A theoretical study on the oxidative metabolism of 4-chloroacetanilide by cytochrome P450: alternative mechanisms for migration of 4-substituents during enzymatic oxidation


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(Received August 18th, 1992)

Abstract. The oxidative metabolism of 4-chloroacetanilide (4-CIAA, 1) by cytochrome P450 has been studied theoretically using ab-initio energy and spin-distribution calculations. A mechanism of oxidation for 4-CIAA is proposed which is in accordance with recent views on the mechanism of metabolic oxidation of substrates by cytochrome P450. An initial one-electron-oxidation step in the metabolic activation of 4-CIAA is suggested to be a hydrogen abstraction from nitrogen in the acetylamo side chain. Spin-delocalisation and subsequent radical recombination reactions between a hydroxyl radical and the reactive centres of the substrate radical can explain the formation of N-HO- and 2-HO-4-CIAA (4, 9), two known metabolites of 4-CIAA. Furthermore, the formation of a 2,5-cyclohexadien-1-imine intermediate (6) is proposed. Hypothetical addition–elimination mechanisms for the decomposition of this intermediate, which are supported by experimental data on analogous compounds, explain the formation of 4-HOAA, 3-HO-4-CIAA (10), and 3-Cl-4-HOAA (11), three other known metabolites of 4-CIAA (1).

Introduction

The cytochrome P450 system is known to catalyze the oxidation of a wide variety of compounds. A large number of P450 isozymes has been characterized in different species and tissues. Despite marked differences between these isozymes in primary amino acid sequence, their spectroscopic behaviour and substrate selectivity, their mechanisms of action and the nature of the oxidizing species are thought to be essentially identical among the different isozymes. This activation mechanism appears to consist of sequential one-electron-oxidation steps rather than of concerted two-electron-oxidation processes. After the first one-electron-oxidation step (abstraction of a hydrogen atom or abstraction of an electron plus proton) the substrate radical can recombine with the activated oxygen species of P450 (a hydroxyl radical) either to yield oxygenated products or to undergo a second one-electron-oxidation step leading to dehydrogenation.

In view of this apparently general mechanism of metabolic oxidation by P450s we have proposed mechanisms of oxidation for paracetamol (4-hydroxyacetanilide, 4-HOAA) and phenacetin (4-ethoxyacetanilide, 4-EtOAA). Quantumchemical calculations were performed to support the hypothesis. In the case of 4-HOAA, the initial one-electron-oxidation step consists of a hydrogen abstraction from the phenolic oxygen yielding a phenoxy radical; this was calculated to be 30 kcal/mole more favourable than initial hydrogen abstraction from the nitrogen in the acetylamo sidechain. Subsequent radical recombination reactions between the reactive centres of the phenoxy radical and a hydroxyl radical or the abstraction of a second hydrogen atom explained the formation of all metabolites of 4-HOAA known to be formed by the enzymatic action of P450.

Analogously, the mechanism of oxidation of 4-EtOAA was proposed to proceed via two, energetically almost equally favourable, initial hydrogen abstractions, i.e. either from nitrogen in the acetylamo side chain or from the α-methylene carbon in the ethoxy side chain. Again, radical recombination reactions between the respective reactive centres of the substrate radicals and the activated oxygen species of P450 (a hydroxyl radical) explained the formation

Abbreviations

AA acetanilide
a.u. atomic unit
4-CIAA 4-chloroacetanilide
GAMESS general atomic and molecular electronic structure system
4-EtOAA 4-ethoxyacetanilide (phenacetin)
4-HOAA 4-hydroxyacetanilide (paracetamol)
3-MC 3-methylcholanthrene
P450 cytochrome P450
SCF self-consistent field
of all metabolites of 4-EtOAA known to be formed by the action of P450. In addition, a hypothetical mechanism for the decomposition of an oxygenated 2,5-cyclohexadien-1-imine (previously named hemiketal) intermediate of 4-EtOAA was proposed which, both in the presence and absence of thiols such as glutathione, explained all presently available experimental metabolic data including those on \(^{15}\)O- and \(^{14}\)C-labeled 4-EtOAA. The above-mentioned quantum-chemical approach involving ab-initio energy and spin-distribution calculations apparently provides simple and useful means to gain insight into the mechanism of formation of several products by P450 from a single substrate. This approach is in accordance with results from Korzekwa et al. on the tendency of carbon–hydrogen bonds from a variety of substrates to undergo hydrogen abstraction by P450. Using quantum-chemical methods, it was shown that the relative ability of substrates to undergo hydrogen abstraction by P450 correlates with the calculated stability of the resulting substrate radical.

In order to investigate whether a uniform mechanism for the oxidative metabolism of acetalidines by P450 can explain the P450-mediated metabolic profile of acetalidines, we have studied the oxidation of 4-chloroacetalidine (4-CIAA, Figure 1, I). This acetalidene (AA) is converted by P450 to 5 different metabolites, namely N-HO-4-CIAA (Figure 1, 4), 2-HO-4-CIAA (Figure 1, 9), 4-HOAA, 3-HO-4-CIAA (Figure 3, 10), and 3-Cl-4-HOAA (Figure 3, 11). The current study was undertaken in order to investigate whether the uniform mechanism of oxidation as hypothesized for 4-HOAA and 4-EtOAA also explains the metabolic profile of 4-CIAA (Figure 1, 1).

**Methods**

In a previous theoretical study on the mechanism of oxidation of 4-HOAA by cytochrome P450, it was shown that, energetically, a planar conformation of 4-HOAA, with the acetylamino side chain lying in the plane of the phenyl moiety, is the most favourable one. Therefore, the conformation of 4-CIAA was optimized starting from a planar geometry. The ab-initio energy and spin distribution calculations were carried out as described previously. In short, the geometries of the parent compound, substrate radical, oxygenated intermediates and products (Figure 1; 1–9) were fully optimized, implying variation of all bond distances, bond angles and torsion angles, using the STO3G minimal basis set. After the geometric optimizations, a SV 6-31G basis set was used for single-point self-consistent field (SCF) energy calculations. These energy data were used to support the hypothetical mechanism of oxidation for 4-CIAA. The optimized geometry of the parent compound 4CIAA was found to have a STO3G and SV 6-31G energy of –886.043 atomic units (a.u.), and –896.218 a.u., respectively. The P450 enzyme complex was substituted by a singlet-oxygen species. The quantum-chemical programme package GAMESS implemented on the Cyber 995 and the CRAY-YMP of the Academic Computer Centre of Amsterdam (SARA) were used.

**Results and discussion**

**Metabolic data of 4CIAA**

The hepatic cytochrome P450-containing mixed-function oxidase system has been shown to be responsible for the oxidation of 4-CIAA to 5 different oxygenated metabolites. Like other N-acyethylamylines, 4-CIAA (I) is N-hydroxylated both in vitro in 3-methylcholanthrene (3-MC)-induced hamster microsomes and in vivo in 3-MC-pretreated hamsters. Furthermore, 4-CIAA is metabolized by P450 to 2-HO-4-CIAA (9) and 3-HO-4-CIAA (10) both in vitro in 3,4-benzopyrene-pretreated rat-liver microsomes and in vivo in 3-MC-induced hamsters and in uninduced rats. The formation of 4-HOAA as a metabolite of 4-CIAA has also been reported. Moreover, the metabolite 3-Cl-4-HOAA (11) has been identified in 3,4-benzopyrene- and 3-MC-pretreated rat-liver microsomes.

The current theoretical study was undertaken to investigate whether the formation of these oxidative metabolites of 4-CIAA can be rationalized in analogy with a previously proposed oxidation mechanism for 4-HOAA and 4-EtOAA. In accordance with recent views on the mechanism of oxidation of substrates by P450 and parallelizing the proposed oxidation mechanism for 4-HOAA and 4-EtOAA, we assumed that the initial one-electron-oxidation step in the case of 4-CIAA consists of a hydrogen abstraction from nitrogen in the acetylamino side chain. The resulting substrate radical is proposed to recombine with the activated oxygen species of cytochrome P450 (a hydroxyl radical) via one of its reactive centres (Figure 1). The methods used in this study should be judged upon their own merit, as the protein environment cannot be taken into account. Therefore, the results of the current study, in which only electronic effects are taken into consideration, have a qualitative significance and do not explain the isozyme-selective metabolism of 4-CIAA.

**Formation of a nitrogen radical from 4-CIAA and subsequent radical recombination reactions with a hydroxyl radical**

Our ab-initio calculations indicate that the unpaired electron of the substrate radical remains primarily localized at the nitrogen atom in the acetylamino side chain and is delocalized to a smaller extent towards the ortho and para carbon atoms relative to the acetylamino side chain and towards the carbononyl oxygen (Figure 2). Radical recombination reactions between the delocalized substrate radical (2) and a hydroxyl radical can explain the direct formation of N-HO-4-CIAA (4; ΔE = 168 kcal/mole) and 2-HO-4-CIAA (9; ΔE = 114 kcal/mole) after rearrangement of intermediate 5 (ΔE = 116 kcal/mole). Recombination involving a hydroxyl radical and the carbononyl oxygen should lead to the formation of a peroxide intermediate (3). However, the formation of this peroxide, not a known metabolite of 4-CIAA, is energetically unfavourable (ΔE = 81 kcal/mole) when compared to the radical recombination reactions yielding 4, 5 and 6 (ΔE = 114, 116, and 123 kcal/mole, respectively).

Figure 1 also indicates the possible formation of a 2,5-cyclohexadien-1-imine intermediate 6 (ΔE = 123 kcal/mole). This oxygenated intermediate can decompose to several minor metabolites of 4-CIAA as discussed in the next section. The above-hypothesized pathway of oxidation for 4-CIAA via initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain thus explains the formation of the metabolites N-HO-4-CIAA (4) and 2-HO-4-CIAA (9). Furthermore, the possible formation of a peroxide intermediate (3) and a 2,5-cyclohexadien-1-imine intermediate (6) is indicated.

**Mechanisms for the decomposition of the 2,5-cyclohexadien-1-imine intermediate**

The formation of the metabolites 3-HO-4-CIAA (10) and 3-Cl-4-HOAA (11) could possibly occur via protonation of the oxygenated 2,5-cyclohexadien-1-imine intermediate (6)
and a subsequent NIH-shift-like rearrangement of the cationic intermediate (Figure 3).\textsuperscript{8,19} Alternatively, however, the 2,5-cyclohexadien-1-imine intermediate (6) could eliminate HCl (8), resulting in the formation of N-acetyl-p-benzoquinone imine (NAPQI; 7; ΔE = +1 kcal/mole). The small energy difference of +1 kcal/mole suggests that small amounts of NAPQI and HCl might be formed, although NAPQI has not as yet been identified as a metabolite of 4-CIAA. We investigated the possible formation of NAPQI from 4-CIAA in liver microsomes of β-naphthoflavone-pretreated rats. However, in the presence of glutathione no NAPQI could be trapped as the 3-(S-glutathionyl)-4-HOAA conjugate (results not shown). This might be due to reduction of NAPQI to 4-HOAA by glutathione, since 4-HOAA has been identified as a metabolite of 4CIAA\textsuperscript{11} and NAPQI has been shown to be extremely sensitive to reduction by glutathione\textsuperscript{20,21}. Another reason for not observing the NAPQI metabolite experimentally could be that HCl adds again (via a Michael-type addition) to NAPQI forming the metabolite 3-CI-4-HOAA (11) as depicted in Figure 4, pathway B. Addition of HCl to NAPQI has recently been studied by Novak et al.\textsuperscript{22} The formation of the regiosomeric 3-HO-4CIAA (10) can be explained in a similar fashion, i.e. by a Michael-type addition of water to the 2,5-cyclohexadien-1-imine intermediate and subsequent elimination of water (Figure 4, pathway A). These types of nucleophilic addition reactions to acetylated quinone imine ketcals have recently been re-

Figure 1. Calculated nitrogen radical pathway and metabolic products of 4-CIAA (1). An initial hydrogen abstraction occurs from the acetylamino nitrogen of 4-CIAA. ΔE (kcal/mole) is the calculated energy difference between reactants, intermediates, and products. Cytochrome P450 is substituted by singlet oxygen (O*)
Figure 2. Calculated Mulliken spin distribution of the nitrogen radical (2) of 4-chloro-acetanilide (4-CIAA). The spin distribution is expressed as the difference in spin between the α and β spins of the radical.

Figure 3. Hypothetical mechanism for the NIH-shift-like decomposition of the 2,5-cyclohexadien-1-imine intermediate 6 of 4-chloro-acetanilide (4-CIAA)\(^\text{16,67}\). Protonation is proposed to occur at the imine nitrogen atom. In pathway [A] the hydroxyl group interacts with the positively charged ring carbon. Subsequent loss of a proton results in formation of 3-HO-4-CIAA (10). In pathway [B] the chlorine atom interacts with the positively charged ring carbon, resulting in formation of 3-Cl-4-HOAA (11) after loss of a proton.

Figure 4. Alternative mechanisms for the decomposition of the 2,5-cyclohexadien-1-imine intermediate 6 (Figure 1) of 4-chloro-acetanilide (4-CIAA). In pathway [A] water adds to intermediate 6 via a Michael-type addition. Subsequent loss of water results in formation of 3-HO-4-CIAA (10). In pathway [B] NAPQI (7) is formed after loss of HCl. This hydrochloric acid adds again to NAPQI under the formation of 3-Cl-4-HOAA (11).

Evidence against initial epoxidation of 4-CIAA by cytochrome P450

The mechanisms hypothesized above for the formation of 4-HOAA, 3-Cl-4-HOAA (11), and 3-HO-4-CIAA (10) (Figures 3 and 4) do not involve initial ring epoxidation or ring oxidation by cytochrome P450. In contrast, oxidation of 4-CIAA is proposed to proceed via initial oxidation of the nitrogen atom in the acetylamino side chain. Experimental evidence against initial epoxidation of acetanilides in the oxidative metabolism by cytochrome P450 has been obtained from studies with 4-HOAA, 4-EtOAA and AA. Initial epoxidation of the aromatic ring of 4-HOAA is not in agreement with the findings that little or no \(^{18}\)O label is lost from \(^{18}\)O-4-HOAA during oxidation by P450 in vivo in hamsters\(^\text{24}\) and in mice\(^\text{25}\). In addition, no \(^{18}\)O from \(^{18}\)O-labelled molecular oxygen was incorporated in the phenoxyl group in a hamster liver microsomal system\(^\text{26}\). Furthermore, metabolism of ring-deuterated analogues of 4-HOAA did not reveal intramolecular migration of deuterium nor a discernable isotope effect\(^\text{27}\). Apparently, initial epoxidation does not contribute to the P450-mediated oxidative metabolism of 4-HOAA.

\(^{18}\)O-labelled 4-EtOAA is metabolized by P450 with 50% loss of the \(^{18}\)O label in the presumed NAPQI metabolite of 4-EtOAA\(^\text{24}\). Initial 3,4-epoxidation of 4-EtOAA by P450 would be expected to rearrange to a significant extent to 3-HO-4-EtOAA. However, this metabolite was only formed in trace amounts\(^\text{27}\). Initial epoxidation of 4-EtOAA by P450 cannot explain the experimental data obtained with \(^{14}\)C]-4-EtOAA (for a detailed discussion also see Ref. 8). Moreover, Baty and Robinson\(^\text{28}\) found no deuterium-isotope in the aromatic hydroxylation of ring-deuterated AA, which is expected in case of an initial ring epoxidation by cytochrome P450.

The above considerations strongly suggest that an initial...
ring epoxidation is not involved in the P450-mediated metabolism of the acetanilides 4-HOAA, 4-EtOAA, and AA. Also in the current study, initial ring epoxidation seems not to be involved in the P450-mediated metabolism of the investigated compound, 4-CIAA. Both the various direct oxygenations of 4-CIAA and the intramolecular migration of para substituents can be explained via initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain. Interestingly, migration of a hydroxyl group from the para towards the meta position of the postulated 2,5-cyclohexadien-1-imine intermediate explains the formation of the minor metabolite 3-HO-4-EtOAA from 4-EtOAA. This was not accounted for in our previous study.

Conclusions

The mechanism of oxidative metabolism of 4-CIAA (1) by P450 is proposed to consist of initial hydrogen abstraction from nitrogen in the acetylamino side chain. The formation of metabolites N-HO-4-CIAA (4) and 2-HO-4-CIAA (9) can be explained via recombination between the reactive centres of the substrate radical and the activated oxygen species of P450. Furthermore, hypothetical mechanisms for the decomposition of the 2,5-cyclohexadien-1-imine intermediate of 4-CIAA (6) are proposed, explaining the formation of the metabolites 4-HOAA, 3-HO-4-CIAA (10), and 3-Cl-4-HOAA (11). These mechanisms do not involve epoxide intermediate NH-shift rearrangements but acid-catalyzed addition–elimination reactions instead. The results of this study and previous studies on 4-HOAA and 4-EtOAA point to a general mechanism of oxidation for acetanilides by cytochromes P450.

References