylation cannot occur as the metabolism of alkanes by P450 enzymes. On the contrary, the first stage in this metabolic process appears to be purely reductive, resulting in the formation of the trichloromethyl radical ($\cdot CCl_3$), which has been confirmed by many experiments.[12,13,15,16,19-29] Much evidence including the production of chloroform ($CHCl_3$)[30,31] and hexachloromethane (C_2Cl_6),[19,32] the covalent binding results and the stimulation of lipid peroxidation, has indirectly proven the formation of $\cdot CCl_3$,[8] and then the successive spin-trapping experiment with phenyl-*tert*-butyl nitrone (PBN) validated its formation directly.[24,33-36] The generated $\cdot CCl_3$ may undergo different reactions depending on the environment.[37] At high oxygen tension, such radical can be oxidized by oxygen to form phosgene ($COCl_2$), which then undergoes hydrolysis to carbon dioxide.[37-39] Under insufficient oxygen or anaerobic condition, this radical may abstract a hydrogen atom from its immediate environment to form chloroform,[23,37,40] and may covalently bind to the lipids and proteins of cells to initiate cell damage.[41,42] Alternatively, it may undergo further reduction by P450 producing another active metabolite, dichlorocarbene ($:CCl_2$),[13,26,43] which was firstly suggested by Wolf *et al*.[44] The formation of $:CCl_2$ has been successively confirmed by the carbene trapping experiment of Pohl *et al*.[26] and by the spectral experiment of Manno *et al*.[13] This active species will then interact with the reduced haem iron leading to reversible inhibition of P450 or undergo hydrolysis to CO, which can bind

to the reduced P450 haem as well, again resulting in P450 inhibition.[13]

The active metabolites of CCl_4 were assumed to not only initiate lipid peroxidation, but also conceivably attack the haem moiety and/or the apoprotein of cytochrome P450 leading to the destruction of the haemoprotein.[13] The finding of almost equimolar loss of cytochrome P450 and haem given by Manno *et al.*,[13] indicated that P450 enzyme was damaged by a direct attack of CCl_4 active metabolite, $\cdot CCl_3$, on its prosthetic moiety, which was in line with the hypothesis of Groot *et al.*[12] However, $:CCl_2$, an alternative active metabolite of CCl_4, was excluded as an irreversible inhibitor of P450, as a consequence of the invalidity of its trapping agent, 2,3-dimethyl-2-butene (DMB), against haem loss.[13] It was just responsible for reversible inhibition of P450 in the same way as its hydrolysis product, CO.[13] Thus, understanding the metabolic mechanism of CCl_4 by P450 has a profound significance on reducing its organism damage more effectively.

Up to now, many groups have speculated the reductively metabolic mechanism of CCl_4 mediated by P450.[13,19,23,45-47] All of them affirmed the key role of the reduced ferrous haem complex in this reductive process. However, there were still some discrepancies about the reaction details among their speculations. For the formation of $\cdot CCl_3$, Ahr *et al.*[23] and Castro *et al.*[46] proposed that the homolytic cleavage of C-Cl bond of CCl_4 with synchronous one-electron reduction yielding $\cdot CCl_3$ and Cl^- was achieved directly by the reduced ferrous haem complex, while Luke *et al.*[48] and Manno *et al.*[13] thought that one more electron should be needed for this reduced complex to achieve such reduction. For the formation of $:CCl_2$, all of experimental investigations indicated that the reduced ferrous haem-$\cdot CCl_3$ complex required one more electron to eliminate a second Cl^-, producing ferrous-$:CCl_2$ complex (Scheme 1). Furthermore, de Visser *et al.*[49] investigated the oxidative metabolism of CCl_4 by a high-valent iron(IV)-oxo heme cation radical species of P450, Cpd I. They also studied the initially reductive metabolism of CCl_4 by reduced P450 to generate chloride anion and trichloromethyl radical, as well as a subsequent oxidation of the obtained trichloromethyl radical initiated by O_2 yielding phosgene (Cl_2CO). Their computational results indicated that the major metabolic pathway was a reductive dechloridation by reduced P450 rather than direct chlorine abstraction by Cpd I. Meanwhile, they also have pointed out that both the C-Cl bond cleavage and the electron transfer occurred synchronously during this reductive dechloridation process. They solely investigated the fate of the generated radical species in the presence rather than in the absence of oxygen. Thus, in the present work, the initial one-electron reduction of CCl_4 to form $\cdot CCl_3$ species was reinvestigated to verify the reliability of our computational approaches and models. The objectives of the present work were to explore the fate of the radical species, which was generated from the initially reductive metabolism, in the absence of oxygen, and particularly to test the hypothesis of a primary, suicidal role played in the P450 inactivation process by haem. To explore these issues, quantum chemical density functional theory (DFT) calculations, which have been successfully used in P450 systems,[50-67] were employed. Our computational results indicate that the reduced ferrous haem complex is responsible for the reduction of CCl_4. The generated $\cdot CCl_3$ can either be further reduced to form another reactive metabolite, $:CCl_2$, leading to reversible P450 inhibition, or covalently bind to *meso*-carbon of the heam group, leading to irreversible P450 destruction.



Scheme 1. Proposed mechanism for CCl_4 biotransformation by P450 enzymes and possible effect of metabolites on P450.

Computational methods

The active species of the P450 enzyme was simplified to the model complex (SH)FePor(L) with L=none, which has been proven a better representation of this enzyme.[55,60] Carbon tetrachloride was selected as the substrate. The spin-unrestricted B3LYP (UB3LYP)[68-71] hybrid density functional was employed for all dispersion corrected density functional theoretical (DFT-D) calculations using Gaussian 09 package of programs.[72] The LACVP(Fe)/6-31G**(C, H, O, N, S) basis set,[73] *i.e.*, LACVP** (denoted B1) was used for geometry optimizations without symmetry constraint and frequency calculations. The transition states were characterized with the sole imaginary frequency for a correct single mode along the reaction path, while all local minima were verified with real frequencies. More accurate energies were determined single point energy (SPE) calculations on the optimized geometries using a higher basis set, which describes Fe by LACV3P and all the rest atoms by 6-311+G** basis set, *i.e.*, LACV3P+** (denoted B2). All calculations were performed in vacuum. Bulk polarity effects of the environment on the barrier heights were explored by single-point calculations on the optimized structures using the self-consistent reaction field (SCRF) method in a polarizable continuum model (PCM) of a nonpolar solvent (chlorobenzene, ε=5.697), at the UB3LYP/B2 level. These computational methods employed have been widely used and proven to be reliable.[50-67]

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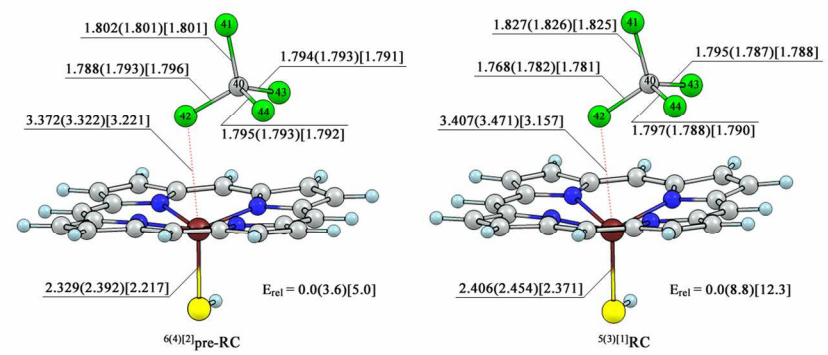

Fig. 1 Geometries of the ferric-CCl_4 complex (pre-**RC**) and the ferrous-CCl_4 complex (**RC**) optimized at the UB3LYP/B1 level in the gas phase (bond distances indicated in Å) and the relative energies of various spin states (in kcal/mol) calculated at the UB3LYP/B2 level including zero-point energy (ZPE) and dispersion corrections.

If not otherwise indicated, the energies reported in the main text are corrected for ZPE, solvation and dispersion corrections. In addition, all of the calculated data and Cartesian coordinates of all fully optimized stationary points are available in the Electronic Supplementary Information (ESI†) document.

Results and discussion

The formation of trichloromethyl radical

According to the well-known catalytic cycle of P450 enzymes,[60,63] we see that the cycle begins with the resting state, *i.e.*, a haem complex with a water molecule as the sixth ligand binding to the ferric ion in the distal side. With the entrance of the substrate, the water will be displaced leaving a penta-coordinated ferric haem complex. This complex with a slightly higher electron-accepting capacity than the resting state, obtains an electron from a reductase protein, yielding the reduced ferrous complex. Thus, the ferric complex was chosen as our initial computational model. To define the geometric and spin information of the ground state, the complexes of substrate CCl_4 with the ferric enzyme ($(SH)Fe^{(III)}Por$) and the reduced ferrous enzyme ($(SH)Fe^{(II)}Por$) were respectively optimized at the UB3LYP/B1 level in the gas phase. The key optimized geometric parameters are depicted in Fig. 1. It is obvious that the substrate is near but not attached to the haem iron with a Fe-Cl distance of more than 3.0 Å, which is in line with the previous findings. In other words, the enzyme in these two complexes keeps penta-coordinate, in which the iron positions below the heme plane as the computational results of Shaik *et al*.[55,60] Comparison of the relative energies indicate that both of the penta-coordianted complexes have high-spin ground states (a sextet ground state for the ferric complex, pre-**RC**, and a quintet ground state for the reduced ferrous complex, **RC**), which is in good agreement with the findings of Shaik *et al*.[55,60] Furthermore, since Luke *et al*.[48] have assumed that the electron transfer occurred fast enough that the parent molecule would not be able to relax during this transfer and the magnitude of vertical electron affinity (VEA) of a molecule could act as an important indication of whether it will accept the electron. Thus, the electron-accepting ability of pre-**RC** species was assessed through the calculation of its VEA. Computational results (see ESI† Table S1) indicated that the one-electron reduction of pre-**RC** to form **RC** would take place quite facilely.

Based on the formation of **RC**, an electron transfers from the ferrous ion to the substrate, and the carbon-chloride bond nearby the haem iron ($C\text{-}Cl_{proximal}$) breaks synchronously, splitting off a trichloromethyl radical (see ESI† Scheme S1). The energetics of the C-Cl bond cleavage in such a case is presented in Fig. 2. We can conclude from this figure that the

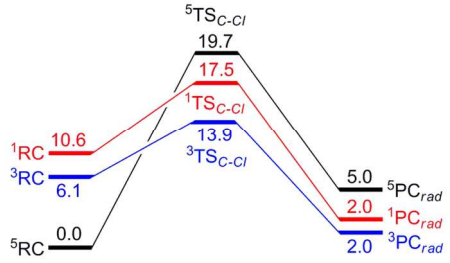

Fig. 2 Energy profiles (in kcal/mol) for the initial reduction of CCl_4 calculated with UB3LYP/B2//B1 on three spin states. All energy data include ZPE, solvation and dispersion corrections.

energy barrier of C-Cl bond cleavage is 19.7/13.9/17.5 kcal/mol for the quintet/triplet/singlet spin state. Compared with **RC**s, the product complexes ($\mathbf{PC}_{rad}$) are thermodynamically unfavorable. This can be due to the high reactivity of the

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Table 1 Mulliken group spin densities and group charges for key moieties during the initial reduction of CCl_4 by P450. All data were computed at the UB3LYP/B1 level.

	Spin densities					*Charges*				
	Fe	Por	SH	Cl	CCl_3	Fe	Por	SH	Cl	CCl_3
^{6}pre-**RC**	3.91	0.60	0.48	0.00	0.00	0.64	-0.57	-0.08	0.12	-0.12
^{4}pre-**RC**	2.48	0.04	0.48	0.00	0.00	0.45	-0.35	-0.11	0.12	-0.11
^{2}pre-**RC**	1.16	-0.11	-0.05	0.00	0.00	0.26	-0.34	0.05	0.12	-0.10
^{5}**RC**	3.65	0.20	0.15	0.00	0.00	0.44	-1.03	-0.38	0.16	-0.19
^{3}**RC**	1.91	-0.11	0.21	0.00	0.00	0.30	-0.89	-0.38	0.12	-0.15
^{1}**RC**	0.00	0.00	0.00	0.00	0.00	0.17	-0.87	-0.29	0.15	-0.15
^{5}**TS**$_{C\text{-}Cl}$	3.28	0.14	-0.09	0.11	0.56	0.34	-0.78	-0.15	-0.20	-0.21
^{3}**TS**$_{C\text{-}Cl}$	1.50	-0.05	0.07	0.08	0.40	0.17	-0.61	-0.20	-0.07	-0.29
^{1}**TS**$_{C\text{-}Cl}$	0.47	-0.10	0.03	-0.10	-0.30	0.05	-0.70	-0.14	0.02	-0.24
^{5}**PC**$_{rad}$	2.57	-0.05	0.36	0.26	0.87	0.34	-0.47	-0.27	-0.44	-0.16
^{3}**PC**$_{rad}$	1.07	-0.07	0.01	0.08	0.92	0.10	-0.54	-0.11	-0.34	-0.11
^{1}**PC**$_{rad}$	1.06	-0.08	0.01	-0.08	-0.91	0.09	-0.54	-0.10	-0.34	-0.11

generated $\cdot CCl_3$, which is essential for achieving the suicidal inactivation of P450 by CCl_4.

Comparison of the group spin densities of pre-**RC**s and **RC**s (Table 1) with those given by Shaik *et al*.,[55] it can be disclosed that for the unreduced ferric haem complex, the sextet should be in a $^6\Delta_{x^2-y^2}$ state possessing a configuration of $a^2_{2u}\ d^1_{x^2-y^2}\ d^1_{xz}\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}\ \sigma^{*1}_{xy}$, the quartet should be in a $^4A_1$ state with a configuration of $a^2_{2u}\ d^2_{x^2-y^2}\ d^1_{xz}\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}$, and the doublet should be in a $^2\prod_{yz}$ state owning a configuration of $a^2_{2u}\ d^2_{x^2-y^2}\ d^2_{xz}\ \pi^{*1}_{yz}$. While for the reduced ferrous haem complex, the quintet ground state should be in a $^5\prod_{yz}$ state with a configuration of $a^2_{2u}\ d^2_{x^2-y^2}\ d^1_{xz}\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}\ \sigma^{*1}_{xy}$, the triplet should be in a $^3\prod_{yz}$ possessing a configuration of $a^2_{2u}\ d^2_{x^2-y^2}\ d^1_{xz}\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}$, and the singlet should be in a $^1A_1$ state with a configuration of $a^2_{2u}\ d^2_{x^2-y^2}\ d^2_{xz}\ \pi^{*2}_{yz}$. Inspection of the spin densities and charges (Table 1), it can be inferred that the C-Cl bond cleavage is homolytic with a spin of *ca*. 1 on the CCl_3 group in **PC**$_{rad}$. To reveal the genuine mechanism involved in the initial reduction process, we also studied the changes of the spin densities on "CCl_4-Cl" (solid line) and "iron+porphine+thiolate" (dash line) moieties (Fig. 3). It is obvious that as the C-Cl distance increases, the spin density on the former moiety increases while that on the latter one decreases gradually. Thus, we conclude that the electron-transfer and the bond breakage are concerted, which is consistent with the finding of de Visser *et al*.[49] The consistence of our results with the previously reported ones also proves the reliability of the computational approaches and models adopted in the present study.

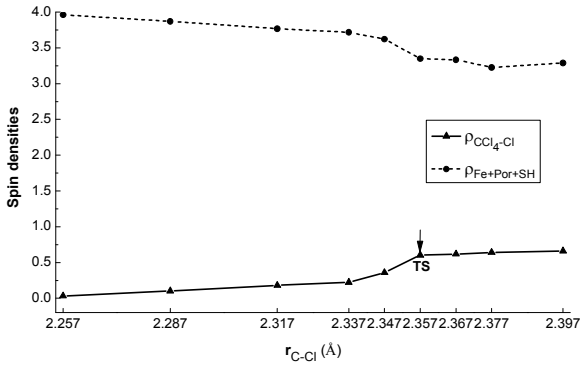

Fig. 3 Changes in the spin densities (ρ) on Iron+Porphine+Thiolate (Fe+Por+SH) and Substrate-Chloride (CCl_4-Cl) moieties as a function of the C-Cl distance, which are calculated at the UB3LYP/B1 level in the gas-phase.

The above results indicate that the reduced ferrous haem complex was responsible for the reduction of CCl_4 to $\cdot CCl_3$, which demonstrates the reasonability of part of experimental speculations. While, some research groups speculated that the reduced ferrous haem complex needed a further one-electron reduction to complete this conversion. Thus, we assessed this kind of speculation *via* calculating the VEA of **RC**. **RC**, with a large negative VEA value (see ESI† Table S1), is a so bad electron-acceptor that the further reduction is less thermodynamically favorable, *i.e.*, a further reduction of the ferrous haem complex is impossible. Meanwhile, the C-Cl bond nearby the haem iron (C-Cl$_{proximal}$) is activated, eliminating a chloride anion from the substrate to produce a free trichloromethyl radical. Whereas, from the optimized geometries of **RC**s (Fig. 1), it is obvious that the C-Cl bond far from the haem iron (C-Cl$_{distal}$) with a longer distance is likely to be broken much easier than the C-Cl$_{proximal}$ bond. Thus, additional calculations were performed to test the possibility of the C-Cl$_{distal}$ bond activation. The scan energies kept increasing (Fig. 4a), which invalidated the activation process of the C-Cl$_{distal}$ bond. Furthermore, we performed computations to reveal the reducing capacity of the unreduced ferric haem complex for the substrate. As with the case of the C-Cl$_{distal}$ bond activation

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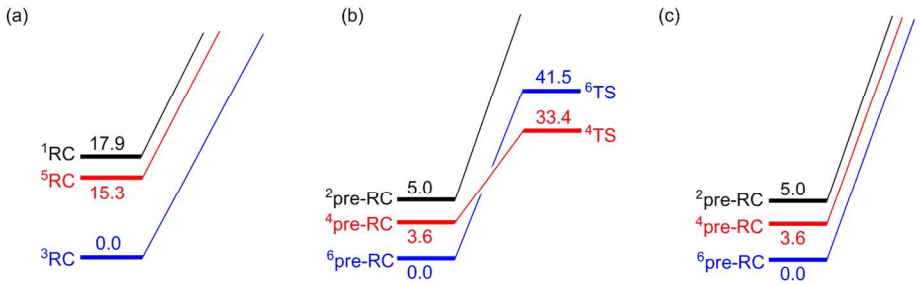

Fig. 4 Scan energy profiles (in kcal/mol) at the UB3LYP/B1 level in the gas phase for the activation of (a) C-Cl_{distal} bond by the ferrous haem complex; (b) C-$Cl_{proximal}$ bond by the ferric haem complex; (c) C-Cl_{distal} bond by the ferric haem complex.

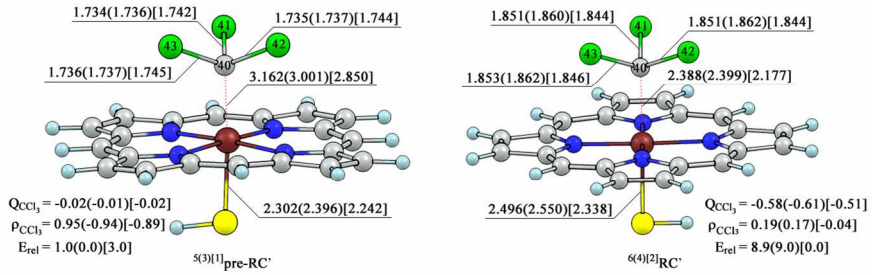

Fig. 5 Geometries of the ferric-·CCl_3 complex (pre-**RC**') and the ferrous-·CCl_3 complex (**RC**') optimized at the UB3LYP/B1 level in the gas phase (bond distances indicated in Å) and the relative energies of various spin states (in kcal/mol) calculated at the UB3LYP/B2 level including ZPE, solvation and dispersion corrections.

by the ferrous haem complex, the scan energies of both C-$Cl_{proximal}$ and C-Cl_{distal} kept increasing (Figs. 4b and 4c), which excluded the direct reduction of CCl_4 by the ferric haem complex.

The formation of dichlorocarbene

Previous experiments have verified the formation of :CCl_2 during the reductive metabolism of CCl_4 by P450 using spectroscopic approaches.[13,19,74] The absorption band at 460nm was attributed to a complex of dichlorocarbene with the ferrous haem. Therefore, the mechanism of :CCl_2 formation was investigated. The geometric features of complexes of ·CCl_3 with the ferric and ferrous haem optimized at the UB3LYP/B1 level, are displayed in Fig. 5. Compared with that in Fig. 1, it is obvious that the ferrous-·CCl_3 complex (**RC**') is hexa-coordinated, in which the carbon atom of ·CCl_3 serves as the sixth ligand binding to the iron. Such hexa-coordinated complex has a doublet ground state, which is also in line with the finding of Shaik *et al.*[55,60] Whereas, the ferric-·CCl_3 complex (pre-**RC**') still keeps penta-coordinated with a C-Fe distance approximating to 3.0 Å. The ferrous-·CCl_3 was obtained after one-electron uptake by the ferric-·CCl_3 complex, and might quickly isomerize to its electromer, a ferric-CCl_3 anion ($^{-}CCl_3$) complex. According to the spin densities (ρ_{CCl_3}) and charges shown in Fig. 5, we can infer that the CCl_3 moiety in pre-**RC**' keeps a radical, while that in **RC**' becomes a carbanion, *i.e.*, the ferrous-·CCl_3 complex stably exists in its electromer form, a ferric-$^{-}CCl_3$ complex. It is worth mentioning that previously reported experimental investigations[13,23,45,48] have proven the necessity of a further one-electron reduction for the ferric-$^{-}CCl_3$ complex to eliminate a second chloride anion, forming a relatively stable ferrous-:CCl_2 complex. But, our calculated results invalidated such speculation, as a consequence of the large negative VEA value of the ferric-$^{-}CCl_3$ complex (see ESI† Table S1).

After the formation of the ferric-$^{-}CCl_3$ complex, a second chloride anion should be eliminated with the assistance of an additional proton, which was mimicked by H_3O^+, *via* a S_E3 mechanism. Since, the direct elimination without any assistance presented a substantial barrier (29.3kcal/mol, see ESI† Fig. S1). Fig. 6 shows the energy profile for such proton-mediated elimination process. We can see clearly that with the assistance of proton, it is nearly barrierless with an energy barrier of 0.5 kcal/mol for the doublet ground state. This is similar to the

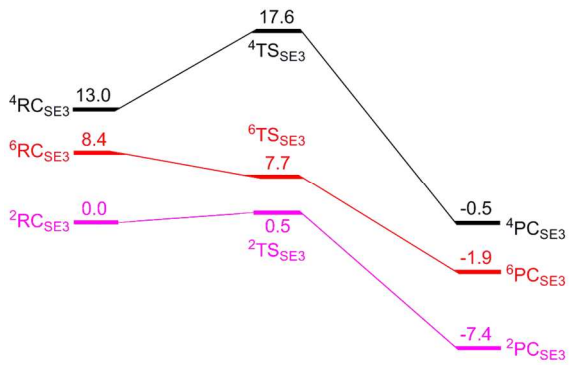

Fig. 6 Energy profiles (in kcal/mol) for the reduction of ·CCl_3 *via* a S_E3 mechanism calculated at the UB3LYP/B2//B1 level on three spin states. All energy data include ZPE, solvation and dispersion corrections.

finding of Hirao *et al*. about the effect of water on the reaction.[75] Meanwhile, the large energy barrier differences among these three spin states indicate that the present reaction is spin-sensitive as the cases of different H-abstraction processes mediated by P450, [62,76,77] but not proceeds *via* a TSR mechanism as the typical alkane hydroxylation reaction by Cpd I.[50,59,60,63] Based on the results mentioned above, we can conclude that the reaction will proceed predominantly *via* the doublet ground state. There is no significant spin density change through $\mathbf{RC}_{S_E3}$, $\mathbf{TS}_{S_E3}$ and $\mathbf{PC}_{S_E3}$ (see ESI† Table S4), thus this process is an elimination of chloride anion. The product complex ($\mathbf{PC}_{S_E3}$) is thermodynamically stable compared with the reactant complex ($\mathbf{RC}_{S_E3}$). However, the spectral experiments have indicated the formation of a ferrous-:CCl_2 complex.[13,19,74] Thus, we assessed the reducing capacity of ferric-:CCl_2 complex *via* calculating its VEA. The computational results indicate that the ferric-:CCl_2 complex is a better electron-acceptor than ferric-$^{-}CCl_3$ (see ESI† Table S1). Therefore, a thermodynamically more stable ferrous-:CCl_2 complex is generated from the one-electron reduction of the ferric-:CCl_2 complex, which is similar to the intermediate complex obtained by Hirao *et al*.[78] The facility of such reduction and the stability of the generated ferrous-:CCl_2 complex further support the findings of spectral experiments.[13,19,74]

Destruction of P450 by CCl_3 radical

Besides the further reduction of the generated ·CCl_3, alternative pathways proposed by experiments involve the covalent binding to the microsomal lipid and protein,[19,79-81] as well as attack and irreversible modification of the prosthetic group or the apoprotein of cytochrome P450.[12,13] Manno *et al*.[13] have proven that CCl_4-dependent P450 destruction was achieved by ·CCl_3 but not the :CCl_2 species, through irreversible modification of the prosthetic group. Thus, the covalent binding of ·CCl_3 to the pyrrole nitrogen and the *meso*-carbon was investigated respectively. Fig. 7 presents the energy profiles for these two possible inactivation processes. Comparison of the

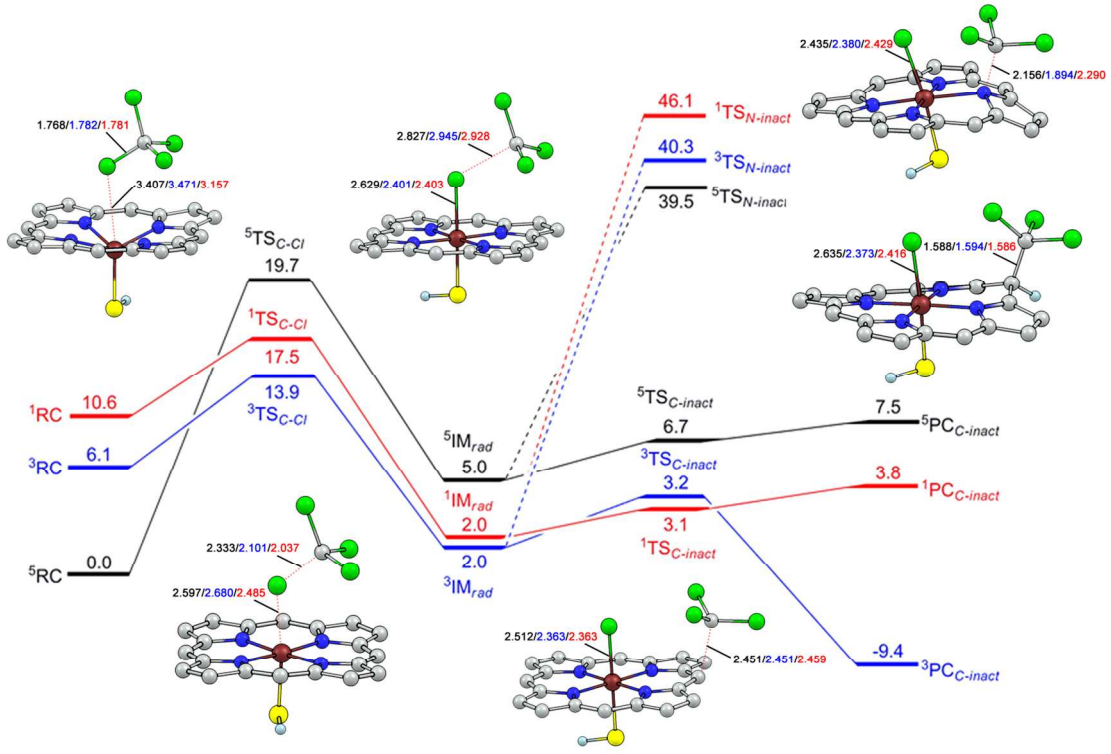

Fig. 7 Energy profiles (in kcal/mol) for the destruction of P450 by ·CCl_3 through covalently binding to the *meso*-carbon (solid line) and pyrrole nitrogen (dash line) for three spin states. All energy data were calculated at the UB3LYP/B2//B1 level including ZPE, solvation and dispersion corrections. The optimized geometries of key species are also shown, with bond distances indicated in Å.

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energy barriers, it is obvious that covalent binding to the *meso*-carbon should be responsible for the CCl_4-dependent P450 destruction with lower energy barriers (3.1 kcal/mol for the singlet state, 3.2 kcal/mol for the triplet and 6.7 kcal/mol for the quintet). Such low energy barriers also demonstrate the high reactivity of $\cdot CCl_3$. Inspection of the geometries depicted in Fig. 7, we can see that the $\mathbf{TS}_{N\text{-}inact}$ with a shorter C-N distance (2.156/1.894/2.290 Å for quintet/triplet/singlet spin state) should occur later than $\mathbf{TS}_{C\text{-}inact}$ with a longer C-C distance (*ca*. 2.5 Å for three spin state), which is in line with the corresponding energy barrier. Furthermore, we speculate that the high activation energy barrier for covalent binding to the pyrrole nitrogen ($\mathbf{TS}_{N\text{-}inact}$) should be due to the higher magnitude of the macrocycle structure breakage, which destabilizes the coordinated iron complex. In addition, during the inactivation process, the generated chloride anion kept coordinating with the iron to stabilize the whole complex.

Conclusion

The initially and further reductive metabolisms of CCl_4 by P450 enzymes as well as the inactivation of P450 by the generated reactive metabolite, trichloromethyl radical, were investigated using quantum chemical calculations in the present study. The computational results indicate that the ferric haem complex should be reduced to the ferrous haem complex to achieve the reduction of substrate CCl_4. In other words, the reducing capacity of the unreduced ferric haem complex was too low to catalyze the reductive dehalogenation of carbon tetrachloride. A reactive metabolite, trichloromethyl radical ($\cdot CCl_3$), was generated after the initial one-electron reduction of CCl_4 by the ferrous haem complex. Based on the formation of such radical species, either a further reduction could take place to yield another reactive metabolite, dichlorocarbene ($:CCl_2$), or covalent binding to the *meso*-carbon atom of the prosthetic group occurred. As with the experimental findings of Manno *et al*.,[13] it is the initial one-electron reduction product of CCl_4, $\cdot CCl_3$, that conceivably attacks the haem moiety leading to the suicidal destruction of P450 enzymes. However, the latter reactive species obtained from two subsequent one-electron reduction of CCl_4, $:CCl_2$, is just involved in the CCl_4-dependent P450 inhibition as its hydrolysis product, CO, but is not significantly responsible for the CCl_4-dependent P450 destruction. Therefore, we can conclude that the reductive metabolism of CCl_4 to reactive intermediates by P450 enzymes is an essential prerequisite for its toxicity.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Proposed reductive mechanism of CCl_4 mediated by P450 enzymes, various energies and the Mulliken population analysis of the S_E3 mechanism, as well as the Cartesian coordinates of the key reaction species. See DOI: 10.1039/b000000x/

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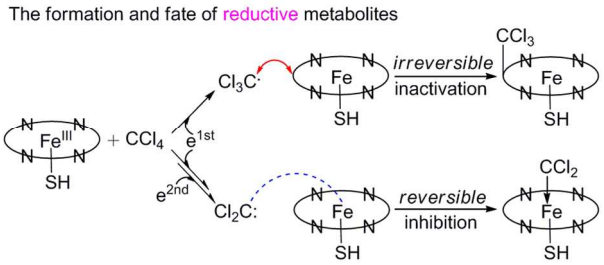
Graphical Abstract



The one-electron reduction product, $\cdot CCl_3$, irreversibly inactivates P450 *via* covalently binding to the *meso*-carbon; whereas the two successive one-electron reductions product, $:CCl_2$, reversibly inhibit P450 by coordinating to iron.