Comparing cytochrome P450 imitators to model semiempirically a possible rate determining step in the hydroxylation of PCDFs

M.J.C. Rozemeijer, M. Van Der Meulen, P. De Voogt, H. Krop, H.A.J. Govers

Department of Environmental and Toxicological Chemistry, ARISE, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands. M.J.C. Rozemeijer@SARA.NL.

Abstract

The rate determining step in the hydroxylation of polychlorinated dibenzofurans (PCDFs) by cytochrome P450 can be modelled with quantum mechanical techniques. When semiempirical techniques are used, it is necessary to define an imitator for the complicated haemstructure. In this study the characteristics of three imitators were assessed for the first step in the hydroxylation. The reaction mechanism can be characterised as an electrophilic aromatic substitution. The heat of reaction (ΔH_f) was calculated as a measure for the rate determining step. The reaction appeared to be predominantly determined by substrate characteristics. A dominating aspect was substitution at the position of attack. Also other features of the substrate appeared of such importance that the influence of the imitator was negligible. One imitator model was choosen and validated with empirical data on halogenated anilines.

1. Introduction

Polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) represent a group of toxic aromatic compounds. They are persistent to degradation, lipophilic and therefore tend to accumulate in the food-chain. Especially in the higher trophic levels such concentrations can be expected that adverse effects can occur. Most observed effects are waisting syndrome, chloracne, thymus atrophy and reduced immunity[1].

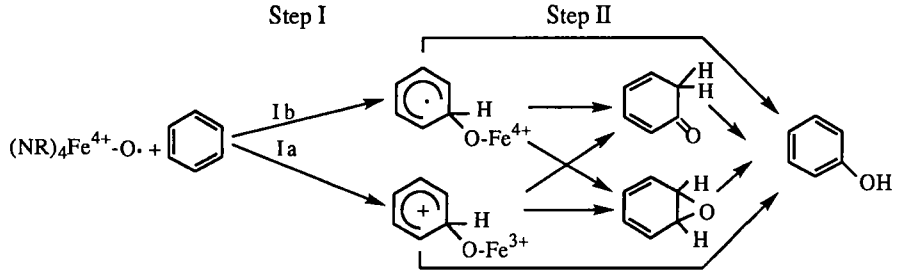

Fig. 1: Proposed mechanisms for the hydroxylation of aromatic compounds by CYP. CYP was symbolised by $(NR)_4Fe^{4+}$. Both oxygen forms (singlet and radical type) were symbolised by -O•.

Because of their lipophilic nature PCDFs cannot readily be excreted from the body. In general the body uses hydrophilic pathways like urine and bile. PCDFs have to be made more hydrophilic by enzymatic transformation. This is done by initial hydroxylation (activation-step) by cytochrome P450 (CYP) and subsequently by conjugating the hydroxy-PCDF to a highly hydrophilic molecule. This enables the use of the bile as excretion route[2].

In general, the first step in hydroxylation by CYP is hydrogen abstraction from the substrate[3]. For aromatics, however, H-abstraction is not likely due to the enormous activation energy required[4]. For PCDFs, in particular, it was shown that H-abstraction did not occur. No difference in rate of hydroxylation could be demonstrated between ^{3}H-TCDF and ^{14}C-TCDF[5]. If H-abstraction was the initial rate limiting step of the metabolism, a pronounced isotope effect should have been observed[6].

As possible mechanism two alternatives have been formulated. The first was an electrophilic substitution by haemgroup with a singlet oxygen on the π-ring. Two electrons are drawn into the binding from which one is used to form the bond and the other is drawn into the Fe (fig. 1, pathway Ia) leaving a positively charged aromatic ring. Similar mechanisms are seen for abiotic electrophilic aromatic substitution. The second mechanism would involve an attack of a haemgroup with triplet type oxygen on the π-ring. The group attaches to the π-ring and forms a bond leaving 4 coupled π-electrons and one radical π-electron (fig. 1, pathway Ib)[5-9]. In our study we will focus on pathway Ia because it appeared impossible to calculate the important aspects of pathway Ib.

The thermodynamic properties of a CYP mediated reaction (activation enthalpy : $\Delta H^{\ddagger}$, entropy: $\Delta S^{\ddagger}$, energy: $\Delta G^{\ddagger}$ and reaction enthalpy : ΔH_r, entropy: ΔS_r and energy: ΔG_r) were modelled with semiempirical methods. For these methods CYP imitators were used because semiempirical methods like AM1, MINDO/3, MNDO are not able to model transition metals. Used imitators for the formation of oxygenated benzenes (step II fig. 1) were oxygen (O) and OH. For the mechanism of H-abstraction *p*-nitrosophenoxy (NP) was used[10,11]. In this study we want to select a good model to calculate one of the possible rate limiting steps: the formation of a cationic intermediate (fig. 1, pathway Ia). The three mentioned imitators which have been used in other circumstances will be tested for our purpose.

2. Material and methods

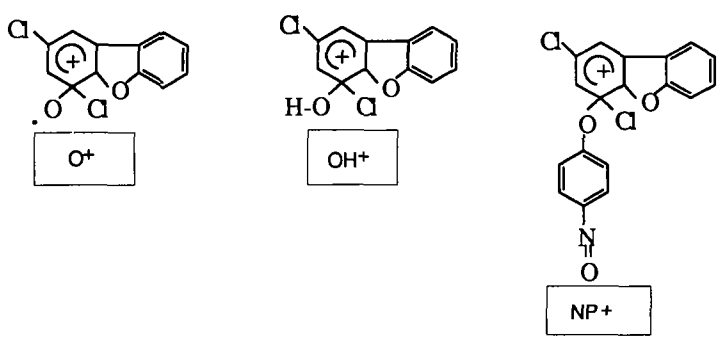

Fig. 2: Molecular structure of the reaction intermediates after step Ia in fig. 1

All molecular structures were drawn and geometrically optimised in SYBYL 6.1. Next the structures were transferred to MOPAC 7.0 where the AM1 Hamiltonian was used to calculate molecular orbitals, geometry and heat of formation (ΔH_f) of the molecular structures. Twenty-five

PCDFs were selected in which all types of substitution were represented. Those PCDFs and three CYP-imitators were calculated. The intermediates were formed by attaching the imitators on the C4 of the PCDF yielding the intermediates O^+, OH^+ and NP^+ (fig. 2).

It was decided to calculate only one position of attack for 25 PCDFs rather than more positions of attack and less PCDFs. Results would be more comparable amongst each other. The C4 position is one of the preferred places to be hydroxylated[1,12]. The positive charge on the intermediates was created by omitting 1 double bond in the attacked PCDF ring and using charge=1. The displaced electron from the PCDF was not placed on the CYP imitator. This would have created more unbalance in the molecule than in the haemgroup which is more apt to accepting and distributing electrons over the Fe and the porphyrine ring[3,13].

Normally the rate of a reaction is related to the $\Delta G^{\ddagger}$ of a reaction whereas equilibrium is connected to ΔG_r. The Brønstedt-theory, however, states that $\Delta G^{\ddagger} \propto \Delta G_r$[6,10]. It was also shown the contribution of ΔS_r is negligible[11]. Therefore it was decided to consider only ΔH_r (calculated according equation 1):

$$\Delta H_r = \Delta H_{f\ intermediate} - (\Delta H_{f\ PCDF} + \Delta H_{f\ CYP\text{-}imitator}) \qquad \text{eq. 1}$$

3. Results and discussion

Preliminary analysis of the data revealed a mayor clustering on basis of the substitution of the C4-atom. Cl-substituted intermediates varied amongst each other but all showed higher ΔH_r values than the H-substituted PCDFs. For each intermediate the data set was divided in two subsets (Cl- and H-substituted). The two mean values of the each intermediate subset varied significantly from each other (table 1). The ΔH_r of the H-substituted PCDFs was more exothermic. This means that according to the Brønstedt approximation the rate of hydroxylation should be much higher. This is in accordance with the fact that PCDFs that are Cl substituted at the 2, 3 or 4 position have a higher retention in the body[1,12]. Thereby all three models prove to be valid in a qualitative sense.

Table 1: Mean ΔH_r values of the formation of the reaction intermediate from a PCDF and a CYP imitator. The PCDFs were divided in two sets: those with H-substitution at the location of attack and those with a Cl- substitution. Mean values were compared with ANOVA.

	H-subst.		Cl-subst.	
	Mean[a]	STD	Mean[a]	STD
O^+	105.3	3.7	120.7[b]	3.4
OH^+	148.0	3.8	158.4[b]	3.3
NP^+	189.9	3.9	203.5[b]	3.8

[a]: ΔH_r in kcal/mol

[b]: significantly different from the H-substituted group at $p<0.0001$

Checking the geometric structures revealed a similar increase in bond lengths for the C-H and the C-Cl bond (data not shown). Since in general the C-Cl bond has less energy content a comparable increase in bond-length (and thereby increase of energy content) should not lead to a higher ΔH_r for the Cl-substituted PCDFs. The higher ΔH_r is due to other causes. The Cl-C4 has a lower net charge (frontier (π) electrons) than the H-C4 (data not shown). Probably more stabilising energy is necessary to perform the electrophilic aromatic substitution. Similar phenomena were observed for abiotic reactions[6] and CYP hydroxylation preferences for PCBs and fluorobenzenes[8,14] and hydroxylation rates for halogenated anilines[15]. A second additional explanation could be a steric effect. The Cl-C-O angle was larger than the H-C-O for all

intermediates. This could also result in higher stabilising energy levels for the Cl-substituted PCDFs.

Table 1 also revealed significant differences between imitators. The O^+ had much lower ΔH_r values than the other two. However, AM1 values cannot be considered in an absolute sense. Results have to be compared in a trend. To choose the appropriate CYP imitator, ΔH_r values were correlated with each other. Again the data sets were divided in PCDFs with Cl- and H-substitution at C4 to prevent that two clusters of points would dominate the correlation rather than the differences between single points. The calculated equations are given in table 2

Table 2: Correlation of ΔH_r values of all cationic intermediates with each other. PCDFs were divided in those that were Cl-substituted at the C-atom of attack, or H-substituted.

Equation		R	n	
Cl-substituted PCDFs:				
$\Delta H_r OH^+ = 0.98\ (\Delta H_r O^+)$	+ 40.2	0.993	13	eq. 2
$\Delta H_r NP^+ = 1.10\ (\Delta H_r O^+)$	+ 70.2	0.993	13	eq. 3
$\Delta H_r NP^+ = 1.10\ (\Delta H_r OH^+)$	+ 25.5	0.997	13	eq. 4
H-substituted PCDFs:				
$\Delta H_r OH^+ = 1.02\ (\Delta H_r O^+)$	+ 40.8	0.998	12	eq. 5
$\Delta H_r NP^+ = 1.05\ (\Delta H_r O^+)$	+ 79.9	0.995	12	eq. 6
$\Delta H_r NP^+ = 1.06\ (\Delta H_r OH^+)$	+ 38.0	0.996	12	eq. 7

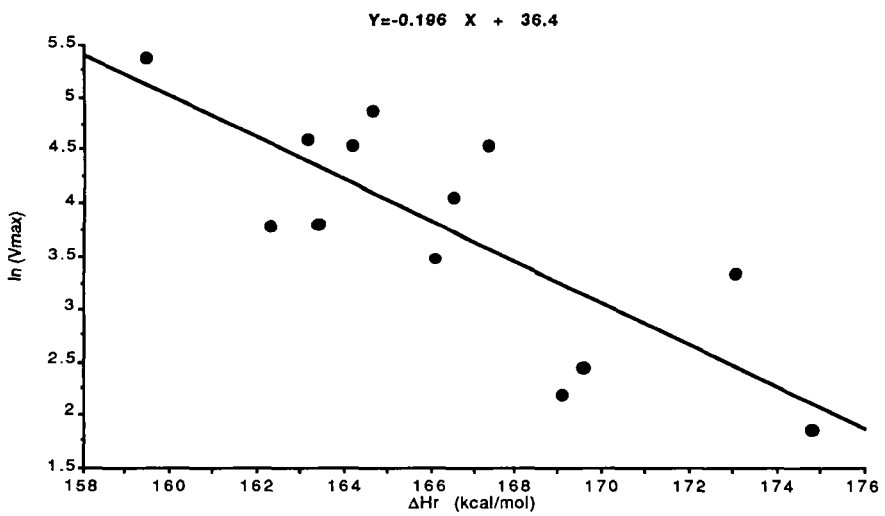

Fig. 3: Correlation of calculated ΔH_r values and the ln V_{max} of halogenated anilines in a iodobenzene supported microsomal assay[15].

All imitators showed excellent correlation's (R>0.99) with each other indicating that ΔH_r is mostly PCDF determined. Also the equations did not differ from each other within one imitator indicating that in principle it is possible to combine both Cl- and H-substituted of one intermediate in one relation. Since it made no difference which imitator was selected it was decided to continue

with NP. This imitator model has been used for the most comparable reaction scheme thus improving comparability[10].

To validate the NP^+-model, the ΔH_f was calculated for the 4 hydroxylation of halogenated anilines. For these substrates the Michaelis-Menten enzyme-kinetic parameters maximum velocity (V_{max}) and substrate affinity (K_m) were available. In this case iodobenzene was used as O-donator because otherwise the splicing of the dioxygen was rate-limiting for this much faster metabolisable substrate[15]. The ln V_{max} was correlated with the ΔH_f revealing a significant correlation (p=0.0014, fig. 3). About 62% of the variation could be explained. Taking the complexity of the reaction into account (caused by the catalytic cycle of CYP itself and by the mixture of CYPs in the microsomes batch used) a R^2 of 0.62 seems quite satisfactory. The enormous simplification of the haemgroup by using NP^+ seems justified. The NP^+ model can be used to elucidate if the rate determining step in PCDF-hydroxylation is the formation of a cationic intermediate.

4. References

1) Safe, S. (1990): Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEF). Crit. Rev. Toxicol.. 21, 51-88.

2) Timbrell, J.A. (1982): Principles of biochemical toxicology. Taylor & Francis LTD, London.

3) Akhtar, M. and J.N. Wright (1991): An unified mechanistic view of oxidative reactions catalysed by P-450 and related Fe-containing enzymes. Natural Products Report 37, 527-551.

4) Griller, D. and K.U. Ingold (1976): Persistent carbon-centered radicals. Acc. Chem. Res. 9, 13-19.

5) Olson, J.R., B.P. McGarrigle, P.J. Gigliotti, S. Kumar and J.R. McReynolds (1994): Hepatic uptake and metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzofuran. Fund. Appl. Toxicol. 22, 631-640.

6) Morrison, R.T. and R.N. Boyd (1983): Organic chemistry, Allyn and Bacon Inc., Boston, London, Sydney, Toronto.

7) Burka, L.T., T.M. Plucinski and T.L. Macdonalds (1983): Mechanism of hydroxylation by cytochrome P-450: Metabolism of monohalobenzenes by phenobarbital-induced microsomes. Proc. Natl. Acad. Sci. USA. 80, 6680-6684.

8) Rietjens, I.M.C.M., A.E.M.F. Soffers, C. Veeger and J. Vervoort (1993): Regioselectivity of cytochrome P-450 catalyzed hydroxylation of fluorobenzenes predicted by calculated frontier orbital substrate characteristics. Biochem. 32, 4801-4812.

9) Riley, P. and R.P. Hanzlik (1994): Electron transfer in P450 mechanisms. Microsomal metabolism of cyclopropylbenzene and p-cyclopropylanisole. Xenobiotica 44, 1-16.

10) Korzekwa, K.R., J.P. Jones and J.R. Gillette (1990): Theoretical studies on cytochrome P450 mediated hydroxylation: a predictive model for hydrogen atom abstraction. J. Am. Chem. Soc., 112, 7042-7046.

11) Korzekwa, K.R., W. Trager, M. Gouterman, D. Spangler. and G.H. Loew (1985): Cytochrome P450 mediated aromatic oxidation: A theoretical study. J. Am. Chem. Soc. 107, 4273-4279.

12) Burka, L.T., S.R. McGown and K.B. Tomer (1990): Identification of the biliary metabolites of 2,3,7,8-tetrachlorodibenzofuran in the rat. Chemosphere 21, 1231-1242.

13) Loew, G.H., J. Collins, B. Luke, A. Waleh and A. Pudzianowski (1986): Theoretical studies of cytochrome P-450. Characterization of stable and transient active states, reaction mechanisms and substrate-enzyme interactions.Enzyme 36, 54-78.

14) Kannan, N., T. Wakimoto and R. Tatsukawa (1989): Possible involvement of frontier (π) electrons in the metabolism of polychlorinated biphenyls (PCBs). Chemosphere 18, 1955-1963.

15) Cnubben N.H.P., S. Peelen, J.-W. Borst, J. Vervoort, C. Veeger and I.M.C.M. Rietjens (1994): Molecular orbital-based quantitative structure-activity relationship for the cytochrome P450-catalyzed 4-hydroxylation of halogenated anilines. Chem. Res. Toxicol. 7, 590-598