Summary and Concluding Remarks

Summary

Cytochrome P450 has received considerable attention of inorganic, organic, and physical (bio)chemists since its discovery due to its unique spectral properties as well as its ability to efficiently catalyze a variety of difficult biotransformations. With the discovery of the involvement of Cytochromes P450 in steroid biosynthesis in the 1970s, joined with its central function in drug metabolism, P450 became one of the most intensively investigated biochemical systems (Chapter 1). However, the catalytic mechanisms by which the Cytochromes P450 operates are still elusive. There is a growing consensus that the oxidative mechanism may be more complex than hitherto assumed and this has recently been formulated in the two-state versus multiple oxidant models (Chapter 2). In the two state model, the classical oxidant Compound I (iron(IV)oxo porphyrin radical \( \pi \)-cation) is presumed to react differently for the doublet and quartet spin states, whereas its precursor Compound 0 (hydroperoxo iron porphyrin) has been proposed as a competing oxidant in the multiple oxidant model. The identity of the oxidizing species has not been established yet definitively and neither has the mechanistic detail for alkane hydroxylation. This thesis describes the theoretical description of the most important parts in the hydrocarbon hydroxylation mechanisms catalyzed by Cytochromes P450 and sheds light on this tantalizing problem.

Before focusing on the catalytic cycle of Cytochrome P450, the question arises which theoretical method is most suitable to address the P450 problem. Due to the size of the P450 active site and the presence of the transition metal iron, accurate computational results can be obtained only with density functional theory (DFT). The electronic energy within the DFT formalism is a function of the electron density that includes approximations for the external potential and for the effects of the Coulomb interactions between the electrons, i.e. the exchange and correlation (XC) interactions. Validation of exchange-correlation DFT functionals for predicting the correct spin ground state of iron complexes is a rather unexplored area. Chapter 3 describes a systematic study on the performance of several xc functionals for seven iron complexes that are experimentally found to have either a low, intermediate or high spin ground
state. Standard xc functionals like LDA, BLYP, and PBE are found to disfavor high spin states, whereas hybrid and some meta-GGA functionals do provide the correct spin ground state for all molecules. Recently improved pure DFT functionals such as Handy’s optimized exchange (OPTX) also perform well. These differences emphasize that great care has to be exercised in choosing the DFT functional to calculate the spin state of biochemically relevant Fe(II) and Fe(III) complexes properly. On the basis of cost efficiency, the combination of the recently improved pure OPTX functional in combination with the PBE correlation functional, abbreviated as OPBE, performs best and further investigations were carried out using this density functional.

The electronic structures of iron(II) and iron(III) porphyrins, the core of the Cytochrome P450 active site, are studied in Chapter 4 using the evaluated OPBE functional. The calculated ground state has intermediate spin character, i.e. triplet for the iron(II) porphyrin and quartet for the iron(III) porphyrin cation. The effects on the energetics of the different spin states upon geometrical deformations of the porphyrin moiety are examined in Chapter 5. As expected for this highly unsaturated molecule, deformation from planarity is unfavorable for the naked iron(II) and iron(III) porphyrin moieties. Nevertheless, at room temperature the porphyrin can go through small deformations, but the electronic ground state remains in its intermediate spin state (Fe(II)-triplet or Fe(III)-quartet).

Complexation by a thiolate ion (SH–) changes the preferred ground state for both species to high spin (quintet-Fe(II) or sextet-Fe(III)). This thiolate complex is used as a mimic for the Cytochrome P450s active site to model the first step of the catalytic cycle of this enzyme (Chapter 6). This first step is believed to concern the removal of an axial oxygen donating ligand from the hexacoordinated aqua-thiolate-porphyrin-iron(III) resting state. The DFT results suggest that this is not a free water molecule, because of its repulsive nature, but that it has instead hydroxy anion character. These calculations are in line with the experimentally observed change in spin state from low to high spin upon removal of this axial hydroxo ligand by binding of the substrate in the heme pocket of Cytochrome P450.

Once the hydroperoxo Compound 0 intermediate is formed after oxygen binding, a second electron reduction, and protonation of the outer oxygen atom, cleavage of the O–O bond is a crucial mechanistic step in the catalytic cycle of Cytochromes P450 and this is described in Chapter 7. This reaction
step toward Compound I has frequently been assumed to proceed spontaneous without an energy barrier. In Chapter 7 it is shown that elimination of electrostatic effects in such gas-phase models results in the presence of a barrier for a slightly endothermic formation of Compound I. The barrier and the endothermicity is found to increase with decreasing acidity of the proton source. Protonation of the iron heme ligand, on the other hand, favors the O–O bond scission and provides an important regulatory component in the catalytic cycle. These calculations demonstrate that the nature of the proton delivery channel is critically important in the energetic relationship between Compound 0 and 1 and that alternative routes starting from Compound 0 are feasible.

After the formation of Compound 0, we found, in Chapter 8, that the catalytic cycle bifurcates into two different pathways. In one pathway, Compound I is formed after heterolytic O–O bond cleavage in Compound 0 and reacts with the substrate via a hydrogen abstraction and oxygen rebound mechanism (pathway A). In the other pathway, the O–O bond splits homolytically, generating a hydroxyl radical that abstracts a hydrogen from the substrate. From this point, this reaction path bifurcates again into two pathways B and C. In pathway B, the generated carbon radical forms a bond with the oxygen atom of the initial generated hydroxyl radical, whereas in pathway C, this C–O bond is formed between the carbon radical and the oxygen atom of the Compound II species. The activation and reaction energies for these three pathways are in the same order of magnitude and all three pathways can be correct descriptions of the chemical behavior of Cytochromes P450. The three different reaction channels (pathways A-C) will lead to one and the same reaction product and illustrates that the “clever” wild type protein will hydroxylate the substrate, irrespective of small perturbations in the vicinity of the reaction center such as disruption of protonation delivery channels.

Finally, an innovative comparison has been made between the alkane hydroxylation by organic peroxy acids and the biological Cytochromes P450 in Chapter 9. The non-catalytic mechanism for alkane hydroxylation by the peroxy acid meta-chloroperoxy-benzoic acid has close resemblance to the hydroxylation mechanism by the hydroperoxo Compound 0 intermediate of Cytochromes P450 (pathway C). Both mechanisms proceed via initial homolytic cleavage of the peroxy oxygen-oxygen bond followed by hydrogen abstraction from the alkane by the generated hydroxyl radical. Subsequently, bond formation between the oxygen of the newly formed water molecule and the
carbon radical followed by deprotonation generates the alcohol product. However, the oxidation reaction by the peracid proceeds via a synchronous non-concerted peroxide oxygen insertion into the C–H bond of the alkane, whereas the enzymatic P450 mechanism is more like a non-synchronous two step reaction due to the high stability of the intermediate species (Compound II).

Concluding Remarks

The results described in this thesis have led to a validated density functional theory methodology to reproduce the experimentally determined electronic ground states of iron complexes (Chapters 3-4). The most important conclusion from this validation study is that the choice of density functional is of crucial importance in predicting the electronic ground state of the selected iron complexes. For that purpose, the OPTX exchange and the PBE correlation functionals were selected from this study based on its superior performance (Chapters 3-4).

The main objective of the research described in this thesis is a better understanding of the hydrocarbon hydroxylation mechanism catalyzed by Cytochromes P450. The consensus view has the Compound I type intermediate (g) as the predominant catalyst. This pathway is supported by previous theoretical calculations, showing that its precursor Compound 0 (f) is a poor oxidant compared to Compound I. However, the research described in this thesis clearly shows that Compound I is not formed spontaneously by heterolytic oxygen-oxygen bond cleavage in Compound 0 and that other pathways are also likely to occur (Chapter 7). We found two new pathways that both involve the initial homolytic cleavage of the peroxy bond in Compound 0 to generate an hydroxyl radical and Compound II (j). This hydroxyl radical subsequently abstracts a hydrogen atom from the substrate molecule. One pathway involves homogenic bond formation between the substrate radical and the oxygen atom of the water (i.e. former hydroxyl radical) species with a simultaneous shift of a hydrogen atom from the water molecule to the iron-oxo species resulting in the alcohol product and a deprotonated resting state. The other pathway shows bond formation between the substrate radical and the oxygen atom of Compound II and leads to the substrate-alkoxy iron(III) porphyrin intermediate. The activation and reaction energies for these three pathways were found to be in the same order of magnitude. Therefore, we conclude that all three pathways can be correct descriptions of the chemical behavior of Cytochromes P450 and
that it shows the great diversity in reactions that are catalyzed by the unique iron-heme core. These two new radical mechanisms together with the “classical” Compound I mechanism provides a clear rationale for the experimental observations, including the main radical nature of the reaction, the diverse kinetic observations, and the different behavior in threonine mutants compared to wild type enzymes (see Chapter 8).

During the time of our research described in this thesis, the research groups of Shaik and Thiel and the group of Bach have also presented their findings using different density functional theory methods. Notwithstanding comparable results, different conclusions were drawn. The groups of Shaik and Thiel concluded that the hydroxylation reaction will be dominated by Compound I, whereas a mechanism comparable to our pathway B is preferred by the group of Bach. However, the present study, using the calibrated OPBE functional, shows that the activation energies for the three pathways A-C are in the same order of magnitude. Therefore, we conclude that the three pathways A-C as found in this work can each be correct descriptions of the chemical behavior of Cytochromes P450.

The present mechanistic findings are based on a general description of the active site core of Cytochromes P450. Therefore, these findings form the basis of the catalytic behavior of these enzymes. In order to gain deeper insight into the actual mechanisms by the specific Cytochrome P450 enzymes, further research should be focused on the systematic mapping of the influence of the neighboring amino acids on the energetics of the three different hydroxylation pathways and on the related calibration with experimental data. These calculations will establish the relative importance of each pathway in the catalytic cycle of Cytochromes P450. Confirmational studies of the results of this comprehensive QM-survey could be performed by using a robust QM/MM methodology. However, these large calculations including the entire enzyme in a QM/MM approach were, to date, shown to be extremely sensitive to the theoretical methodology and identifying the local minima has appeared to be rather difficult. Further improvements of the QM/MM methodologies are therefore indispensable for these confirmational studies.