A Theoretical Study on the Metabolic Activation of Paracetamol by Cytochrome P-450: Indications for a Uniform Oxidation Mechanism

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The cytochrome P-450 mediated activation of paracetamol (PAR) to the reactive electrophilic intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI) has been studied by use of SV 6-31G ab initio energy calculations and spin distributions. A simplified model for cytochrome P-450 has been used by substituting the proposed biologically active ferric–oxene state of cytochrome P-450 by a singlet oxygen atom. The results indicate that an initial hydrogen abstraction from the phenolic hydroxyl group is favored by 30.1 kcal/mol over an initial hydrogen abstraction from the acetylamino nitrogen atom. Metabolic activation of PAR via primary formation of a phenoxy radical seems the most likely mechanism. The calculated ab initio spin densities indicate that the radical formed by hydrogen abstraction from the phenolic hydroxyl group stays predominantly localized at the phenolic oxygen. A second hydrogen abstraction from the acetylamino nitrogen atom, giving rise to the reactive intermediate NAPQI, is then favored in terms of energy differences. The unpaired electron of the phenoxy radical was found to delocalize only to a small extent toward the carbon atoms at the ortho and para positions relative to the hydroxyl-containing ring carbon, but nevertheless a recombination reaction between a hydroxyl radical and these radicalized carbon atoms at the ortho or para positions could explain the formation of the minor metabolites 3-hydroxy-PAR and *p*-benzoquinone plus acetamide.

Introduction

Paracetamol (*N*-acetyl-*p*-aminophenol, PAR,[1] Figure 1) is a widely used analgesic and antipyretic drug, which upon overdosing can cause hepatic necrosis in man and experimental animals (*1–3*). Over the past decades PAR has frequently been used in suicides and suicidical attempts (*4*). Furthermore, it is known that alcohol may predispose to liver damage caused by therapeutic doses of PAR (*5*). Because of these toxic side effects PAR has been subject to many toxicological studies during the last 20 years (*6, 7*).

A hepatotoxic dose of PAR generally results in saturation of the detoxifying glucuronidation and sulfation biotransformation routes, after which PAR is more extensively bioactivated by the hepatic cytochrome P-450 containing mixed-function oxidase system to an electrophilic intermediate. This reactive intermediate is subsequently conjugated with glutathione, thus resulting in hepatic glutathione depletion. The molecular mechanism underlying the hepatotoxic effect has been the subject of many investigations (*8–14*). Initially, covalent binding of the elusive reactive metabolite of PAR to thiol groups of proteins was solely thought to be responsible for the initiation of the PAR-induced hepatotoxicity (*8, 9*). More recent studies, however, strongly suggest that the PAR-induced hepatotoxicity more probably is due to cellular oxidative stress, resulting in lipid peroxidation, in protein and nonprotein thiol oxidation, and in changes in the intracellular calcium homeostasis, all being processes fatal to cells (*10–14*).

Evidence has been presented that *N*-acetyl-*p*-benzoquinone imine (NAPQI, Figure 1) is likely to be the reactive electrophilic intermediate of PAR (*15–20*). NAPQI has been detected upon oxidation of PAR with purified cytochrome P-450 using cumene hydroperoxide as oxidizing agent (*18*) and, recently, as a metabolite of a NADPH-dependent cytochrome P-450 catalyzed oxidation of PAR in a study with purified and reconstituted isozymes (*21*). Initially, the formation of the reactive intermediate of PAR was thought to be the result of oxygenation of PAR to *N*-hydroxy-PAR or 3,4-epoxy-PAR, in both cases followed by dehydration to NAPQI. However, several studies provided evidence against the involvement of *N*-hydroxy-PAR or 3,4-epoxy-PAR in the formation of NAPQI (*22–26*). Instead of these monooxygenase reactions usually carried out by cytochrome P-450, direct oxidation mechanisms have also been proposed for the formation of NAPQI from PAR. Hinson et al. (*7*) and Nelson et al. (*27*) have proposed the formation of a ferric–oxyamide complex, which would rapidly decompose to NAPQI and H_2O (Figure 1, route A). However, in view of the stability of *N*-hydroxy-PAR, with a half-life of 15 min in aqueous solutions at pH 7.4 (*22*), one would expect this complex to decompose also to some extent to *N*-hydroxy-PAR, a

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[1] Abbreviations: PAR, paracetamol; NAPQI, *N*-acetyl-*p*-benzoquinone imine; MNDO, modified neglect of differential overlap; LCAO, linear combination of atomic orbitals; MO, molecular orbital; SCF, self-consistent field; GAMESS, general atomic and molecular electronic structure system; UHF, unrestricted Hartree–Fock; CASSCF, complete active space SCF.

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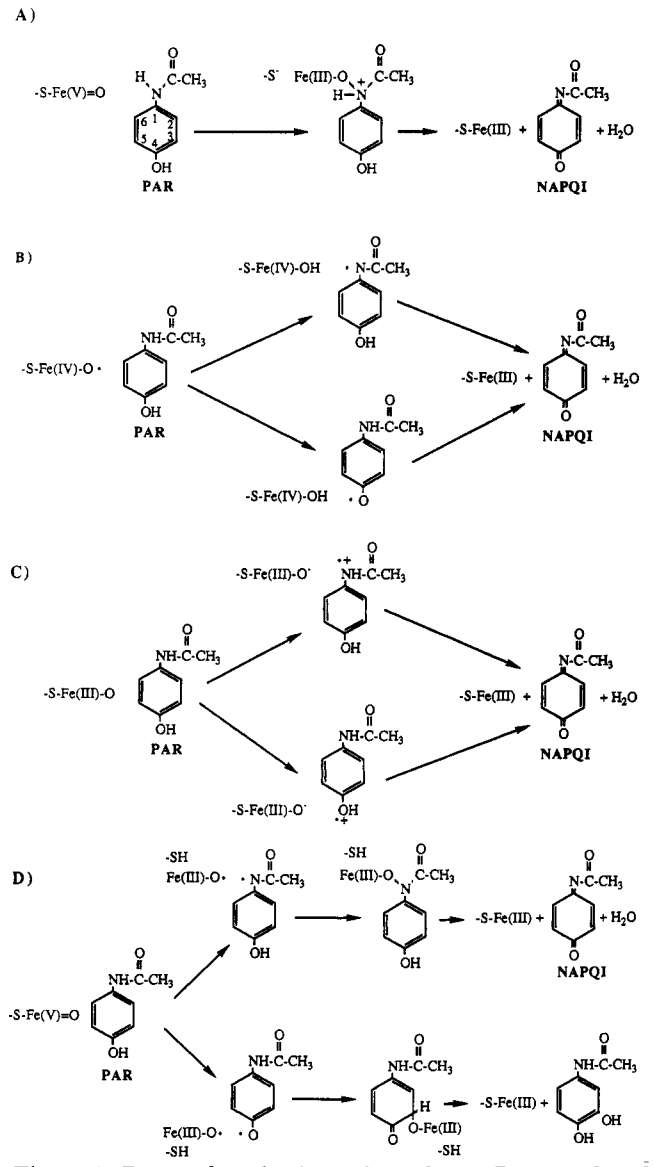

Figure 1. Proposed mechanisms of cytochrome P-450 mediated biotransformation of paracetamol to NAPQI and 3-hydroxy-PAR. Route A: Reaction of a cytochrome P-450–oxene complex with the lone pair of the nitrogen atom of PAR and formation of a ferric–oxyamide complex (*7*, *27*). Route B: Peroxidase-like reaction, in which PAR undergoes two successive hydrogen abstractions (*27*). Route C: Direct two-electron oxidation of PAR by a cytochrome P-450–oxene complex with a semiquinone radical cation of PAR as a one-electron oxidized intermediate (*20*). Route D: Formation of NAPQI and 3-hydroxy-PAR through distinct radical intermediates; NAPQI is formed via an initial hydrogen abstraction from the nitrogen atom of PAR, followed by a radical recombination reaction to a ferric–oxyamide complex; 3-hydroxy-PAR, via an initial hydrogen abstraction at the phenolic oxygen atom of PAR, followed by a radical recombination reaction at the radicalized carbon atom at the 3-position (*21*).

metabolite that is apparently not formed from PAR in a cytochrome P-450 dependent oxidation reaction. An alternative hypothesis by Nelson et al. (*27*) was based on a peroxidase-like reaction of a cytochrome P-450–oxene complex with PAR (Figure 1, route B). It suggested that PAR might undergo two successive hydrogen abstractions or one-electron oxidation reactions to yield NAPQI and H_2O. More recently, Van de Straat et al. (*20*) proposed a direct two-step two-electron oxidation mechanism for PAR by a ferric–oxene complex of cytochrome P-450 (Figure 1, route C); upon an initial one-electron oxidation of PAR to a semiquinone radical cation of PAR, no oxygen transfer would take place as a second step, but instead of this an additional one-electron-transfer reaction would rapidly take place within the heme pocket of the enzyme. Furthermore, it was suggested by Van de Straat et al. (*28*) that the phenolic hydroxyl group of PAR would be more essential for the oxidation of PAR and that it would be closer to the heme iron ion than the nitrogen atom in the acetylamino side chain. These observations were based on spectrophotometric binding studies and on NMR relaxation rate measurements, using two purified hepatic cytochrome P-450 isozymes with different catalytic activities toward PAR oxidation.

However, none of the above-mentioned "direct transformation" mechanisms (Figure 1, routes A–C) also explains the occurrence of 3-hydroxy-PAR as a (minor) phase I metabolite, resulting from the metabolism of PAR by cytochrome P-450 (*29–31*). Recently, Harvison et al. (*21*) demonstrated a selective formation of 3-hydroxy-PAR and NAPQI by cytochrome P-450 isozymes. They proposed mechanisms for the formation of 3-hydroxy-PAR and NAPQI through distinct radical intermediates (Figure 1, route D). NAPQI was proposed to be formed via initial one-electron oxidation at the acetylamino nitrogen atom followed by a rapid recombination of the radical pair to form a cytochrome P-450 heme site bound ferric–oxyamide complex which would decompose rapidly to generate NAPQI and a ferric cytochrome P-450 complex. In contrast, 3-hydroxy-PAR was thought to arise via initial one-electron oxidation at the phenolic oxygen atom followed by a radical recombination reaction between the activated enzyme-bound oxygen and the carbon atom at the 3-position.

As far as the oxidation of PAR to NAPQI is concerned, only one theoretical study has been published (*32*). In this study the semiempirical MNDO method (*33*) was used to calculate the reaction thermodynamics for the oxidation of PAR by both radical and nonradical mechanisms. From all mechanisms of NAPQI formation considered here, a peroxidase-like mechanism leading directly to NAPQI via radical intermediates (Figure 1, route B) was calculated to be thermodynamically favored. However, it is now known that the semiempirical MNDO method underestimates conjugation effects (*34*) as present in PAR and that peroxidases are heme containing proteins different from cytochrome P-450.

In the current quantum chemical study use was made of ab initio energy calculations and spin distributions to support a new hypothetical mechanism of oxidation of PAR (Figures 2 and 3). This mechanism would explain the formation of both NAPQI and 3-hydroxy-PAR from PAR and, in addition, the formation of *p*-benzoquinone plus acetamide. In this hypothetical mechanism it is assumed that a hydrogen abstraction from either the phenolic oxygen atom (Figure 2) or the acetylamino nitrogen atom (Figure 3) occurs as initial step in the metabolic activation of PAR. All principally possible subsequent hydrogen abstraction, radical recombination, and/or rearrangement reactions are considered.

Experimental Procedures

The ab initio calculations used in this study were performed at the LCAO–MO–SCF level by using the STO–3G (*35*) minimal basis set for the geometry optimizations and a SV 6-31G (*36*) basis set for the subsequent SCF energy calculations. The quantum chemical program package GAMESS (*37–39*) was used on the Cyber 995 and the Cyber 205 of the Academic Computer Centre of Amsterdam (SARA).

The geometries of the parent compound, intermediates and reaction products were fully optimized, implying variation of all bond distances, bond angles, and torsion angles, using the STO-3G

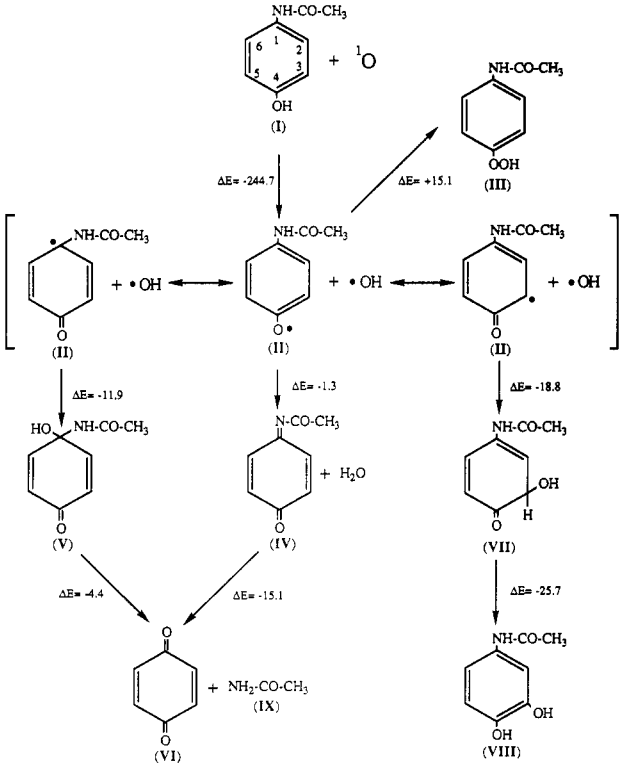

Figure 2. Hypothesized phenoxy radical pathway for the oxidation and oxygenation of PAR. An initial hydrogen abstraction is assumed to take place at the phenolic hydroxyl group of paracetamol. Possible subsequent hydrogen abstraction, radical recombination, and rearrangement reactions, explaining the formation of NAPQI (IV), 3-hydroxy-PAR (VIII), *p*-benzoquinone (VI), and acetamide (IX), are also indicated. ΔE (kcal/mol) is the calculated energy difference between reactants, intermediates, and products.

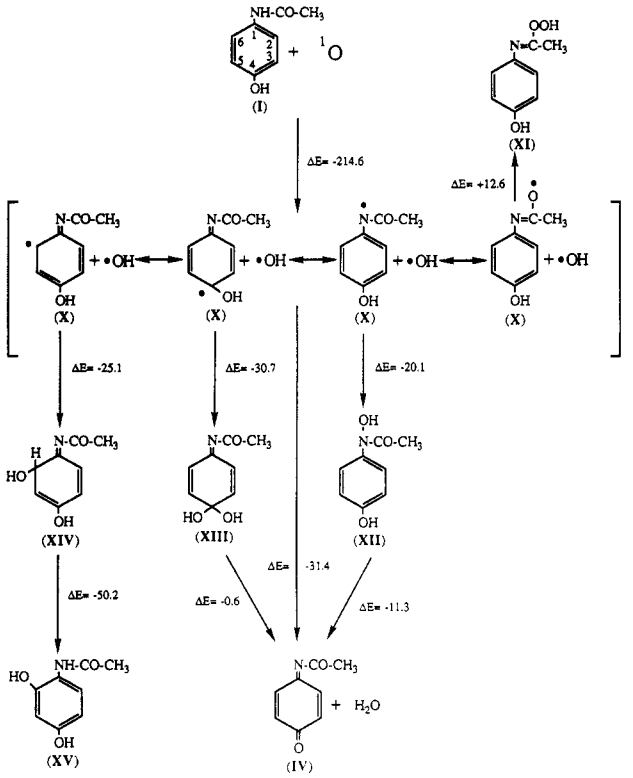

Figure 3. Hypothesized nitrogen radical pathway for the oxidation and oxygenation of PAR. An initial hydrogen abstraction is assumed to take place at the nitrogen atom in the acetylamino side chain of PAR. Possible subsequent hydrogen abstraction, radical recombination, and rearrangement reactions are considered. ΔE (kcal/mole) is the calculated energy difference between reactants, intermediates, and products.

minimal basis set. The initial geometry was taken from a full MNDO (*33*) geometry optimization. The MNDO calculations were performed at a μVAX-II. In the study of Loew et al. (*32*), MNDO calculations revealed a minimal energy conformation of PAR, in which the acetylamino side chain was rotated nearly perpendicular to the benzene ring (τ-CNC$_1$C$_2$ = 90–95°). In this geometry the π-electron systems of the acetylamino side chain and the benzene ring are independent. In addition to this "perpendicular" conformation we found with MNDO a second minimum energy conformation with the side chain in the plane of the ring. The "perpendicular" conformation was energetically favored over the "flat" conformation by 3.3 kcal/mol. It is known, however, that the semiempirical MNDO method underestimates conjugation effects (*34*). An ab initio SV 3-21G SCF energy calculation on the two geometries favored the flat "conjugated" geometry by 4.4 kcal/mol. Therefore, we used the "flat" MNDO geometries as starting points for the ab initio geometry optimizations. For the geometry optimization of radical species the UHF formalism was used, which assumes different orbitals for different spins. After the STO-3G geometry optimization an SV 6-31G SCF energy calculation was performed. These SCF energies were used to support the hypothetical mechanism.

As to the spin distributions of the phenoxy- and acetylamino nitrogen radical it is known that in the UHF calculations admixture of higher spin states may distort the results, leading to too negative energies and unrealistic spin densities (*34*). Therefore, SV 6-31G RHF spin distributions were calculated for the UHF geometries of the radicals. The Mulliken spin distributions were expressed as the difference between the α- and β-spins of the respective radicals (parts A and B of Figure 4). The results of subsequent CASSCF calculations on the radicals, in which the four highest occupied molecular orbitals and the lowest unoccupied molecular orbital of the radicals were permitted variable occupancy, did not change the overall picture.

A cytochrome P-450 enzyme reaction usually consists of a transfer of an activated oxygen atom from the enzyme to the enzyme-bound substrate. The reactive oxygen atom of the proposed biologically active ferric–oxene state of the cytochrome P-450 enzyme is a triplet (^{3}P)O species. We used a simplified model system for our calculations by substituting the cytochrome P-450 enzyme complex by an oxygen atom. As the energy difference between a singlet and a triplet oxygen atom is too small to influence the energy calculations of the proposed hypothetical activation of PAR, use was made of a singlet oxygen atom. This simplified model system is used to reduce the computational efforts and should not be construed as implying that the activated enzyme-bound oxygen is a free atomic oxygen.

Results

Figure 2 represents the hypothetical mechanism for the metabolic activation of PAR by cytochrome P-450, in which as the initial step a hydrogen atom is abstracted from the phenolic oxygen atom of PAR. The hydrogen atom is supposed to react with the enzyme-bound reactive oxygen species to yield a hydroxyl radical and the phenoxy radical of PAR. Possible subsequent hydrogen abstraction, radical recombination, and/or rearrangement reactions are also given in Figure 2. Apart from the formation of NAPQI, this hypothetical mechanism also includes the formation of 3-hydroxy-PAR and *p*-benzoquinone plus acetamide from PAR. An analogous hypothetical mechanism for the metabolic activation of PAR with an initial hydrogen abstraction from the acetylamino nitrogen atom and the corresponding subsequent reactions is depicted in Figure 3.

The Phenoxy Radical Pathway. As is depicted in Figure 2, a hydrogen abstraction from the phenolic hydroxyl group of PAR yields a phenoxy radical (Figure 2, II) and a hydroxyl radical. The calculated Mulliken spin distribution of the phenoxy radical is given in Figure 4A.

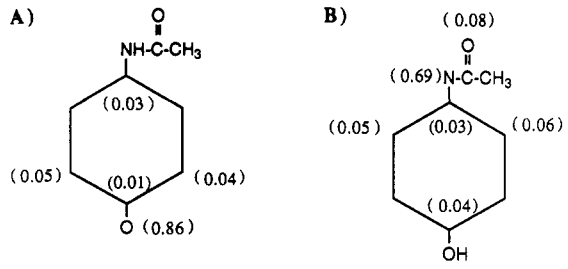

Figure 4. Calculated Mulliken spin distributions of the phenoxy radical (A) and the nitrogen radical of paracetamol (B), respectively. Spin distributions are expressed as the difference in spin between the α- and β-spins of the respective radicals.

Most of the unpaired electron of the phenoxy radical remains localized at the phenoxy oxygen, whereas only small parts are delocalized toward the carbon atoms at the ortho and para positions of the C_4 carbon atom (Figure 4A).

A recombination reaction between the phenoxy radical and the hydroxyl radical, which would yield a peroxide (Figure 2, III), was calculated to be energetically unfavored (ΔE = +15.1 kcal/mol). A secondary hydrogen abstraction from the acetylamino nitrogen giving rise to the reactive intermediate NAPQI (Figure 2, IV), however, was found to be more favorable (ΔE = −1.3 kcal/mol).

A recombination reaction between the hydroxyl radical and one of the radicalized carbon atoms ortho relative to the C_4 carbon atom would yield intermediate VII. This radical recombination reaction was found to be favored over a secondary hydrogen abstraction from the acetylamino nitrogen atom to NAPQI by 17.5 kcal/mol. Intermediate VII might gain substantial energy (ΔE = −25.7 kcal/mol) by rearranging to the stable 3-hydroxy-PAR (VIII), one of the (minor) phase I metabolites of PAR. A recombination reaction between the hydroxyl radical and the carbon atom at the para position relative to the C_4 carbon atom would result in ipso intermediate V. This radical recombination reaction was also found to be energetically more favorable (ΔE = −10.6 kcal/mol) than a secondary hydrogen abstraction from the acetylamino nitrogen atom, yielding NAPQI. Ipso intermediate V is supposed to rearrange rapidly to *p*-benzoquinone (VI) and acetamide (IX) (ΔE = −4.4 kcal/mol).

The Nitrogen Radical Pathway. As is depicted in Figure 3, a hydrogen abstraction from the nitrogen atom in the acetylamino side chain of PAR yields an acetylamino nitrogen radical (Figure 3, X) and a hydroxyl radical. The calculated Mulliken spin distribution of the nitrogen radical is depicted in Figure 4B. Apparently, the unpaired electron remains predominantly localized at the nitrogen atom. Only small portions are delocalized toward the carbonyl oxygen in the acetylamino side chain and toward the carbon atoms at the ortho and para positions relative to the C_1 carbon atom.

A recombination reaction between the hydroxyl radical and the different reactive sites of the nitrogen radical would yield intermediates XI–XIV, respectively (Figure 3). The formation of all these four intermediates was calculated to be energetically unfavored when compared to a secondary hydrogen abstraction from the phenolic oxygen to yield the reactive intermediate NAPQI. In the case of intermediate XIV, a rearrangement to the stable 2-hydroxy-PAR (XV) could take place (ΔE = −50.2 kcal/mol). In contrast to 3-hydroxy-PAR (Figure 2, VIII), however, this product is unknown as a phase I metabolite of PAR.

A comparison of the phenoxy and nitrogen radical pathway reveals that an initial hydrogen abstraction from the phenolic hydroxyl group of PAR (Figure 2) is favored by 30.1 kcal/mol over an initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain (Figure 3).

Discussion

The NADPH and molecular oxygen dependent biotransformation of substrates by the cytochrome P-450 containing mixed-function oxidase system usually consists of a monooxygenation reaction. As a result of this, an oxygen atom from molecular oxygen is incorporated via a ferric–oxene cytochrome P-450–substrate complex into substrates, leading to hydroxylations, heteroatom oxygenations, or epoxidation reactions. In the case of PAR, however, a direct transformation reaction of PAR to NAPQI by electron transfer to the cytochrome P-450 system has been suggested as an alternative possibility (*7, 20, 21, 27*). Until now, however, it has not been possible to detect experimentally as yet elusive intermediates, such as the semiquinone radical of PAR, that in principle might be formed during the transformation of PAR to NAPQI. In such cases the potential contribution of a theoretical study might be helpful to further understand the molecular mechanism of a biotransformation reaction. The main object of the current theoretical study was to investigate a hypothetical mechanism of the oxidative biotransformation of PAR to its reactive metabolite NAPQI and the other, oxygenated, metabolites. This mechanism involves an initial hydrogen abstraction either from the phenolic hydroxyl group or from the nitrogen atom in the acetylamino side chain (Figures 2 and 3, respectively). The proposed mechanisms are discussed in terms of energy differences between reactants, intermediates, and products.

In contrast to previous MNDO results (*32*), our ab initio SV 6-31G calculations revealed that a flat "conjugated" conformation of PAR, with the acetylamino side chain in the plane of the aromatic nucleus, is favored over a conformation with the acetylamino side chain perpendicular to the plane of the ring. We therefore assume that PAR will also interact with the cytochrome P-450 enzyme system in a flat "conjugated" conformation. The optimized conformations of the nitrogen radical and the phenoxy radical were also found to be "flat" and closely resembled the optimized conformation of the parent compound PAR. The cytochrome P-450 enzyme system was substituted in our calculations by a singlet oxygen atom in order to reduce the computational efforts.

Our present results further suggest that an initial hydrogen abstraction from PAR occurs at the phenolic hydroxyl group, since this hydrogen abstraction reaction is favored by 30.1 kcal/mol (Figure 2) when compared to an initial hydrogen abstraction from the nitrogen in the acetylamino side chain (Figure 3). A metabolic activation of PAR via a phenoxy radical pathway is consistent with recent findings of Van de Straat et al. (*28*), who suggested that the phenolic oxygen group of PAR rather than the acetylamino side chain would be essential for the oxidation of PAR. From proton NMR longitudinal relaxation rate measurements and spectrophotometric binding studies it was concluded that in the case of the isozyme cytochrome P-450c, active in the oxidation of PAR to NAPQI, the phenolic hydroxyl group was in closest proximity of the heme iron ion, whereas in the case of the isozyme cytochrome P-450b, inactive in this oxidation reaction, the acetylamino side chain more closely approached the central heme iron ion.

The calculated spin distribution of the phenoxy radical of PAR revealed that the unpaired electron is primarily localized at the phenolic oxygen atom (Figure 4A). The

probability of finding the unpaired electron at the carbon atoms at the ortho and para positions relative to the C_4 carbon atom of the aromatic nucleus was found to be small. These findings are in contrast with previous MNDO results by Loew et al. (*32*), who calculated that the phenoxy radical of PAR was not primarily an oxygen-centered radical but that the unpaired spin was delocalized mainly on the ring carbon atoms at the ortho position relative to the C_4 carbon atom. Our ab initio calculations on the spin distribution of the phenoxy radical, however, are in agreement with a study of West et al. (*40*), who detected a free radical produced from PAR in the presence of horseradish peroxidase and hydrogen peroxide by direct fast-flow ESR spectroscopy. The free radical detected was characterized as an oxygen-centered phenoxy free radical with a dominant and large coupling with the two equivalent ortho hydrogens.

In case the unpaired electron remains localized at the phenoxy oxygen, theoretically a subsequent hydrogen abstraction from the acetylamino nitrogen atom to yield NAPQI as second step in a direct two-electron oxidation of PAR appeared to be most likely in terms of energy differences (Figure 2). A direct two-electron oxidation pathway for the oxidation of PAR to NAPQI by cytochrome P-450 is in agreement with a proposal of Hinson et al. (*25*, *26*), who showed that little or no ^{18}O label was lost from [p-^{18}O]PAR during metabolic activation in vivo and, in addition, that no oxygen atom from ^{18}O-labeled molecular oxygen was built in at the phenoxy oxygen position in a hamster liver microsomal system.

The unpaired electron of the phenoxy radical was found to delocalize only to a small extent toward the ortho carbon atoms relative to the C_4 carbon atom of the aromatic ring (Figure 4A). In this case, apart from a secondary hydrogen abstraction, a recombination reaction between the hydroxyl radical and one of the ortho carbon atoms could take place. Via formation of intermediate VII this reaction would yield the (minor) phase I metabolite 3-hydroxy-PAR (VIII), and it was calculated to be energetically favored over a secondary hydrogen abstraction from the acetylamino nitrogen atom under the formation of NAPQI. The formation of 3-hydroxy-PAR through a phenoxy radical mechanism is consistent with the current view on the mechanism of the normal monooxygenase activity of cytochrome P-450 as well as with the findings of Hinson et al. (*29*) in a hamster liver microsomal system that the oxygen at the 3-position of 3-hydroxy-PAR originates from molecular oxygen. It is also consistent with the fact that purified epoxide hydrolase has no effect on covalent binding of NAPQI nor on the formation of 3-hydroxy-PAR (*29*). In addition, it is known that NAPQI-reducing compounds such as glutathione and ascorbic acid inhibit the covalent binding of NAPQI, formed from PAR, to microsomal protein, whereas under similar conditions these compounds do not inhibit the formation of 3-hydroxy-PAR (*29*). This might be explained by assuming that these NAPQI-reducing compounds do not influence the spin distribution of the phenoxy radical and thus do not influence the formation of 3-hydroxy-PAR. Our results support the recent proposal of Harvison et al. (*21*) that 3-hydroxy-PAR is formed via an initial hydrogen abstraction from the phenolic hydroxyl group of PAR and a subsequent radical recombination/rearrangement reaction at the carbon atom at the 3-position (Figure 2 and Figure 1, route D).

A radical recombination reaction between the hydroxyl radical and the delocalized radical at the carbon atom at the para position relative to the C_4 carbon atom, via formation of ipso intermediate V, would give rise to p-benzoquinone (VI) and acetamide (IX) as products (Figure 2). In this way, p-benzoquinone plus acetamide, analogously to the formation of 3-hydroxy-PAR from PAR, could be viewed as resulting from oxygenation of PAR after primary one-electron oxidation by cytochrome P-450. However, the formation of p-benzoquinone plus acetamide has also been reported to result from hydrolysis of NAPQI by various authors (*15*, *16*, *18*). The present ab initio calculations were also found to be consistent with this alternative possibility (ΔE = −15.1 kcal/mol).

The formation of a nitrogen radical via an initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain of PAR (Figure 3) was found to be energetically unfavored by 30.1 kcal/mol when compared to the initial formation of a phenoxy radical (Figure 2). Once the nitrogen radical is formed, a second hydrogen abstraction from the phenolic oxygen atom of PAR to yield NAPQI is favored over radical recombination/rearrangement reactions at the different reactive centers of the nitrogen radical. This would be consistent with the findings that none of the intermediates (Figure 3, XIII–XIV) nor any of the products (Figure 3, XII–XV) theoretically derived from the nitrogen radical pathway have ever been detected chemically, either in a liver microsomal or in an in vivo system. The results are also in agreement with the findings of Van de Straat et al. (*28*) that in the case of the isozyme cytochrome P-450b, shown to be inactive in the oxidation of PAR to NAPQI, the acetylamino side chain was in closest proximity to the heme iron ion. Our findings, however, are in contrast with previous MNDO calculations (*32*), which favored an initial hydrogen abstraction from the nitrogen radical over an initial hydrogen abstraction from the phenolic hydroxyl group of PAR. Our results are also in contrast with a recent study of Harvison et al. (*21*), who without further experimental evidence proposed that an initial hydrogen abstraction from the acetylamino nitrogen atom would be essential for the formation of NAPQI from PAR.

The above proposed phenoxy radical pathway for the oxidation of PAR, in contrast to the nitrogen radical pathway, in principle explains the formation of all known metabolites of PAR (NAPQI, 3-hydroxy-PAR, and p-benzoquinone plus acetamide) by one mechanism, i.e., one involving only an initial hydrogen abstraction from the phenolic hydroxyl group of PAR. Interestingly, the finding that NAPQI is the major metabolite of PAR (*15–20*) and that 3-hydroxy-PAR and p-benzoquinone plus acetamide are only minor metabolites (*15*, *16*, *29–31*) is reflected by the calculated spin distribution of the phenoxy radical. Moreover, this mechanism also fits with recent NMR longitudinal relaxation rate measurements, suggesting that the phenolic function of PAR plays an essential role in the isoenzyme-selective oxidative bioactivation of PAR to NAPQI, as well as with similar mechanisms recently proposed for amine hydroxylation and N-demethylation (*41*, *42*). However, our calculations do not yet fully explain the selective formation of 3-hydroxy-PAR and NAPQI by cytochrome P-450 isozymes, as demonstrated by Harvison et al. (*21*). One possible explanation might be an isozyme-selective influence of the spin distribution of the phenoxy radical of PAR. The exact nature of the isozyme-selective formation of the metabolites of PAR remains to be established, however.

Conclusions

Ab initio SV 6-31G calculations in a model system for cytochrome P-450 have demonstrated that a metabolic activation of PAR by cytochrome P-450 via an initial hy-

drogen abstraction from the phenolic oxygen atom (Figure 2) is favored by 30.1 kcal/mol over an initial hydrogen abstraction from the nitrogen atom in the acetylamino side chain (Figure 3). The occurrence of NAPQI, 3-hydroxy-PAR, and *p*-benzoquinone as phase I metabolites of PAR could be explained with the aid of a phenoxy radical pathway, this in contrast to a mechanism involving a nitrogen radical pathway. The unpaired electron of the phenoxy radical remains primarily localized at the phenolic oxygen atom. In this case, the intermediate NAPQI is formed as major product of PAR via a second hydrogen abstraction from the acetylamino nitrogen atom in the side chain. The probability to find the unpaired electron of the phenoxy radical at the carbon atoms ortho or para relative to the C_4 carbon atom is small, but nevertheless it could explain the formation of the minor metabolites 3-hydroxy-PAR and *p*-benzoquinone plus acetamide. These findings are supported by ab initio energy calculations and spin distributions and are consistent with available experimental data and with current ideas about the mechanisms of action of cytochrome P-450.

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