Conformational Dynamics Detected by Nuclear Magnetic Resonance NOE Values and J Coupling Constants[⊥]

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Abstract: Conformational relevant NMR parameters such as NOE values and coupling constants depend in a nonlinear way on distances and dihedral angles. This may be misleading in the determination of molecular conformations when a conformational equilibrium exists with rates that are fast on the NMR time scale: short nuclear distances are overemphasized when distances obtained by NOEs are used as a tool for modelling the conformation in solution. Antamanide, a cyclic decapeptide, is shown to be such a case. However, molecular dynamics calculations with NOE constraints can be used to identify crucial NOE values and prove the evidence of a conformational equilibrium. In addition, homonuclear and heteronuclear coupling constants provide additional support for the existence of "the" conformation or the conformational equilibrium, respectively.

Conformational analysis of biomolecules in solution by NMR spectroscopy is mainly based on atom–atom distance information obtained from the quantitative evaluation of NOESY spectra. Having obtained a set of atom–atom distance constraints, the next step is to obtain a conformation that satisfies all the constraints. This can be done by performing a molecular dynamics (MD) simulation in which a restraining term is added to the potential energy function which drives the molecule toward a conformation that satisfies the constraints. The obtained conformation is checked for violations of the NOE distance constraints. Further independent NMR parameters which provide conformational information such as homonuclear[1-3] and heteronuclear[4,5] coupling constants, which have become available recently for larger molecules, are normally not included as restraints in the MD calculations (but see ref 6). The conformation obtained from MD, however, is also checked for agreement with those parameters. If there is good agreement with all NMR parameters, the conformation is assumed to represent the solution conformation, although there may be other low-energy conformations satisfying the NMR parameter set. If the obtained conformation does not fit all NMR parameters this may be due to errors in the experimental parameters or to the occurrence of multiple conformations of the molecule, which generate an NMR parameter set that cannot be satisfied by *one* conformation. Such a situation was postulated several times[7,8] but up to now there has been no experimental evidence found. We will present here the conformational analysis of the cyclic decapeptide antamanide for which the available NOE and J coupling data cannot be satisfied by one conformation of this molecule. At least two conformations are required.

Conformational homogeneity means that one set of signals in the NMR spectrum corresponds to one molecular conformation. We call a conformation a class of structures of a molecule that transform into each other via changes of torsion angles with rates larger than or equal to the inverse global correlation time τ_{rot}, determined by the rotational rate of the molecule. Two classes of structures which interconvert with a rate *smaller* than the inverse of this correlation time are called two conformations in dynamic exchange. When they interconvert fast on the NMR time scale they give rise to a single set of signals in the NMR spectrum.[9] The "NMR time scale" is to be interpreted as the inverse of the frequency difference between the resonances of both conformations, or as the spin–spin relaxation rate if no chemical shift differences occur.

Generally, the experimental NMR parameters L_{exp}, such as NOE values or J coupling constants, are the weighted (by the populations p_i) mean values of the NMR parameters L_i of the individual conformations i:

$$L_{exp} = \sum_i p_i L_i \tag{1}$$

NMR parameters L_i usually depend in a nonlinear way (function f) on their associated structural parameters S_i. For example, NOE $= f(r) \sim r^{-6}$ where r denotes the distance between cross-relaxing nuclei. In that case L cannot be obtained from the weighted mean S of the structural parameters S_i of the individual conformations involved:[7]

$$L = \sum_i p_i L_i = \sum_i p_i f(S_i) \neq f(\sum_i p_i S_i) = f(S) \tag{2}$$

When calculating NMR parameters from MD trajectories, the parameters are not calculated from the time-averaged MD structure, but they are calculated for each time step of the trajectory and subsequently averaged.[10] The effective NOE atom–atom distance $r_{eff}(i,j)$ between i and j is obtained from

$$r_{eff}(i,j) = \langle r_{ij}^{-3} \rangle^{-1/3} \tag{3}$$

where $\langle ... \rangle$ denotes averaging over a trajectory.

Equation 3 is based on the fact that the NOE effect is determined by dipolar interactions that persist over the time scale of the rotational motions of the molecule. Fast fluctuations on the picosecond time scale of MD simulations do not contribute significantly and should be averaged out. Following Tropp[11] and simplifying for the case of isotropic rotational diffusion with

[⊥] Dedicated to Prof. Dr. Th. Wieland on the occasion of his 75th birthday.

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0002-7863/88/1510-3393$01.50/0

Table I. Effective Proton–Proton Distances r_{eff} in Antamanide which Deviate Most from the Starting Conformation (X-ray) or ⟨MD⟩ Trajectory Calculated Distances[a] (Strong Violations Are Underlined)

	5 PheNH–4 AlaC$_\alpha$	5 PheNH–5 PheH$_\alpha$	5 PheH$_\alpha$–10 PheH$_\alpha$	6 PheNH–5 PheH$_\alpha$	6 PheNH–10 PheH$_\alpha$	10 PheNH–9 PheH$_\alpha$	10 PheNH–10 PheH$_\alpha$
exptl[b]	2.4	2.5	2.5	2.5	3.0	2.7	2.2
X-ray[c]	3.7	2.3	3.0	3.2	4.0	3.6	2.1
MD[d]	2.2 (0.2)	2.8 (0.1)	2.4 (0.2)	2.2 (0.1)	2.5 (0.3)	2.3 (0.2)	2.8 (0.1)
MD ⟨A⟩[e]	3.5 (0.1)	2.0 (0.1)	2.6 (0.2)	3.4 (0.1)	5.5 (0.2)	3.5 (0.1)	2.0 (0.1)
MD ⟨B⟩[f]	2.2 (0.1)	2.8 (0.1)	2.3 (0.2)	2.1 (0.1)	2.5 (0.2)	2.3 (0.2)	2.8 (0.1)
MD ⟨AB⟩[g]	2.5 (0.7)	2.3 (0.4)	2.4 (0.3)	2.5 (0.7)	3.0 (1.8)	2.6 (0.6)	2.3 (0.4)
MD ⟨C⟩[h]	2.3 (0.2)	2.8 (0.1)	2.2 (0.2)	2.3 (0.2)	2.9 (0.4)	3.4 (0.1)	2.2 (0.1)
MD ⟨D⟩[i]	3.5 (0.1)	2.0 (0.1)	2.2 (0.2)	3.3 (0.2)	5.0 (0.2)	2.3 (0.2)	2.8 (0.1)
MD ⟨CD⟩[j]	2.6 (0.7)	2.3 (0.4)	2.2 (0.2)	2.6 (0.6)	3.4 (1.2)	2.6 (0.6)	2.4 (0.3)
MD ⟨ABCD⟩[k]	2.6 (0.7)	2.3 (0.4)	2.3 (0.3)	2.6 (0.7)	3.2 (1.5)	2.7 (0.6)	2.3 (0.4)

[a] All distances and fluctuations (in parentheses) are given in Å. [b] Experimental values obtained from 500 MHz NOESY spectra at two different mixing times in $CDCl_3$ ($T = -25$ °C). [c] Distances calculated from the published X-ray structure.[16] [d] Calculated distances from the trajectory between 13 and 33 ps using all 40 constraints. [e] Calculated distances starting from the X-ray structure omitting the 7 constraints given in this table. Mean from 10 to 20 ps. [f] Calculated distances starting from the ⟨MD⟩ structure omitting the 7 constraints given in this table. Mean from 10 to 20 ps. [g] Mean distances calculated from the trajectories mentioned under *e* and *f* assuming a 1:1 equilibrium. [h] Calculated distances starting from a conformation, in which $\phi_5 = -80°$ and $\phi_{10} = +80°$ were initially restrained for 5 ps. After that the dihedral restraining was removed and the 33 constraints were included (5 ps with k_{dc} = 4000 kJ/(mol·nm), 5 ps with k_{dc} = 1000 kJ/(mol·nm)). Averaging over the following 10 ps including the 33 constraints (k_{dc} = 1000 kJ/(mol·nm)) yields ⟨C⟩. [i] Same as *h*, but started from $\phi_5 = +80°$, $\phi_{10} = -80°$, yields ⟨D⟩. [j] Mean over ⟨C⟩ and ⟨D⟩; each 50%. [k] Mean over ⟨A⟩, ⟨B⟩, ⟨C⟩, ⟨D⟩; each 25%.

correlation time τ_{rot} longer than the inverse angular Larmor frequency, we can write for the NOE cross relaxation rate σ_{ij}

$$\sigma_{ij} = -\frac{2\pi}{5}(\gamma_H{}^2\hbar)^2 \int_0^\infty \exp(-t/\tau_{rot}) f(t)\, dt \qquad (4)$$

where $f(t)$ is the correlation function of the dipolar interaction in a molecule-fixed coordinate frame

$$f(t) = \frac{1}{5}\sum_{n=-2}^{2}\left\langle \frac{Y_2^{-n}\{\Theta(0),\varphi(0)\}Y_2^{n}\{\Theta(t),\varphi(t)\}}{r^3(0)r^3(t)}\right\rangle \qquad (5)$$

Here $Y_2^n\{\Theta,\varphi\}$ are normalized spherical harmonic functions of the orientation angles of the H–H interaction vector r in the molecular frame. The angular term does not fluctuate significantly compared to the fluctuation in r^{-3} and can be considered as essentially constant. (Although the full angular term could be retained,[12] this assumption simplifies the calculations from MD considerably). The sum over the angular terms now becomes constant, so that a correlation function remains that decays rapidly from its initial value $\langle r^{-6}\rangle$ to a final value $\langle r^{-3}\rangle^2$, with a time constant τ_c. Assuming exponential decay for the fast relaxation, $f(t)$ has the form

$$f(t) = \langle r^{-3}\rangle^2 + \{\langle r^{-6}\rangle - \langle r^{-3}\rangle^2\}\exp(-t/\tau_c) \qquad (6)$$

and the integral in eq 4 becomes

$$\langle r^{-3}\rangle^2\tau_{rot} + \{\langle r^{-6}\rangle - \langle r^{-3}\rangle^2\}\tau_{rot}\tau_c/(\tau_{rot} + \tau_c) \qquad (7)$$

For τ_c in the ps range (as sensed by MD) the second term can be neglected, so that $\sigma_{ij} \propto \langle r^{-3}\rangle^2$, as used in eq 3. For motions that are much slower than the rotational rate, $\tau_c >> \tau_{rot}$, expression 7 and hence σ_{ij} becomes proportional to $\langle r^{-6}\rangle$. So if there are different conformations exchanging on a time scale longer than the rotational correlation time, the weighted average of r_{eff}^{-6} determines the NOE.

The ^3J coupling constants are calculated from[13]

$$^3J_{KLMN} = \langle A\cos^2\Theta + B\cos\Theta + C\rangle \qquad (8)$$

where Θ denotes the dihedral angle between K and N. The experimentally adjusted parameters A, B, and C have been taken from ref 13. They are $A = 9.4$, $B = -1.1$, and $C = 0.4$ for $^3J_{HNC_\alpha H}$ and $A = 9.0$, $B = -4.4$, and $C = -0.8$ for $^3J_{C'NC_\alpha H}$.

Antamanide, the cyclic decapeptide cyclo(-Val[1]-Pro[2]-Pro[3]-Ala[4]-Phe[5]-Phe[6]-Pro[7]-Pro[8]-Phe[9]-Phe[10]-),[14] was analyzed with 2D-NMR methods.[15] NOEs taken from a 2D-NOESY spectrum and also from 1D-NOE difference spectra provided a set of 40 distance constraints.

The available X-ray structure[16] satisfies 35 distance constraints, while violating 5 NOE distances. These are underlined in Table I. Therefore, the molecule was simulated over a period of 33 ps using the GROMOS molecular dynamics simulation package and applying an atom–atom distance restraining potential to the 40 NOE distances in the usual way.[17-20] Pseudoatom sites representing nonspecifically assigned NOEs were defined as in ref 20. In this study we used the NOE distance constraints as an upper limit r_0 to the proton–proton distances, that is, we applied a one-sided harmonic oscillator potential V_{NOE}, only attractive beyond the distance r_0. A repulsive part, $r > r_0$, could also be applied,[21] in a case where very accurate distances r_0 are available. The proton–proton potential, V_{NOE}, is only applied to guide the molecule to a conformation that satisfies the proton–proton distance constraints. Since the complete interatomic interaction function already contains repulsive van der Waals terms, the addition of a repulsive term based on NOE lower limit proton–proton distances does not significantly improve the obtained convergence to $V_{NOE} = 0$. Moreover in the presence of motional averaging as observed here a physically correct choice of a lower limit r_0 is not well possible: The experimental lower limits r_0 represent an average, and single molecular configurations of a trajectory may well violate these limits ($r < r_0$), while the average over the trajectory equals r_0. The effective NOE distances r_{eff} calculated from the atomic trajectories satisfy 38 NOE distances, only two are violated (underlined in row 3, Table I) (Note that distances larger than the experimental ones are violations. Also some shorter distances are found, but their deviations from r_0 disappear in the final averaging process, see below.) Comparing X-ray and ⟨MD⟩ NOE distances, it is observed that NOEs are

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Table II. Homonuclear Coupling Constants $^3J_{HNC^{\alpha}H}$ (in Hz) in Antamanide in $CDCl_3$ Solution[a] (Fluctuations Are Given in Parentheses)

	1 Val	4 Ala	5 Phe	6 Phe	9 Phe	10 Phe
exptl	7.3	8.6	6.8	6.6	8.3	6.7
X-ray	10.3	10.2	8.0	6.5	10.1	8.4
MD	7.4 (1.5)	7.9 (1.9)	7.5 (1.9)	10.2	6.7 (1.6)	7.1 (1.4)
MD ⟨A⟩	4.2 (1.3)	4.7 (2.0)	8.2 (0.2)	1.0 (0.7)	4.2 (1.4)	8.1 (0.3)
MD ⟨B⟩	10.3 (0.3)	7.7 (1.5)	8.6 (1.3)	10.1 (0.6)	6.8 (1.7)	7.5 (1.1)
MD ⟨AB⟩	7.3 (2.4)	6.2 (2.3)	8.4 (0.9)	5.6 (4.6)	5.5 (2.0)	7.8 (0.9)
MD ⟨C⟩	10.0 (1.2)	7.5 (1.8)	7.0 (1.5)	10.4 (0.7)	3.3 (2.5)	7.5 (1.2)
MD ⟨D⟩	10.0 (1.4)	5.7 (2.5)	8.5 (0.3)	1.2 (1.6)	8.8 (1.5)	6.3 (1.4)
MD ⟨CD⟩	10.0 (1.3)	6.6 (2.3)	7.7 (1.3)	5.8 (4.7)	6.1 (3.4)	6.9 (1.4)
MD ⟨ABCD⟩	8.7 (2.9)	6.5 (2.4)	8.2 (1.2)	5.8 (4.7)	5.8 (2.9)	7.5 (1.3)

[a] For explanation see Table I.

Table III. Heteronuclear Coupling Constants $^3J_{C'NC^{\alpha}H}$ (in Hz) in Antamanide in $CDCl_3$ Solution[a] (Fluctuations Are Given in Parentheses)

	1 Val	4 Ala	5 Phe	6 Phe	9 Phe	10 Phe
exptl[b]	–	–	+ (4.4)	–	–	+ (6.6)
X-ray	3.6	3.5	12.1	1.1	3.5	12.6
MD	3.4 (0.5)	2.0 (1.2)	1.7 (1.2)	3.5 (0.4)	1.3 (1.0)	1.5 (0.9)
MD ⟨A⟩	−0.2 (0.9)	0.1 (1.0)	12.4 (0.3)	−0.9 (0.7)	−0.2 (0.7)	12.3 (0.4)
MD ⟨B⟩	3.6 (0.3)	1.9 (1.0)	2.4 (0.8)	3.5 (0.5)	1.3 (1.0)	1.7 (0.7)
MD ⟨AB⟩	1.7 (2.0)	1.0 (1.4)	7.4 (5.0)	1.3 (2.3)	0.5 (1.2)	7.0 (5.3)
MD ⟨C⟩	3.2 (0.8)	1.6 (1.1)	1.3 (0.9)	3.5 (0.5)	−0.2 (1.1)	11.0 (1.6)
MD ⟨D⟩	3.2 (0.9)	0.6 (1.4)	12.3 (0.4)	−0.4 (1.2)	2.4 (0.9)	0.9 (0.8)
MD ⟨CD⟩	3.2 (0.8)	1.1 (1.3)	6.8 (5.5)	1.5 (2.1)	1.1 (1.7)	5.9 (5.2)
MD ⟨ABCD⟩	2.5 (1.7)	1.1 (1.3)	7.1 (5.3)	1.4 (2.2)	0.8 (1.5)	6.5 (5.3)

[a] For explanation see Table I. [b] Experimental values. Qualitative information is obtained from the appearance of cross peaks in the COLOC spectrum.[4,5] (−) No cross peak in COLOC spectrum, coupling constant smaller than about 4–5 Hz; (+) cross peak in the COLOC spectrum, assigning relatively large coupling constants. J values are determined via heteronuclear J-resolved 2D spectroscopy using soft 180° pulses.[23]

either violated by the X-ray structure or by the MD trajectory.

Homo- and heteronuclear coupling constants have been calculated by averaging the MD trajectories with the KARPLUS relationships of eq. 8. The thus obtained $^3J_{HNC_{\alpha}H}$ coupling constants are compared with the experimental values in Table II. It seems that the observed J values except those for Phe[6] are in agreement with the ⟨MD⟩ values and further away from the values expected for the X-ray structure. However, striking evidence for the presence of the X-ray structure in the conformational equilibrium in solution is given by the heteronuclear coupling constant $C'_{i-1}H^{\alpha}_i$. The inspection of COLOC spectra[22] with respect to peak intensities provides qualitative information about such coupling constants as has been shown for $^3J_{CC^{\alpha}C^{\beta}H}$, couplings[4,5] and for heteronuclear coupling constants for the determination of torsion angles of the peptide backbone.[5,23] In combination with $^3J_{HNC^{\alpha}H}$ homonuclear coupling constants $^3J_{C'NC^{\alpha}H}$ can be used to determine φ angles.

The COLOC spectrum of antamanide (see Figure 11 in ref 14) shows that there are no $C'_{i-1}NC^{\alpha}H^{\alpha}_i$ cross peaks except for the Phe^5H^{α}/Ala^4C' and $Phe^{10}H^{\alpha}/Phe^9C'$. This result suggests $\varphi_5 \approx 80°$ instead of −87 (fluctuation 13.0) and $\varphi_{10} \approx 40°$ instead of −83 (fluctuation 9.1) as found in the ⟨MD⟩ conformation. These two φ values are exactly those by which the crystal and ⟨MD⟩ conformations are distinguished.

This discrepancy between the "NOE structure" and the "J structure" led us to investigate the NOE values in more detail. With the assumption that two different conformations are in equilibrium (fast on the NMR time scale), the findings become explainable. We assume one conformation (A) which resembles more or less the X-ray structure and another (B) which resembles the MD trajectory. In order to generate trajectories representing the two distinct conformations A and B we have performed two more MD simulations from which the 7 constraints listed in Table I were omitted: one denoted by MD (A) which was started from the X-ray structure and another denoted by MD (B) which was started from the ⟨MD⟩ structure. The NMR parameters obtained from the trajectories belonging to conformations A and B are displayed in the tables. The NOE distances and a few J coupling constants are quite different in both, A and B, and neither of the conformations matches the experimental values. However, if we calculate the NMR parameters from both A and B trajectories, that is, if we for the first approximation assume that conformations A and B are equally populated ($p_A = p_B = 0.5$), all NOE distances are satisfied and the largest deviations from the experimental vicinal coupling constants have vanished. Only two homonuclear ^3J values are not reproduced within the fluctuation: those for Phe[5] and Phe[9]. However, one should bear in mind that J values are rapidly changing functions of the torsion angle, so a slight change in the torsion angle or in the populations may change the J value by a few units, especially for intermediate J values. The fluctuations obtained by averaging over conformations A and B are much larger than the ones belonging to the individual conformations A and B. This indicates the distinctness of both conformations.

Kinetic measurements with ultrasonic methods showed already 13 years ago[25] that in solution antamanide adopts at least two conformations which transform into each other by a backbone reorientation with rates of about 10^6 s^{-1}. The two conformations A and B found in our present study may correspond to the observed ones. They can interconvert by a flip of the peptide unit between Phe[9] and Phe[10] and the one between Ala[4] and Phe[5], involving both adjacent angles ψ_i and φ_{i+1}. Such conformational changes are expected to occur easily in peptides. Conformational differences of this type have been observed in different crystals of the same peptide.[26] A full analysis of the simulated conformations will be given elsewhere.

Due to the fact that NMR parameters are sensitive only to short range effects, both amide bond flips may occur independently, actually resulting in four different conformations. This possibility has been investigated in the following way. The A conformation, which is close to the X-ray one, may be characterized by (φ_5,φ_{10}) = (+,+), in fact the X-ray structure has (φ_5,φ_{10}) = (+73,+58). The B conformation, which is close to the ⟨MD⟩ with (φ_5,φ_{10}) = (−89,−83), may be characterized by (−,−). Now, two other conformations are generated: C (−,+) and D (+,−). MD simulations were performed just like for the A and B conformations, and the average NOE distances and J values assuming $P_A = P_B = P_C = P_D$ are found in the last rows of Tables I, II, and III. The

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results show that the experimental data are even slightly better explained by the assumption of an equilibrium between the four conformations A, B, C, and D. However, the alternative of an equilibrium between equal amounts of C and D cannot be excluded experimentally.

In principle, an estimate of the relative populations of the four conformations could be obtained by the calculation of the relative free energies. Different techniques could be used.[27] The configurational entropy of a conformation can be obtained by directly integrating the distribution function, generated by the MD trajectory using harmonic approximation.[28,29] This technique does not allow for incorporation of free energy contributions of the solvent.[27] An alternative is to use umbrella sampling techniques along the pathway leading from one conformation to the other. This method requires a pathway to be postulated but allows the inclusion of solvent contributions to the relative free energy in case a MD simulation in solvent is performed. However, we think that such an approach does not contribute further to the principle of the detection of a conformational equilibrium. Finally, we conclude that when performing conformational analysis on the basis of NMR parameters one must be on the watch for multiple mutually incompatible conformations contributing to the set of NMR parameters. As was shown here, MD simulations may be used to trace such a situation and to get a crude estimate of the conformations involved and their relative populations.

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Acknowledgment. We gratefully acknowledge discussions with Prof. R. Kaptein, Utrecht. Financial support was given by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft.

Theoretical Investigation of the Anaerobic Reduction of Halogenated Alkanes by Cytochrome P-450. 2. Vertical Electron Affinities of Chlorofluoromethanes as a Measure of Their Activity

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Received June 8, 1987

Abstract: The electron-accepting ability of chlorofluoromethanes is determined by both ab initio and MNDO methods. For the full set of molecules (CH_3F, CH_2F_2, CHF_3, CF_4, CH_3Cl, CH_2Cl_2, $CHCl_3$, CCl_4, CH_2FCl, CHF_2Cl, $CHFCl_2$, CF_3Cl, CF_2Cl_2, $CFCl_3$), the vertical electron affinity (VEA) is calculated. The VEA is defined as the negative of the energy change on going from the neutral molecule, at its equilibrium geometry, to the metastable anion, leading to dissociative electron attachment, at the same geometry. The geometry of the neutral molecule is totally optimized at the ab initio and MNDO levels. The ab initio optimization uses 3-21G and 6-31G* basis sets. Single-point calculations are performed at the HF/3-21G optimized geometry on a 6-31G* basis with second-order Moller-Plesset perturbation theory. A direct correlation is found between the VEA of a compound and its activity toward reductive metabolism. Of the nine species that have been experimentally examined, these results correctly predict their relative activity and allow a prediction of the activity of the remaining five compounds.

Several halogenated hydrocarbons are known to be reductively dehalogenated by cytochrome P-450 under anaerobic conditions.[1] As discussed in the first paper of this series, hereafter referred to as I,[2] the first step in this process is the binding of the halocarbon to the enzyme (Figure 1 in I). While large halogenated hydrocarbons are known to act as "type I" substrates binding to a hydrophobic site of the enzyme that is near, but not attached to, the heme unit, halomethanes may loosely interact with the iron. Evidence for this comes from the recent observation that the binding of camphor to the hydrophobic site of $P\text{-}450_{cam}$ from *Pseudomonas putida* does not affect the rate of reductive dehalogenation of CCl_4 and $BrCCl_3$.[3] Wade and Castro have proposed that CH_3I can form a loose affiliation with the iron, producing a change in the heme visible spectrum but no change in the NMR spectrum.[4]

Under reducing conditions, and in the absence of molecular oxygen, an electron can be transferred from the enzyme to the substrate. It has been suggested that the concentration of O_2 may be quite low in the center of liver lobules.[5] This reduced halocarbon dissociates to a halide anion and a haloalkyl radical. The radical can, after a second reductive dehalogenation, form an iron-carbene complex or leave the enzyme and abstract a lipid hydrogen, initiating lipid peroxidation and tissue damage. It may also directly inactivate the enyzyme through further reactions, though the mechanism for this is not well understood.[6]

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0002-7863/88/1510-3396$01.50/0