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Prediction of Activation Energies for Hydrogen Abstraction by Cytochrome P450

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Abstract

We have estimated the activation energy for the hydrogen abstraction by compound I in cytochrome P450 for a diverse set of 24 small organic substrates using state-of-the-art density functional theory (B3LYP). We then show that they can be reproduced by computationally less demanding methods, e.g. by using small organic mimics of compound I with both B3LYP and the semiempirical AM1 method (mean absolute error of 3–4 kJ/mol), or by calculating the bond dissociation energy, calculated without relaxation of the radical (B3LYP) or estimated from three-point fit to a Morse potential (AM1; errors of 4 and 5 kJ/mol, respectively). We can assign an activation energy of 74, 61, 53, 47, and 30 kJ/mol to primary carbons, secondary/tertiary carbons, carbons with adjacent sp^2 or aromatic groups, ethers/thioethers, and amines, respectively, which gives a very simple and predictive model. Finally, some of the less demanding methods are applied to study the CYP3A4 metabolism of progesterone and dextromethorphan.

Introduction

The cytochromes P450 form a ubiquitous protein family with functions including synthesis and degradation of many physiologically important compounds, as well as degradation of xenobiotic compounds, e.g. drugs.¹ Much effort has been put into the study of these enzymes, because they influence the transformation of pro-drugs into their active form and the bioavailability and degradation of many drugs.

The cytochrome P450 enzymes catalyze several different types of reactions, of which the hydroxylation of CH groups is the most studied.^{2,3} Recent investigations suggest that the hydroxylation follows a two-step mechanism, in which the first step involves a hydrogen abstraction from the substrate by a $\text{Fe}^{\text{V}}=\text{O}$ species (formally), denoted compound I, and in the second step, the radical rebounds to the $\text{Fe}^{\text{IV}}-\text{OH}$ intermediate. This gives a Fe^{III} -alcohol complex, from which the hydroxylated product may dissociate. Density functional theory (DFT) calculations indicate that the highest energy barrier along this pathway is observed for the hydrogen-abstraction step,⁴⁻⁷ and this is also supported by experimental results.⁸

Considering the importance of the cytochromes P450 in the metabolism of drugs, it would be highly desirable to have a method that could predict if and in what way a drug candidate will be metabolized by these enzymes. Most previous studies have focused on how a compound is metabolized, based on quantum chemical studies on isolated substrates, pharmacophore models, docking, molecular dynamics simulations, chemical rules, or quantitative structure-activity relationships (QSAR) from physicochemical, topological, or 3D structures.⁹⁻¹³ The consensus is that not one single computational approach can reliably predict metabolism by the P450s, but for most enzymes, a combination of both the intrinsic reactivity of various parts of the substrate (electronic factors) and the accessibility of the groups to the reactive $\text{Fe}^{\text{V}}=\text{O}$ group in the enzyme (steric effects) need to be taken into account.

The intrinsic reactivity of the various groups have normally been estimated by QM methods at the Hartree-Fock, semiempirical, or DFT levels. It has typically been estimated from the stability of the radicals formed after hydrogen abstraction,¹⁴ the ionization potential of these radicals (estimated from the energy of highest occupied molecular orbital, HOMO),^{11,15,16} or from the density of the HOMO on

the various atoms¹⁷. More sophisticated methods involve the direct estimation of the activation energy for the hydrogen abstraction using simplified models of the $\text{Fe}^{\text{V}}=\text{O}$ state of cytochrome P450, e.g. an isolated O atom^{18,19}, a methoxy^{16,20,21}, or a *p*-nitrosophenoxy radical.^{15,22} Recently, it has even become possible to calculate activation energies with DFT and full models of the active porphyrin species, giving near quantitative results.^{3,23,24} However, such calculations are still quite time-consuming, especially for molecules of the size of a typical drug (weeks of CPU time). On the other hand, they can be used to develop and calibrate more approximate methods. A first such attempt has recently been published,⁶ but it used only 11 substrates, without any external validation, and tested relatively few methods. It should also be mentioned that Park & Harris have compared the relative reactivity of two sites in methoxyflurane predicted from a full DFT optimization of the transition state and from simpler models, showing that both the radical stability and the *p*-nitrosophenoxy radical model gave excellent results at the DFT level, but not at the semiempirical AM1 level.²²

In this paper, we extend this work. We have used DFT calculations on a full model of compound I and determined the activation energy for the hydrogen abstraction from 24 substrates (cf. Figure 1), involving primary, secondary, and tertiary aliphatic carbon atoms and nitrogen, oxygen, sulfur, as well as sp^2 or aromatic carbon atoms next to the reactive atom. This covers the most common types of atoms in drug-like molecules. The 24 substrates were divided into training (14 compounds) and test sets (10 compounds), which were used to validate the ability of different computationally less demanding methods to predict the activation energies in a systematic manner. These models include DFT calculations with different basis sets, calculations with small radical models of compound I at both the DFT and semiempirical levels, as well as a set of 20 different descriptors of the substrate or the radical (e.g. bond dissociation and orbital energies). The results shows that it is possible to predict the DFT activation energies with an accuracy of 2–5 kJ/mol at a modest computational cost.

Methods

Model complexes

We have modeled the active site of compound I in cytochrome P450 as iron (formally Fe^{V}) porphine (i.e. a porphyrin without side chains) with CH_3S^- and O^{2-} (formally) as axial ligands (cf. Figure 2). Shaik and coworkers have argued that SH^- is a better model of Cys than CH_3S^- .^{23,25} However, a recent QM/MM study,²⁶ where a larger reference model for Cys was used ($\text{HCONHCH}(\text{CH}_2\text{S})\text{CONH}_2$), showed that SCH_3^- gives better geometries than does SH^- . SH^- gives better spin densities in vacuum, but the opposite is true in the protein. Therefore, we have used SCH_3^- as a model of the Cys ligand. We also tested to model compound I by a methoxy, phenoxy, or *p*-nitrosophenoxy radical (Figure 2), as has been suggested before.^{15,16,20-22,27}

We have studied the hydrogen abstraction from 24 different substrates (Figure 1). The studied substrates involve primary, secondary, and tertiary aliphatic carbon atoms, which have nitrogen, oxygen, sulfur, as well as sp^3 , sp^2 , or aromatic carbon atoms next to them. For the test of various simplified methods to predict the activation energy, the substrates were divided into a training set (**1-14**) and test set (**15-24**), which both still contain all the various functional environments.

Three different states along the hydrogen-abstraction reaction were studied (Figure 2), viz. the isolated compound I and substrate, the transition state (TS) for the hydrogen abstraction, and the intermediate after the hydrogen abstraction, where the substrate radical weakly interacts with the $\text{Fe}^{\text{IV}}-\text{OH}$ complex (protonated compound II), i.e. the intermediate before the radical-rebound step. All energies are given relative to the sum of the energies of the isolated compound I model and the isolated substrate.

All three states were studied in the quartet (intermediate-spin) state. Previous studies have shown that the low- and intermediate-spin states are very close in energy for both compound I and the hydrogen-abstraction TS.^{6,28} Therefore, it is enough to study one of the spin states, and we have selected the quartet for computational reasons. The next step in the reaction, the radical-rebound step is barrierless in the doublet state, but it has a small barrier for the quartet state, 4–12 kJ/mol.^{4,5,7,23,29} This means that the

TS for the hydrogen abstraction normally has the highest energy on the potential energy surface.^{4,5,7}

Therefore, it is enough to study only the TS of the hydrogen-abstraction step.

The quantum chemical calculations were performed with the density functional method B3LYP³⁰⁻³² (unrestricted formalism for open shell systems) or with the semiempirical AM1 method.³³ In the B3LYP calculations, we have used for iron the double- ζ basis set of Schäfer et al.³⁴, enhanced with a p function with the exponent 0.134915. For the other atoms, the 6-31G(d) basis set³⁵ was used. The final energies were determined at the B3LYP/6-311++G(2d,2p) level. These energies also include the zero-point vibrational energy, calculated at the B3LYP/6-31G(d) level. The frequency calculations also verified that the structures represented true minima or transition states.

Molecular Descriptors

We have extracted 20 descriptors for the substrates and radicals from the DFT and AM1 calculations. These are the Mulliken charges on the carbon (q_C) and hydrogen (q_H) atoms involved in the reaction, the spin on the carbon atom in the radical (S_C), the energies of the HOMO and LUMO (lowest unoccupied molecular orbital), the energy difference between these two orbitals, as well as the coefficients for the hydrogen 1s and carbon 2p atomic orbitals in the HOMO and LUMO.

Moreover, we studied the bond dissociation energies (BDEs), calculated in three different ways: First, we have used the standard definition of the BDE, i.e. the difference in energy between the substrate and the sum of the radical and hydrogen atom, using optimized geometries for all three species:

$$BDE = E(\text{substrate}) - E(\text{radical}) - E(\text{H}) \quad (1)$$

Of course, this is also a direct measure of the stability of the radical (the energy of a hydrogen atom is a constant). We can save some computer time by omitting the optimization of the radical (i.e. by calculating the $E(\text{radical})$ for the geometry of the substrate, but with the hydrogen atom removed)⁶. This will be denoted BDE_{fr} (frozen radical) in the following. The structures were optimized using B3LYP/6-31G(d), and the final energies were determined with B3LYP/6-311++G(2d,2p) with the zero-point

vibrational energy included (or with AM1). Finally, we also tested to obtain the BDE by a fit to a Morse potential, as has been proposed by Lewin and Cramer³⁶: The energies of the substrate was calculated in three points (the equilibrium C–H distance and ± 0.3 Å the equilibrium C–H distance), with the rest of the geometry fully optimized and they were then used to fit the dissociation energy by a Morse potential:

$$E(r) = BDE_{Morse}[1 - \exp(\alpha_{Morse}(r - r_{eq}))]^2 \quad (2)$$

The calculations were performed with the B3LYP/MIDI! ³⁷ and AM1 methods.

Programs

The non-linear fits to the Morse potential were done with the Grafit program³⁸. Partial least squares of latent variables (PLS) were generated in the Simca program³⁹ and all B3LYP and AM1 calculations were performed with the Gaussian03 software.⁴⁰

Results and Discussion

Calculated Activation Energies

We have studied 24 different hydrogen-abstraction reactions (Figure 1) using the Fe(porphine)(SCH₃)O model of compound I (Figure 2a). In Table 1, the B3LYP/6-311++G(2d,2p)//B3LYP/6-31G(d) energies (relative to the isolated substrate and compound I; in kJ/mol) and O–H and C–H bond lengths in the TS and the intermediate are shown. The amines (**13**, **14**, **23**, and **24**) have the lowest activation energies for the hydrogen abstraction, 28–32 kJ/mol. The ethers and thioethers (**10–12**, **22**) have activation energies of 45–55 kJ/mol, and compounds with *sp*² hybridized (**5**, **6**, **18**, **19**) or aromatic (**7–9**, **20**, **21**) groups next to the reactive carbon have activation energies in the 48–56 kJ/mol range. Substrates with only *sp*³-hybridized atoms have the highest barriers. Abstractions from secondary (**3**) and tertiary (**4**) carbon atoms or from carbon with F atoms bound (**16**) have activation energies of 60–62 kJ/mol, whereas abstractions from primary carbon atoms (**1**, **2**, **15**,

17) have even larger activation energies (>72 kJ/mol). This follows the qualitative stability of the radicals, as has been noted before.^{15,16}

The C–H and O–H bond lengths in the TS are clearly correlated to the activation energy: the longer C–H and shorter O–H (cf. Figure 3a) bond length, the larger activation energy. One extreme is methane which has the highest activation energy and a very late TS with an O–H bond length of 1.15 Å, indicating that the H atom is almost completely transferred to the O atom already in the TS. The other extremes are the amines (**13**, **14**, **23**, **24**) which have O–H bond lengths of ~1.3 Å and the lowest activation energies. A similar relation between the bond lengths and activation energies for hydrogen-abstraction reactions has been previously noted.^{6,15}

Seven of the substrates (**1–3**, **5**, **7**, **17**, and **24**) have previously been studied by Shaik and co-workers using similar methods (structures at the B3LYP/LACVP level and energies at the B3LYP/LACVP3+* level, using a SH[–] model of the Cys ligand).^{6,28} The two sets of activation energies differ by less than 10 kJ/mol, which show that the model of the Cys ligand and the other details of the calculations are not crucial in terms of energies.

Simplified DFT calculations

The energies in Table 1 constitute state-of-the-art DFT estimates of the activation energy for hydrogen abstraction from various substrates by any cytochrome P450. They should provide a consistent set of best computational estimates of the intrinsic reactivity for various substrates with compound I. Similar methods have been used to study many different enzymes, with absolute errors in the activation energy of less than ~20 kJ/mole and even smaller relative errors.^{41,42} In particular, such methods have been successfully applied also to cytochromes P450,³ giving kinetic isotope effects²⁸ and regioselectivity²² in good accordance with experimental results, for example. The aim of this article is to see if we can predict these energies with computationally less demanding methods (the DFT calculations took several CPU-weeks for each substrate; cf. Table 2.)

A first step in this direction is to see if the zero-point energies are really needed, because the frequency calculations take almost as long time as the geometry optimizations. The results in Table 2 show that this is not really the case: The activation energies with zero-point corrections correlate almost perfectly with those without the corrections ($R^2 = 1.00$; mean absolute error between energies predicted from the correlation line and the activation energies with zero-point corrections, MAE = 0.7 kJ/mol). Likewise, the time-consuming calculation with the large 6-311++G(2d,2p) basis set can also be avoided (it also takes about the same time as the geometry optimization), but the effect is appreciably larger: $R^2 = 0.78$ and MAE = 6 kJ/mol for the test set if the B3LYP/6-31G(d) energies are used without both the zero-point energies and the calculation with the big basis set. De Visser *et al.*⁶ reached a similar conclusion.

Another possibility is to study the intermediate after the hydrogen abstraction (but before the radical rebound; it is easier to optimize than the TS). The results in Figure 3b show that for most of the substrates, the energies of the intermediates correlate well with that of the TS ($R^2 = 0.96$). However, for substrates with aromatic or sp^2 hybridized groups next to the reactive carbon (**5–9**, **18–21**), this correlation disappears ($R^2 = 0.50$) **xxx Delete 0.50 from Figure 3b – it is not the same line and the deviating points are not shown; it is enough to have it in the text.** For these substrates, the interaction between the radical and compound II is weak (C-H distance larger than 2.40 Å), and the radical is essentially dissociated from the compound II model (whereas it forms a stable interaction to the Fe^{IV}–OH group, with C–H distances of 2.11–2.35 Å for the other substrates). This is probably because the radical is delocalized, which stabilizes it. Thus, there is little gain to study the intermediates.

Smaller models of compound I

In previous work, simplified organic radicals have been used to model compound I, e.g. methoxy^{16,20,21} or *p*-nitrosophenoxy radicals^{15,22}. Therefore, we also tested how well activation energies, calculated with the *p*-nitrosophenoxy, phenoxy, and methoxy radicals (Figure 2b) correlate with the activation energies obtained with the Fe(porphine)(SCH₃)O model. The activation energies and the O–H and C–H bond

lengths in the TS obtained with these models at both the B3LYP and AM1 levels are collected in Tables S1 and S2 in the supplementary material. The correlation to the full DFT activation energies are shown in Table 3 for both the training and test sets. It can be seen that these models work well at both B3LYP and AM1 level: The MAE of the predicted activation energies is only 3–4 kJ/mol for B3LYP ~4 kJ/mol for AM1 (with one outlier excluded for the *p*-nitrosophenoxy radical). However, most of the models give a large systematic error in the absolute activation energies (see Figure 4 and Tables S1 and S2). The phenoxy radical gives both the smallest MAEs and the best absolute energies.

As for the full porphyrin model, significant computer time can be saved by omitting the frequency and big basis set calculations (Table 2) and even though the phenoxy radical gives slightly better results, much computer time can be saved by using the methoxy radical. Even more computer time can be saved by using AM1 for these calculations (Table 4).

Molecular descriptors

All calculations up to now have involved the optimization of TS geometries. This is quite tedious and not guaranteed to always converge. It would be preferable if a correlation could be found between the activation energies and the properties of the isolated substrates or radicals alone (geometry optimizations of ground states are much easier and more stable). To this aim, we have studied the correlation between the activation energies and various molecular descriptors of the substrates and their corresponding radicals. The descriptors include the Mulliken charges on the carbon (q_C) and hydrogen (q_H) atoms involved in the reaction, the spin on the carbon atom in the radical (S_C), the energies of the HOMO (which is an approximation to the ionization potential, according to Koopmans' theorem) and LUMO, and their energy difference, the coefficients for the hydrogen $1s$ and carbon $2p$ atomic orbitals in the HOMO and LUMO, as well as the BDE of the reactive C–H bond, calculated by three different methods. The results (R^2 and MAE for the training and test sets) are collected in Tables 5 (B3LYP) and 6 (AM1).

Most of the descriptors correlate quite well with the activation energies for the compounds that do not have a sp^2 hybridized or aromatic atom next to the reactive carbon (substrates **5–9**, and **18–21**), as is illustrated in Figure 5a. However, including the latter substrates strongly weakens the correlations. The best correlations for all compounds are obtained for the descriptors related to the BDE. Interestingly, with B3LYP the best results are obtained for BDE_{fr} (MAE = 4 kJ/mol; Table 5 and Figure 5b), which is the cheapest BDE descriptor, whereas the other two gave twice as large errors. De Visser *et al.*⁶ have obtained similar results for a smaller set of substrates (e.g. not involving N, O, and S), and the models were not externally validated. As before, the B3LYP results are similar if the frequency and big basis set calculations are omitted (cf. Table 2). With AM1, all three BDE descriptors gave similar MAEs for the test set (6–8 kJ/mol), but α_{Morse} gave slightly better results (MAE = 5 kJ/mol). α_{Morse} is associated with the width of the Morse potential, that is related to the force constant and BDE_{Morse} , which may explain why there is a correlation with the height of the energy barrier.

All the other descriptors gave much worse correlations and predictions (MAE > 8 kJ/mol). Descriptors based on the radicals gave slightly better results than those based on the substrates. In general, the HOMO energy of the radical (Figure 5c), the HOMO–LUMO energy gap, the carbon $2p$ coefficient in HOMO, and the spin density on the radical carbon atom (AM1) gave the best results. The ionization potential of the radical has previously been related to calculated activation energies (together with the radical stability, i.e. the BDE).^{15,16,20,22,27} Our results in Table 4 and Figure 5c confirm that this is a reasonable approximation.

Finally, we applied the partial least squares of latent variables (PLS) method to make models of the activation energies using all the molecular descriptors at either B3LYP or AM1 level. The score plot in Figure 6a shows that the first component separates the compounds according to the activation energy, e.g. compounds **1** and **13/14** are found in different parts of the plot. The second component separates the compounds that contain sp^2 hybridized or aromatic atoms (compounds **5–9**) next to the reactive carbon. The loadings plot in Figure 6b shows that the BDE descriptors, the $2p$ coefficient in HOMO, the HOMO-LUMO energy gap, and the spin on the carbon of the radical ($2P(\text{HOMO})$, $E(\text{HOMO})$)-

$E(\text{LUMO})$, and S_C , respectively) are found relatively close to the activation energy, and are therefore positively correlated with it. This means that the larger bond dissociation energy, the larger is the activation energy. On the other hand, the HOMO and LUMO energies, and α_{Morse} are negatively correlated to activation energy, because these descriptors are found in the opposite part of the plot. The fact that the LUMO energies are located in the lower region of the loadings plot is also one of the reasons why the second component separates the compounds with and without aromatic rings in the score plot (LUMO is more stable for the aromatic compounds).

At the B3LYP level, a PLS model that contains all descriptors with $\text{VIP} > 0.8$ (variable influence on projection) is of the same quality as BDE_{fr} alone (the MAEs of the test set are both ~ 4 kJ/mol). Likewise, at the AM1 level, the PLS model is not significantly better than α_{Morse} alone (MAE of the test set are 4 and 5 kJ/mol, respectively). Therefore, we see no reason to employ the more complicated and computationally demanding PLS models.

A simple qualitative model

As mentioned above, we observe a clear grouping of the activation energies according to the chemical function and environment of the reactive carbon atom. Based on this observation and the average energies in the training set, we can set up the following very simple predictive model for the activation energies (energies in brackets are the averages obtained for both the training and test sets together):

- Primary carbon atoms in a sp^3 environment; no F atom bound to reactive C (**2; 15,17**) – 73.8 (74.4) kJ/mol
- Secondary or tertiary carbon in a sp^3 environment or F directly bound to reactive C (**3,4; 16**) – 60.9 (61.1) kJ/mol
- Aromatic or sp^2 hybridized atoms next to the reactive C (**5-9; 18-21**) – 52.6 (52.2) kJ/mol
- O or S atoms next to the reactive C (**10-12; 22**) – 47.3 (49.1) kJ/mol
- N atoms next to the reactive C (**13,14; 23,24**) – 29.8 (30.1) kJ/mol

Interestingly, this very simple model gave the best predictions obtained in this paper: the MAE for the test set was 2.5 kJ/mol, with the largest error arising from methoxybenzene (**22**; 7.2 kJ/mol; MAE = 2.1 kJ/mol without this compound). Thus, the data in this paper allows for a prediction of the activation energy by visual inspection. Of course, such predictions can be easily automatized. However, for most typical drug candidates with many nearby heteroatoms and complicated ring systems, this qualitative model may be hard to apply. If so, B3LYP calculations of BDE_{fr} or AM1 calculations of the α_{Morse} descriptor may be needed. Even more accurate results are obtained with explicit TS calculations using a methoxy or phenoxy radical model at AM1 level.

Applications on drug-like molecules

Our previous results have established correlations between various theoretical descriptors and activation energies for the hydrogen abstraction calculated by state-of-the-art DFT methods. In this section, we will illustrate the applicability of these methods on drug-like molecules and compare with experimental data. Since we study only the intrinsic reactivity of the various sites, we will concentrate on metabolites cytochrome P450 CYP3A4, because this enzyme has a very broad specificity (over 50% of the marketed drugs are metabolized by this enzyme) and therefore does not seem to put severe restrictions on the orientation of the ligand in the binding pocket^{43,44}. In addition, we combine the calculated activation energies with estimates of the solvent-accessible surface area (SASA)⁴⁵ of the various hydrogen atoms, which has been used as measure of the intrinsic accessibility of the various sites.⁴⁶ We have studied two CYP3A4 substrates, progesterone⁴⁷ and dextromethorphan⁴⁸(cf. Figure 7).

There are 30 hydrogen atoms in progesterone, all of which may react by hydrogen-atom abstraction, except H4, which is expected to react via epoxide formation instead. We have studied all these 29 atoms with seven of our suggested methods (it is too time demanding to do a full DFT optimization of all the TS energies). Our simple qualitative model directly suggests that the four C atoms next to sp^2 hybridized atoms, positions 2, 6, 17, and 21, should be more reactive than the tertiary (in positions 8, 9, 14), secondary (in positions 1, 7, 11, 12, 15, 16), and primary (in positions 18, 19)

carbon atoms in a sp^3 environment. This is in good agreement with experimental results, which show that CYP3A4 performs 2β , 6β , and 16α hydroxylations, whereas other CYP2C9 and CYP2C19 are responsible for hydroxylation of the H atoms in the 17 and 21 positions⁴².

However, the qualitative model cannot discriminate between the α and β positions. The intrinsic accessibility may be the explanation why H16 α is more reactive than H (the SASA is ~55% larger for this atom, cf. Table 3 in supplementary information), but for H2 and H6, the difference is smaller and goes in the wrong direction.

Apparently, the qualitative model is not accurate enough to correctly predict such details in the metabolism. However, all the other descriptors (the activation energy predicted with the methoxy radical and BDE_{fr} calculated at the B3LYP or AM1 levels, as well as BDE_{Morse} and α_{Morse} , calculated at the AM1 levels; see Table S3 in the supplementary information), predict that the H2 β and H6 β atoms are appreciably more reactive than the corresponding α atoms (see Figure 8). These H atoms are almost perpendicular to the π electron system, which probably stabilize the TS and intermediate, since the radical would be in conjugation with the π system.

The secondary and tertiary C atoms are other possible sites of hydroxylation. The tertiary C atoms (position 8, 9, and 14) have smaller predicted TS barriers than the secondary C atoms, but they are not very accessible (the SASA is less than 8 Å²), which may explain why they are not hydroxylated.⁴³ The secondary sp^3 hybridized C atoms have larger predicted TS energies, but they are significantly more accessible (> 10 Å²). The most accessible secondary sp^3 hybridized H atom is that in 16 α position, which may explain why it is metabolized by CYP3A4 (see Table 3 in supplementary information).

Dextromethorphan contains 25 hydrogen atoms, three of which are in the benzene ring and are not expected to react by H abstraction. Our qualitative model predicts that the carbon atoms next to the nitrogen atom are most reactive, followed by the C atom of the methoxy group, the C atom next to the aromatic ring, and the tertiary C atom xxx **This is hard to read without atom numbers. Can you**

include atom numbers in Figure 7 and here? The IUPAC name is 3-methoxy-17-methyl-

(9 α ,13 α ,14 α)-morphinan. which may help the numbering somewhat. Experimentally, only the H atoms of the free methyl group that is next to the amine are metabolized by CYP3A4. This can be explained by the higher accessibility of these atoms. (Figure 9 or Table S4 in supplementary information). **xxx I would prefer to move Tables S3 and S4 back to the article and delete Figures 8 and 9 (it is hard to see any details).** Interestingly, CYP2D6 catalyses O-dealkylation (C20), which is also predicted by the qualitative model and the methoxy hydrogen atoms have rather low predicted activation energies and a high solvent accessibility.

Conclusions

With DFT calculations, we have determined the TS for the reaction of a realistic model of compound I in cytochrome P450 with 24 different substrates. We have then tested in a systematic way if these activation energies can be predicted by computationally less demanding methods. To this aim, the 24 substrates were divided into a training and a test set, which both contain primary, secondary, and tertiary aliphatic carbon atoms and nitrogen, oxygen, sulfur, as well as sp^2 or aromatic carbon atoms next to the reactive atom.

First, we showed that the zero-point energy corrections and the big basis set (B3LYP/6-311++G(2d,2p)) energies normally employed in such calculations change the activation energies by only 1 and 6 kJ/mol on the average, but they increase the computational effort by a factor of 2–3. On the other hand, it is not possible to study the intermediate after the hydrogen abstraction, rather than the TS for the hydrogen abstraction.

Second, we have evaluated the use of small radicals instead of a full iron-porphine model for compound I. We have tested three such models: *p*-nitrosophenoxy, phenoxy, and methoxy radicals. All give excellent correlations to the activation energies obtained with the full compound I model, with MAEs of 3–4 kJ/mol for B3LYP and 4 kJ/mol with AM1. The phenoxy radical gave the best correlation and the smallest absolute errors. Furthermore, the O–H and C–H bond lengths of the atoms directly

involved in the reaction correlate well with those obtained by using the full porphine model, which means that optimizations, that contain compound I, can be started from reasonable structures that will converge faster.

Third, we have tried to find a correlation between the activation energies and molecular descriptors of the substrate or the corresponding radicals. The best results are obtained with the BDE obtained without relaxing the geometry of the radical (BDE_{fr}) with the B3LYP method, without zero-point and big basis set corrections (MAE = 4 kJ/mol). At the AM1 level, only slightly worse results (MAE = 5 kJ/mol) can be obtained from a three-point fit of the BDE, using a Morse potential (α_{Morse}). Interestingly, the results are not significantly improved by a PLS model involving all the 20 studied descriptors.

However, the fastest and most accurate results can actually be obtained directly from the DFT activation energies and a classification of the chemical function and environment of the reactive carbon atom: substrates with nitrogen atoms next to the reactive carbon have activation energies of 30 kJ/mol. Those with oxygen and sulfur atoms next to the reactive carbon have activation energies of 47 kJ/mol. The activation energy of substrates with aromatic or sp^2 hybridized atoms next to the reactive C is 53 kJ/mol. Finally, primary carbon atoms have a activation energy of 74 kJ/mol, whereas secondary and tertiary, as well as carbons with bound F atoms have activation energies around 61 kJ/mol. Using these five groups, the activation energies in the test set can be predicted with a MAE of only 2.5 kJ/mol.

Thus, our investigation allows us to define a hierarchy of methods (in terms of accuracy and computational load) to estimate the intrinsic reactivity of each aliphatic carbon atom in a general drug candidate: For simple groups, an accurate estimate of the activation energy can be directly obtained by visual inspection of the chemical environment. This should be enough for most screenings of drugs. Only if the drug contains atoms with complicated or conflicting environments or functional groups not covered in this investigation does it seem to be justified to use more demanding methods to estimate the activation energy. If so, reasonably accurate results can be obtained from α_{Morse} , calculated at the AM1 level. These calculations can easily be automatized and takes a few minutes for a typical drug candidate. If a higher accuracy is needed or if the AM1 method is not accurate enough for the substrate of interest,

the BDE_{fr} can be calculated at the B3LYP level in less than an hour for a typical drug. Even more accurate results are obtained with explicit TS calculations using a methoxy or phenoxy radical model, calculations which take ~10 minutes at the AM1 level and a few hours at the B3LYP level. Finally, the most accurate results are obtained by a DFT optimization of the TS with a full porphyrin model. Such calculations take several weeks, however.

We have illustrated the applicability of our results on two drug-like molecules, progesterone and dextromethorphan. We show that we can predict most experimental sites of metabolism with the simple qualitative model, combined with an estimate of the intrinsic accessibility of each site using the SASA. However, for a more detailed view of the reactivities (e.g. the preference between the α and β atoms, DFT or AM1 calculations are needed.

The most interesting result in this investigation is perhaps that all activation energies are quite low, <80 kJ/mol (excluding methane). This means that essentially all CH groups in a drug can be metabolized by cytochrome P450 within a reasonable time (an activation energy of 80 kJ/mol corresponds to a rate constant of $\sim 0.1 \text{ s}^{-1}$). This illustrates the extreme reactivity of compound I in cytochromes P450. This reduces the fundamental question whether and how a drug will be metabolized by these enzymes to two simpler ones: What groups are sterically accessible to the oxygen atom of compound I and which of these groups have the highest intrinsic reactivity. The present investigation solves the second issue and gives a few predictive rules that are directly applicable to nearly any drug candidate. The first issue is what remains to be solved. It involves steric factors of the enzyme, which of course differ between different types of cytochromes P450 and may be approached by docking and MD simulations.⁴⁹⁻⁵²

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Supporting Information Available: B3LYP or AM1 energies, C–H, and O–H bond lengths in the TS with small organic radical models of compound I. **xxx Also Tables S3-4 if we keep them there**

Figure Captions

Figure 1. Compounds used to study the TS energies for the aliphatic hydrogen abstraction reaction. The arrows indicate the carbon atom from which the hydrogen was abstracted for compounds with more than one possible aliphatic site of abstraction. Compounds **1–14** were used as training set and compounds **15 – 24** as test set for the models of the TS energies.

Figure 2. Energies (kJ/mol), O–H, and C–H distances (Å) for the hydrogen-atom abstraction of methane by a (a) Fe(porphine)(SCH₃)O model and (b) smaller organic radicals at the B3LYP/6-311++G(2d,2p)//B3LYP/6-31G(d) level with the zero-point vibrational energy at the B3LYP/6-31G(d) included. Numbers in brackets are the corresponding AM1 energies.

Figure 3. Activation energies of the Fe(porphine)(SCH₃)O model related to (a) O–H bond length in the TS, and (b) energy of the intermediate (compounds with *sp*² hybridized, aromatic atoms, or a F atom next to the reactive carbon atom omitted, i.e. with 9 and 4 compounds in the training and test sets, respectively). Closed and open symbols represent training and test sets, respectively. *R*² is given for the training set.

Figure 4. (a) Comparison of activation energies (kJ/mol) using *p*-nitrosophenoxy (PNP), phenoxy, or methoxy radicals to abstract the H atom with Fe(porphine)(SCH₃)O as model for compound I. Comparison of the (b) energies, (c) O–H, and (d) C–H bond lengths (Å) in the TS using a *p*-nitrosophenoxy radical instead of the Fe(porphine)(SCH₃)O model at B3LYP or AM1 level. The full and open symbols represent the training and test sets, respectively. *R*² is given for the training set.

Figure 5. Activation energy for the Fe(porphine)(CH₃S)O model versus (a) BDE , (b) BDE_{fr} , and (c) $E(\text{HOMO})$ of the radical determined at B3LYP level. R^2 for the training set is also indicated and without the compounds that are highlighted in the plot in brackets.

Figure 6. PLS analysis of B3LYP descriptors: (a) score plot and (b) loadings plot.

Figure 7. Progesterone and dextromethorphan.

Figure 8. (a) Reactivity of the hydrogen atoms (BDE_{fr} determined with B3LYP/6-31G(d)) and (b) the SASA of the H atoms in progesterone. Green H atoms: High reactivity (low TS) and high SASA. White H atoms: Low reactivity (high TS) or low SASA. For further details, see Table S3 in the supplementary information.

Figure 9. (a) Reactivity of the hydrogen atoms (BDE_{fr} determined with B3LYP/6-31G(d)) and (b) the SASA of the H atoms in dextromethorphan. Green H atoms: High reactivity (low TS) and high SASA. White H atoms: Low reactivity (high TS) or low SASA. For further details, see Table S4 in the supplementary information.

Table 1. Relative energies and C–H and O–H bond lengths in the transition state for hydrogen

abstraction and following intermediate. The energies were determined at B3LYP/6-

311++G(2d,2p)//B3LYP/6-31G(d) level with the zero-point vibrational energy at the B3LYP/6-31G(d)

included.^a

Substrate	Transition state			Intermediate		
	Energy ^b	C–H ^c	O–H ^c	Energy ^b	C–H ^c	O–H ^c
1 Methane	86.7	1.39	1.15	51.8	2.35	0.98
2 Propane(1)	73.9	1.35	1.18	34.2	2.25	0.98
3 Propane(2)	62.0	1.32	1.21	15.0	2.23	0.98
4 Isobutane	59.7	1.31	1.24	4.5	2.33	0.98
5 Propene	53.9	1.31	1.25	-27.2	2.44	0.98
6 Propionaldehyde	47.9	1.35	1.21	-22.4	2.59	0.97
7 Toluene	54.6	1.31	1.24	-15.0	2.43	0.98
8 Ethylbenzene(2)	50.6	1.29	1.27	-29.5 ^d	2.63	0.97
9 1-Methylethylbenzene	55.8	1.30	1.26	-34.0 ^d	2.70	0.97
10 Methoxymethane	50.9	1.31	1.24	3.8	2.24	0.98
11 Dimethylsulfane	45.9	1.33	1.22	-4.0	2.27	0.98
12 Methyl(phenyl)sulfane	45.4	1.34	1.21	-2.6	2.32	0.98
13 Dimethylamine	31.9	1.27	1.30	-14.7	2.11	0.99
14 Trimethylamine	27.9	1.27	1.31	-18.4	2.12	0.99
15 Fluoroethane (2)	77.2	1.38	1.15	35.2	2.70	0.97
16 Fluoroethane (1)	61.6	1.34	1.19	20.3	2.24	0.98
17 Ethylbenzene(1)	72.2	1.35	1.18	34.8	2.29	0.98
18 2-fluoroprop-1-ene	55.2	1.33	1.23	-19.4	2.48	0.97
19 prop-1-en-2-ol	49.1	1.32	1.25	-52.5 ^e	3.43	0.97
20 p-xylene	53.0	1.31	1.25	-17.8	2.40	0.97
21 1-methyl-4-nitrosobenzene	49.5	1.33	1.21	-20.8 ^d	2.52	0.97
22 methoxybenzene	54.5	1.34	1.21	9.6	2.34	0.98
23 N-methylaniline ^e	31.9	1.30	1.26	-12.4 ^f	-	-
24 N,N-dimethylaniline	28.9	1.29	1.28	-21.2	2.20	0.98

^a All energies are relative to the sum of the energies of the isolated substrate and the compound I model. The numbers in brackets for the substrates indicates the position from which the hydrogen is abstracted.

^b In kJ/mol

^c In Ångström (Å)

^d RMS forces were converged to at least 0.000030 a.u. (the maximum and RMS distances were not converged).

^e In the optimized structure, a hydrogen bond between the OH groups of the substrate and compound II was formed.

^f The intermediate was not stable, and the result of the optimization is the corresponding imine. The energy is calculated as the sum of the isolated radical and protonated compound II

Table 2. Correlation of the activation energy calculated with the full method and with calculations with smaller basis sets or without zero-point energy (ZPE) corrections.^a

	Fe(porphine)(CH ₃ S)O B3LYP/6-31G(d) ^b	Methoxy	Phenoxy	BDE _{fr}
R ² (training set)	0.90	0.91	0.91	0.84
MAE (training set)	3.81	2.84	3.59	4.73
R ² (test set)	0.78	0.89	0.96	0.90
MAE (test set)	6.31	4.09	2.88	3.60
CPU time	6d. 10h	1h. 30min.	5h. 36min	20min.
	B3LYP/6-311++G(2d,2p) ^c			
R ² (training set)	1.00	0.89	0.89	0.86
MAE (training set)	0.72	3.50	3.98	4.32
R ² (test set)	1.00	0.94	0.96	0.94
MAE (test set)	0.74	3.70	3.36	3.21
CPU time	12d. 15h.	2h. 12min.	7h. 19min.	48min.
	B3LYP/6-311++G(2d,2p) and ZPE corrections ^d			
CPU time	17d. 17h	3h. 40min	13h. 5min	1h. 50min

^a CPU estimates for 1-methyl-4-nitrosobenzene (compound **21**) are also included for each method. MAE in kJ/mol.

^b Geometry optimization at B3LYP/6-31G(d) level. An initial frequency calculation at HF/3-21G(d) was carried out before the TS optimizations.

^c TS optimization as described above and single point energy calculation at B3LYP/6-311++G(2d,2p) level.

^d TS optimization as described above, followed by a frequency calculation with B3LYP/6-31G(d) level and single point energy calculation at B3LYP/6-311++G(2d,2p) level.

Table 3. Correlation between activation energies (kJ/mol), O–H, and C–H bond lengths (Å) obtained with the Fe(porphine)(SCH₃)O model and with small organic radical models of compound I.^a

		Training set		Test set	
		R^2	MAE	R^2	MAE
B3LYP					
p-nitroso-phenoxy	Energy	0.89	3.50	0.94	3.68
	O–H	0.90	0.01	0.81	0.01
	C–H	0.82	0.01	0.63	0.01
phenoxy	Energy	0.89	3.98	0.96	3.36
	O–H	0.92	0.01	0.97	0.01
	C–H	0.85	0.01	0.95	0.01
methoxy	Energy	0.89	3.50	0.94	3.70
	O–H	0.93	0.01	0.98	0.01
	C–H	0.77	0.01	0.92	0.01
AM1					
p-nitroso-phenoxy ^b	Energy	0.86	4.24	0.31	9.08
	O–H	0.79	0.02	0.77	0.02
	C–H	0.39	0.02	0.48	0.01
phenoxy	Energy	0.88	3.96	0.89	3.95
	O–H	0.80	0.02	0.84	0.01
	C–H	0.37	0.02	0.74	0.01
methoxy	Energy	0.87	4.55	0.84	4.43
	O–H	0.86	0.01	0.79	0.01
	C–H	0.07	0.02	0.38	0.02

^a Regression lines were obtained by fitting the activation energies by using smaller radical mimics at B3LYP and AM1 level with those obtained using the Fe(Porphine)(CH₃S)O model at B3LYP level.

^b If compound **21** is omitted from the test set, $R^2 = 0.91$ and MAE = 3.78.

Table 4. CPU-time estimates at AM1 level.^a

	CPU time
Methoxy	8 min.
Phenoxy	7 min.
α_{Morse}	5 min.

^a The CPU estimates are with 1-methyl-4-nitrosobenzene (compound **21**) as substrate. The CPU estimates for the TS calculations include an initial frequency calculation.

Table 5. Correlations of various descriptors at B3LYP level with activation energies obtained using the full porphine model at the B3LYP level^a.

	Training set		Test set	
	R^2	MAE	R^2	MAE
Descriptors related to BDE				
BDE_{Morse}	0.65	6.53	0.76	7.30
α_{Morse}	0.73	6.21	0.57	8.60
BDE	0.37	9.85	0.58	7.69
BDE_{fr}	0.86	4.32	0.94	3.21
Substrate descriptors				
q_C	0.08	11.06	0.02	12.20
q_H	0.01	10.87	0.05	10.77
$E(\text{HOMO})$	0.80	5.69	0.55	8.97
$E(\text{LUMO})$	0.10	9.95	0.17	11.13
$2p(\text{HOMO})$	0.50	8.88	0.33	11.83
$1s(\text{HOMO})$	0.04	10.09	0.01	10.89
$2p(\text{LUMO})$	0.12	10.93	0.14	12.53
$1s(\text{LUMO})$	0.08	9.80	0.16	11.66
$E(\text{HOMO})-E(\text{LUMO})$	0.49	8.32	0.44	10.24
Radical descriptors				
q_C	0.00	10.46	0.01	10.86
S_C	0.43	9.10	0.42	9.70
$E(\text{HOMO})^b$	0.41	8.65	0.64	8.25
$2p(\text{HOMO})$	0.40	8.81	0.53	9.83
$E(\text{LUMO})$	0.04	10.67	0.02	11.84
$2p(\text{LUMO})$	0.25	8.69	0.16	12.55
$E(\text{HOMO})-E(\text{LUMO})$	0.28	9.80	0.55	8.40

^a The following descriptors were used: BDE_{Morse} and α_{Morse} : Bond dissociation energy and alpha parameter from Eqn. (2); BDE is defined in Eqn (1), where the geometry of the radical is relaxed. BDE_{fr} is determined as BDE, except that the geometry of the radical is not relaxed; q_C and q_H : Mulliken charges on the reactive C and H atoms; $2p$ and $1s$: Orbital coefficients for either the HOMO or LUMO on the reactive C or H atom. E : Energies of the HOMO or LUMO orbitals. S_C : Spin on the reactive C atom.

^b If the propionaldehyde (**6**) is omitted from the training set, $R^2 = 0.82$ and MAE = 5.02 kJ/mol

Table 6. Correlations of various descriptors at AM1 level with activation energies obtained using the full porphine model at the B3LYP level^a.

AM1	Training set		Test set	
	R^2	MAE	R^2	MAE
Descriptors related to BDE				
BDE_{Morse}	0.72	6.30	0.76	6.16
α_{Morse}	0.73	5.17	0.82	5.19
BDE	0.43	9.16	0.71	7.56
BDE_{fr}	0.68	7.34	0.68	6.25
Substrate descriptors				
q_C	0.02	10.82	0.14	10.96
q_H	0.05	10.24	0.24	10.80
$E(\text{HOMO})$	0.63	7.68	0.52	8.73
$E(\text{LUMO})$	0.14	9.38	0.23	11.08
$2p(\text{HOMO})$	0.45	8.48	0.26	11.28
$1s(\text{HOMO})$	0.09	9.52	0.03	10.69
$2p(\text{LUMO})$	0.08	10.40	0.26	10.29
$1s(\text{LUMO})$	0.37	8.25	0.26	9.76
$E(\text{HOMO})-E(\text{LUMO})$	0.37	8.81	0.42	10.01
Radical descriptors				
q_C	0.05	9.36	0.44	10.18
S_C	0.54	7.75	0.60	7.69
$E(\text{HOMO})^b$	0.53	7.61	0.60	8.30
$2p(\text{HOMO})$	0.32	9.42	0.45	9.55
$E(\text{LUMO})$	0.01	10.35	0.04	11.28
$2p(\text{LUMO})$	0.13	9.46	0.11	10.97
$E(\text{HOMO})-E(\text{LUMO})$	0.25	9.73	0.67	8.46

^a The following descriptors were used: BDE_{Morse} and α_{Morse} : Bond dissociation energy and alpha parameter from Eqn. (2); BDE is defined in Eqn (1), where the geometry of the radical is relaxed. BDE_{fr} is determined as BDE, except that the geometry of the radical is not relaxed; q_C and q_H : Mulliken charges on the reactive C and H atoms; $2p$ and $1s$: Orbital coefficients for either the HOMO or LUMO on the reactive C or H atom. E : Energies of the HOMO or LUMO orbitals. S_C : Spin on the reactive C atom.

^b If the propionaldehyde (**6**) is omitted from the training set, $R^2 = 0.86$ and MAE = 5.09 kJ/mol.

Table 7. The results of the PLS model, using either B3LYP or AM1 descriptors.^a

	#PC ^b	Training set		MAE	Test set
		R^2	Q^2		MAE
		B3LYP			
All	2	0.90	0.79	3.66	4.39
All (VIP>0.8) ^c	2	0.92	0.87	3.28	3.95
BDE _{fr}		0.86		4.32	3.21
AM1					
All	2	0.88	0.76	3.40	4.38
All (VIP>0.8) ^c	2	0.91	0.85	3.36	3.98
α_{max}		0.73		5.17	5.19

^a The descriptors are those listed in Tables 5 and 6.

^b Number of principal components in the model.

^c VIP: (variable influence on projection)

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