Density Functional Calculations

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Nitrogen Inversion Barriers Affect the N-Oxidation of Tertiary Alkylamines by Cytochromes P450**

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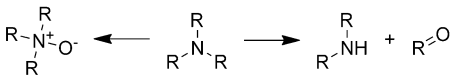
The cytochrome P450 enzymes (CYPs) form a protein superfamily of monooxygenases. They are involved in the synthesis of many physiologically important endogenous compounds, but also contribute to the degradation of xenobiotic compounds and are involved in the metabolism of up to 90% of all drugs in use today.[1]

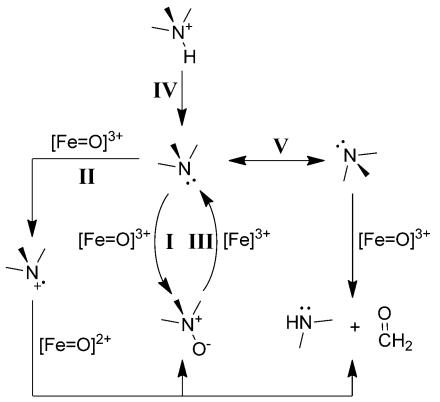
There are at least two reasons why it is complex to rationalize which of the many possible reactions with a certain substrate are likely to occur. First, many of the CYP enzymes are structurally flexible[2] and thus, there could be many possible orientations of the substrates. Second, these enzymes are capable of catalyzing a number of chemically different reactions, for example, the aliphatic and aromatic hydroxylation, N- and O-dealkylation, epoxidation, and hetero-atom oxidations like N-oxidations. Thus, almost any atom in the substrate may react with the enzyme, thus illustrating the complexity of rationalizing CYP metabolism.

Many CYP substrates are amines for which reaction on the α-carbon atoms usually is preferred,[3] which can lead to N-dealkylation. However, also formation of an *N*-oxide is possible (see Scheme 1).[4] In particular for the tertiary amines, it seems that the formation of *N*-oxides occurs more frequently than for primary and secondary amines.[5] These *N*-oxides can either increase or decrease the potency of their parent compounds, but generally the *N*-oxides of tertiary alkylamines are more polar and more easily excreted than their parent compounds.[6]



Scheme 1. The possible *N*-oxide and dealkylation products formed by reactions of CYPs with tertiary alkylamines.

In this study we investigate what influences the *N*-oxide formation by CYPs, and what factors determine the relative amount of *N*-oxides and dealkylated products that are formed. To do this, we use density functional theory (DFT) calculations to investigate previously proposed mechanisms and introduce the N-inversion hypothesis, which seems to describe the experimental data most accurately. These mechanisms (I–V in Scheme 2) are based on: N-oxidation reactivity, I) through direct σ-bond formation or II) through a preceding single electron transfer (SET), which has been proposed also to lead to N-dealkylation;[8] III) N-oxidation reversibility, since it is has been shown that CYPs can reduce some *N*-oxides of tertiary amines,[9] and it is also known that binding of *N*-oxides of arylamines to CYPs can lead to the formation of Compound I, the highly reactive intermediate that is responsible for most oxidation reactions mediated by CYPs;[10] IV) pK_a, since deprotonation of the amine nitrogen atom is required for N-oxidation; and finally, V) the inversion of the amine nitrogen atom, which is required to change the binding conformation of the amine nitrogen between the poses most liable to N-oxidation versus N-dealkylation reactions.



Scheme 2. The investigated mechanisms (I–V) in CYP metabolism of tertiary amines.

It appears that oxidation of the nitrogen atom in tertiary alkylamines appears consistently for some specific fragments

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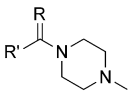
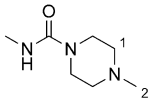
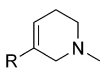
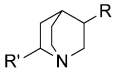
(Table 1).[11] As a control group to investigate what factors dictate whether N-oxidation occurs, we also extracted five common tertiary alkylamine fragments from our data sets for which we could not find any experimental evidence of

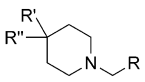
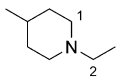
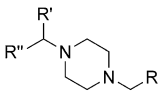
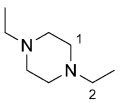
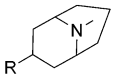
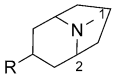
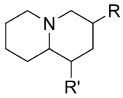
Table 1: Examples of typical tertiary alkylamines that can become N-oxidized by CYPs (denoted group 1 consisting of fragments **A**–**E**). Numbering of atoms in the fragments refer to calculations presented below.

Group 1	Fragment	Fragment used in calculation	Compounds containing the fragment
A			clozapine,[12] k_11777,[13] mianserin,[14] mirtazapine,[15] zopiclone,[16] olanzapine[17]
B			Lu25_109[18]
C			riddelliine,[7b] senecionine[[19]
D			zatosetron[20]
E			quinidine,[4a] dolasetron mesilate[21]

significant N-oxidation by CYPs (shown in Table 2). This observation does not necessarily prove that N-oxidations of these fragments cannot occur (a few occasions have been observed[5]), but that if they do, they occur most likely to such a minor extent that they are not quantified in most experiments.

To investigate whether the N-oxidation occurs from direct oxidation by Compound I (mechanism I, Scheme 2), we

Table 2: Examples of typical tertiary alkylamines for which N-oxidation by CYPs is rarely observed (denoted group 2 consisting of fragments **F**–**J**). Numbering of atoms in the fragments refer to calculations presented below.

Group 2	Fragment	Fragment used in calculation	Compounds containing the fragment
F			imipramine[22]
G			terfenadine[23]
H			perazine[24]
I			granisetron[25]
J			sparteine[26]

computed the transition states for that type of reaction for fragments **A**–**J.** We also computed the transition states for the competing H-abstraction from neighboring carbon atoms (labeled with numbers in Table 1 and Table 2).

While the reaction barriers explain why we rarely observe N-oxidations for fragments **F**–**J** (the H-abstraction barriers are consistently lower than the N-oxidation barriers; see Table 3), the correlations for the fragments that do get N-

Table 3: Energy barriers (in kJ mol^{-1}) for N-oxidations (doublet state) and H-abstractions (quartet state).

Fragment	N-oxidation	H-abstraction[a]
Group 1: N-oxidation observed		
A	44.2	61.1/43.7
B	45.7	48.3/38.6/46.0
C	37.9	28.3/33.7
D	50.1	86.8/31.5
E	42.2	67.7
Group 2: No N-oxidation observed		
F	42.3	38.8/37.1
G	49.9	35.9/37.5
H	50.8	44.5/39.6
I	43.5	22.9/72.5
J	68.4	37.5/44.7

[a] Multiple sites in order as numbered in Tables 1 and 2, and separated by /.

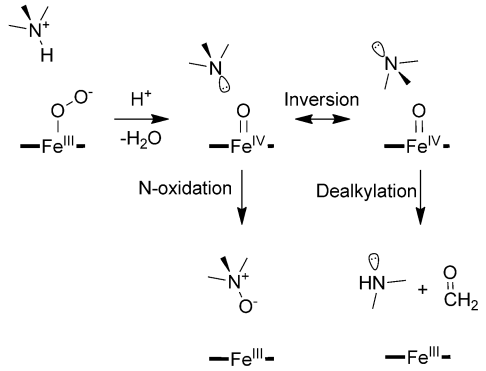
oxidized are not obvious. The only fragment that has a preference for N-oxidation is fragment **E**, which also is the only fragment for which hydroxylation next to the nitrogen atom is never seen. The high H-abstraction barrier for this fragment is due to its symmetric shape, which causes all hydrogen atoms to point in the same direction as the nitrogen lone pair, thereby increasing the activation energy.[27] Hence, there is probably at least one more factor beyond reactivity that causes the formation of *N*-oxides.

One possible explanation is that the reaction with the tertiary amines occurs through the SET pathway. It was, however, not possible to identify the SET state (mechanism II, Scheme 2), thus generating the complex with a cationic amine radical and the reduced Compound I. This result agrees well with previous DFT studies, where it was concluded that the energy of the SET state is significantly higher than the reaction barrier of the H-abstraction or the direct oxygen transfer to the amine.[3,27–28] If the SET pathway is the initial step of the *N*-oxide formation, there could be a correlation between the occurrence of *N*-oxides and either the energy required to form an aminium radical ion or the stability of the products (the stability of the *N*-oxide versus the formation of the carbinolamine, see Figure S1 in the Supporting Information). There is, however, in neither case a clear correlation with the experimentally observed metabolites (Tables S1 and S2 in the Supporting Information). Hence, we cannot find a clear correlation that would support the participation of the SET pathway in the N-oxidation of tertiary alkylamines.

Reduction of *N*-oxides to amines (mechanism III, Scheme 2) has been shown to lead to the formation of Compound I for some *N*-oxides of arylamines.[10] To inves-

tigate if this would be a possible reaction path for the *N*-oxides of tertiary alkylamines, we computed the energies of the *N*-oxide products relative to the substrates (Table S3 in the Supporting Information), and also added the N-oxidation barrier to get the reverse barrier (Figure S2 in the Supporting Information). However, no correlations to the observed N-oxidations were found for the product energies or for the product energies with the oxidation barriers added.

Another possible cause of the formation of *N*-oxides could be related to the protonation of the amine nitrogen atoms (mechanism IV, Scheme 2). For N-oxidation to occur, the nitrogen atom needs to be deprotonated, and tertiary amines are known to commonly be protonated in water. While this has been shown to be an unlikely reason for determining if N-oxidation is possible,[29] it has not been shown if it affects the amount of *N*-oxide formed relative to possible dealkylation or hydroxylation products. However, the computed pK_a values show similar distributions for the two groups of fragments (see Figure S3 in the Supporting Information). Thus, the deprotonation process does not seem to affect the rate of N-oxidation. This can most likely be explained by the fact that protons are used during the CYP catalytic cycle in the formation of the active Compound I (shown in the first step in Scheme 3).



Scheme 3. Illustration of the N-inversion hypothesis.

Another possible contribution to the formation of *N*-oxides could be how easily the inversion of the nitrogen atom occurs (mechanism V, Scheme 2). When a neutral amine (possibly deprotonated in the active site as indicated in Scheme 3) binds close to the heme, it can essentially bind in two orientations. Either the nitrogen lone pair is pointing towards the heme iron center, or away from it (see Scheme 3). If the lone pair is pointing towards the heme iron center, then the geometry is set up for an N-oxidation, which occurs through the formation of a σ bond between the lone pair of the nitrogen atom and the iron–oxo oxygen atom. An H-abstraction is not favorable in this geometry owing to the fact that the nitrogen lone pair will not be able to stabilize the transition state without undergoing an inversion. If, on the other hand, the lone pair points away from the heme iron center, the geometry is set up for an H-abstraction reaction, and N-oxidation is unlikely. To change one of these conformations into the other, an inversion of the nitrogen is required. If a protonated amine binds in the active site and is deprotonated by the CYP, then it will most likely bind in the conformation that prefers N-oxidation. Then, the formation of an *N*-oxide will be in competition with an inversion process, and the likelihood of observing *N*-oxides could be related to the inversion barrier.

To evaluate if this hypothesis is reasonable we have computed the inversion barriers for all ten fragments, and the resulting energies are shown in Figure 1 and Table S4 in the Supporting Information. An example of the energetics for

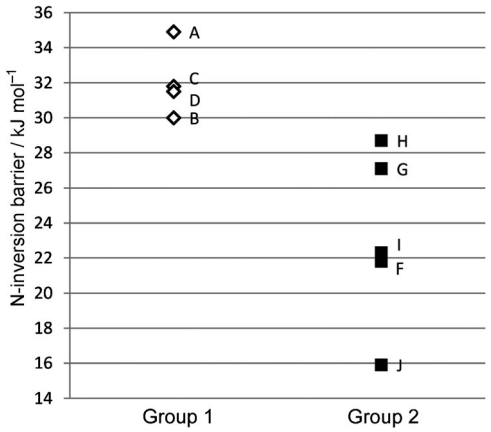


Figure 1. N-inversion barriers for nine fragments (**E** excluded, since the inverted complex is unstable). N-oxidized fragments are shown in white (group 1), others in black (group 2).

fragment **H** is shown in Figure S4 in the Supporting Information. The results show that the inversion barrier is consistently lower for the fragments that are not N-oxidized (**F**–**J**). While the difference between the highest barrier in this group (**H**, 28.7 kJ mol^{-1}) and the lowest barrier in the N-oxidized group (**B**, 30 kJ mol^{-1}) is very small, the possibility to classify the fragments into two groups by a cutoff value shows that the inversion process is an important determinant in the discrimination between N-oxidation and H-abstraction reactions. It is also worth noting that in Lu25_109[18] (which contains fragment **B**, with the lowest inversion barrier among the fragments that get N-oxidized), the *N*-oxide is a minor metabolite in CYPs. Two other products are generated in larger amounts (a demethylated product, and a product in which the nitrogen-containing ring has become aromatic owing to the removal of three hydrogen atoms).

To investigate whether such an N-inversion is possible inside the protein cavity, the inversion barrier of olanzapine (containing fragment **A**) was determined in the presence of the protein. A scan of the nitrogen inversion process was performed, with the protein kept fixed during the optimizations. The same scan was also performed in the gas phase to check whether it is realistic to observe a nitrogen inversion in the protein.

As shown in Figure 2a,b, the geometry-optimized structures in the protein and gas phase are quite similar. The superimposition of the atoms of the piperazine ring reveals that there is only a small structural difference (Figure 2c). Energetically, these small structural differences between the

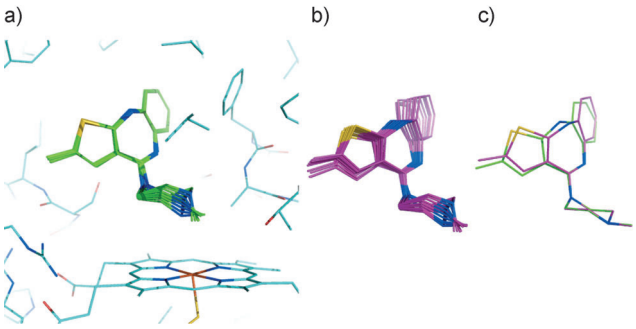


Figure 2. The N-inversion for olanzapine a) inside CYP 3A4 (C atoms of substrate in green) and b) in the gas phase (C atoms of substrate in magenta). c) The structures in the energy minima in the protein and gas phase.

protein cavity and the gas phase translates into an N-inversion barrier difference of less than 3 kJ mol^{-1}. Thus, it seems possible to make the inversion inside the protein cavity without steric constraints from the protein.

We considered a larger set of 475 CYP 3A4 substrates[5] to see if the knowledge gained in this study can be used to rationalize whether N-oxidation occurs. This set of compounds contains 145 tertiary amines, of which 12 are N-oxidized. Applying the reactivity method implemented in the SMARTCyp program on the 475 CYP 3A4 substrates,[11a–c] 64 %/76 %/81 % of the experimentally observed sites of metabolism are within the top 1/2/3 ranked sites, respectively (see Table S5 in the Supporting Information). Thus, the most reactive sites are usually also likely to be oxidized by CYP 3A4. However, while oxidation at the α-carbon atom (which leads to N-dealkylation) is usually ranked first in silico, also the oxidation of the nitrogen atom is often highly ranked. In fact, for 93 out of the 145 tertiary amines the nitrogen atom is among the top 3 ranked reactive sites (Table S6 in the Supporting Information), thus indicating that these amines are likely to be N-oxidized, although this is not often observed experimentally.

By using the knowledge on which types of alkylamines that have high N-inversion barriers, a fragment-based rule for determining if a tertiary alkylamine is unlikely to form an *N*-oxide or not was created. Tertiary alkylamine nitrogen atoms that are unlikely to become N-oxidized get a penalty to their score (Table S6 in the Supporting Information). This empirical correction improves the top 3 accuracy (see Table S5 in the Supporting Information). In particular, the false positive rate is reduced significantly.

To summarize the findings, the N-inversion barrier is higher for all compounds being N-oxidized by CYPs (fragments **A**–**E**) than for those compounds that are not N-oxidized (fragments **F**–**J**). This indicates that the competing and often low-energy-barrier abstraction of the α-hydrogen atom can occur for compounds with a low *N*-inversion barrier. Without the N-inversion, the probability is higher that the α-hydrogen atom is on the same side as the nitrogen lone pair in the CYP reaction, thereby increasing the energy barrier for H-abstraction and thus, favoring the N-oxidation reaction.

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