Theoretical studies of mononuclear non-heme iron active sites

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Abstract

The quantum chemical investigations presented in this thesis use hybrid density functional theory to shed light on the catalytic mechanisms of mononuclear non-heme iron oxygenases, accommodating a ferrous ion in their active sites. More specifically, the dioxygen activation process and the subsequent oxidative reactions in the following enzymes were studied: tetrahydrobiopterin-dependent hydroxylases, naphthalene 1,2-dioxygenase and \( \alpha \)-ketoglutarate-dependent enzymes. In light of many experimental efforts devoted to the functional mimics of non-heme iron oxygenases, the reactivity of functional analogues was also examined.

The computed energetics and the available experimental data served to assess the feasibility of the reaction mechanisms investigated. Dioxygen activation in tetrahydrobiopterin- and \( \alpha \)-ketoglutarate-dependent enzymes were found to involve a high-valent iron-oxo species, which was then capable of substrate hydroxylation. In the case of naphthalene 1,2-dioxygenase, the reactivity of an iron(III)-hydroperoxo species toward the substrate was investigated and compared to the biomimetic counterpart.
List of Papers


IX. A. Bassan, M. R. A. Blomberg, P. E. M. Siegbahn, L. Que, Jr., Theoretical Studies on Olefin Oxidation by Biomimetic Non-Heme Iron(III)-Hydroperoxo Complexes, in manuscript.
René Magritte, Les promenades d’Euclide, 1955
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Preface

Computational chemistry offers valuable mathematical tools to characterize reaction mechanisms, and in particular, theoretical investigations of biological reactions may provide a basic understanding of the catalytic power of enzymes. In the present thesis we have tried to shed light on catalysis of mononuclear non-heme iron oxygenases by means of a theoretical approach based on hybrid density functional theory (DFT). The quantum chemical investigations aim at elucidating reaction pathways by deriving the corresponding activation energy barriers, which represent the major guideline to judge the feasibility of the probed mechanism.

Mononuclear non-heme iron enzymes are involved in a diverse range of key biological processes, which start with O₂ activation. Interestingly, these metalloproteins often share similar structural themes for the catalysis of different chemistry. We undertook several theoretical studies to explore the strategy adopted by the mononuclear non-heme iron centers to carry out dioxygen activation and the corresponding oxidative transformations. In light of many experimental efforts devoted to the functional mimics of non-heme iron oxygenases, we also paid particular attention to the reactivity of synthetic complexes, which have proved to be a useful playground to gain insight into the corresponding enzymatic reactions.

The first chapter of this thesis presents an introduction of the methodology employed in our investigations. It is compulsory to include in this chapter a discussion on the performance of DFT. This will help to estimate the errors affecting the calculations. Transition-state theory is also briefly treated due to its fundamental role in the analysis of chemical reactivity. The details of the approach adopted to build the computational models are also discussed.

The second chapter deals with the basic concepts of enzymatic catalysis and it also provides an introductory overview on mononuclear non-heme iron oxygenases, with major emphasis on those metalloproteins con-
taining the 2-His-1-carboxylate motif in the coordination environment of iron. This pattern of ligands characterizes the enzymes investigated in this thesis: tetrahydrobiopterin-dependent hydroxylases, α-ketoglutarate-dependent enzymes and naphthalene 1,2-dioxygenase.

The third chapter summarizes our investigation on tetrahydrobiopterin-dependent hydroxylases. First, the mechanism of dioxygen activation leading to an iron(IV)-oxo intermediate is presented (Paper I). Then the pathway describing the subsequent substrate hydroxylation is illustrated (Paper II). Importantly, this chapter also discusses the more recent structural insights into the active site of these enzymes together with some preliminary calculations undertaken after the availability of the new experimental structures.

The fourth chapter analyzes the strategy of dioxygen activation in another family of non-heme iron oxygenases, the α-ketoglutarate-dependent enzymes (Paper V). Furthermore, the dioxygen activation mechanism in a functional analogue (Paper VI) is discussed.

The fifth chapter reviews our investigation on naphthalene 1,2-dioxygenase, where dioxygen activation at the mononuclear iron center involves an electron transfer from a neighboring iron-sulfur cluster. After introducing the investigation on the electron transfer process (Paper IV), our proposed mechanism for naphthalene dioxygenation is summarized (Paper III). This chapter also describes some preliminary calculations on alternative reactions (i.e., monohydroxylation and sulfoxidation) catalyzed by this enzyme.

The sixth chapter focuses on the reactions (i.e., alkane hydroxylation, olefin cis-dihydroxylation and epoxidation) catalyzed by a family of biomimetic non-heme iron complexes, namely, the TPA family. The formation of an iron(V)-oxo species with the TPA ligand is discussed (Paper VII) together with its reactivity toward alkanes and olefins (Paper VIII). The properties (e.g., spin splittings, reactivity) of the catalysts in relation to the topology of the ligand are mentioned (Paper IX).
Theoretical background and methods

Quantum theory provides the mathematical tools for describing the motion of electrons in a molecule. All the properties of a system, and particularly the energy, can, in principle, be derived from the solution of the appropriate Schrödinger equation ($\hat{H}\Psi = E\Psi$), the basic equation of quantum mechanics\(^1\). Unfortunately, solving the Schrödinger equation exactly is far too complicated and to make the calculations feasible, various approximations are needed. Nowadays, calculations of chemical properties through a quantum mechanical approach are possible thanks to the availability of efficient computational formalisms and to the outstanding technological development of computing facilities. In particular, quantum chemistry allows the exploration of reaction mechanisms by locating intermediates and transition states, and the computed energy barriers can be used to assess the feasibility of the mechanisms under investigation.

1.1 Elementary quantum chemistry

Computational quantum chemistry revolves around the solution of the time-independent, non-relativistic Schrödinger equation\(^2\). Because the equations describing the interactions of a many-body system are compli-

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\(^1\) As George Hall once said: 'We know how electrons behave and we have to deduce how molecules behave' [1].

\(^2\) I will only present in a qualitative way the basics of quantum chemistry trying to highlight the major concepts which are encountered in the computational formalisms.
cated, some simplifications are required. One important approximation assumes that in a molecule electrons move in a static nuclear framework; this approximation, known as the Born-Oppenheimer approximation, is justified by the significant difference of mass of electrons and nuclei and it practically allows the separation of the motion of the electrons from the motion of the nuclei. The electronic energy is obtained for a frozen conformation of the nuclei and a new electronic energy is calculated changing the nuclear arrangement. The dependency of the energy on the conformation gives rise to a potential-energy surface, which defines the equilibrium conformations of a molecule. The notion of potential-energy surface is meaningful under the validity of the Born-Oppenheimer approximation.

The Born-Oppenheimer approximation is not enough to solve the electronic Schrödinger equation for a many-body system, and it is necessary to resort to other approximations. One of the most important approximations is the Hartree-Fock (HF) method, which is based on the independent-particle model or molecular orbital (MO) model\(^3\). Each electron is associated with a one-electron wave function, which is the product of a spatial function that depends on the coordinate of the electron, and a spin function that depends on its spin (the one-electron wave functions are called spin orbitals). In order to satisfy the antisymmetry principle for electrons, the solution of the Schrödinger equation is chosen to be a single Slater determinant of the N one-electron wave functions (N is the number of electrons). After having selected the form of the wave function, the variational principle provides a method to numerically solve the Schrödinger equation. The expectation value of the energy is minimized with respect to some parameters of the trial wave function and by imposing the appropriate normalization condition. With these simplifications the N-particle problem is reduced to a set of one-particle eigenvalue problems:

\[ \hat{f}_i \chi_i = \xi_i \chi_i \quad (1.1) \]

where \( \hat{f}_i \) is an effective one-electron operator, in which the electron-electron repulsion is treated in an average way (each electron feels only the average field generated by the other electrons); \( \chi_i \) is the corresponding

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Exhaustive discussions and mathematical formulations of quantum theory can be found in several text books [2–5].

\(^3\)The Hamiltonian of the Hartree-Fock method includes only electrostatic effects and neglects relativistic and the magnetic effects.
1.2 Density Functional Theory

eigenfunction (MO) and the electron in the MO is considered to have an orbital energy $\xi_i$. The HF equation is non-linear and it has to be solved iteratively (the procedure is called the self-consistent field method). In practice, the solution of the HF equation starts by introducing a finite set of spatial basis functions, which are used to expand the spatial part of the spin orbitals. A way to construct molecular orbitals for many-electron molecules is to use a linear combination of the orbitals of the parent atoms; this procedure is known as the linear combination of atomic orbitals (LCAO) molecular orbital (MO) method.

The independent-particle model, which treats each electron in an average field, sets the major limit of the HF method, since the 'local' distortion of the distribution of the electrons is neglected. It is said that the HF scheme neglects electron correlation. It should be specified that the single-determinant approximation to the wave function accounts for the exchange interaction, which means that the motion of electrons with parallel spins is correlated. It is customary to define the correlation energy as the difference between the HF energy and the exact non-relativistic energy. Although the correlation energy is usually a small fraction of the computed total energy, when neglected, it gives substantial errors in the evaluation of interaction energies. Various computational methods have been developed to account for the electron correlation problem, some of them are based on perturbation theory and some others on the variational principle \cite{6}. Accurate energies can be obtained from, for example, the configuration interaction (e.g., CISD and QCISD) and the coupled cluster (e.g., CCSD and CCSD(T)) approaches, which, however, are computationally very expensive. With standard ab initio methods, reaching a high accuracy (i.e., the so called chemical accuracy of about 1-2 kcal mol$^{-1}$) is very demanding and thus only relatively small system can be treated.

1.2 Density Functional Theory

In the early 1990s the application of density functional theory (DFT) for the understanding of chemical problems provoked a remarkable revolution

\footnote{The HF method leads to a reasonably accurate description of N$_2$ around the equilibrium geometry. Nevertheless, the neglected correlation energy, which is estimated to be about 0.5% of the total HF energy, causes an error of a few electronvolts in the computed bond dissociation energy for N$_2$.}
in quantum chemistry\footnote{Again, a qualitative discussion of DFT is presented; a comprehensive treatment of DFT can be found in the classical book by Parr and Yang \cite{parryang} or in the DFT guide for chemists \cite{parr}.}. The development of DFT methods opened up a new era where relatively large systems, containing transition metals as well, could be investigated quantum mechanically \cite{parr}. The theoretical pillars of DFT were laid by Hohenberg and Kohn, who showed that the total energy is a unique functional of the electron density, that is, $E = E[\rho(r)]$ \cite{parr}. This means that the properties of interest can be derived from the ground-state density and thus one does not need to know the complicated many-electron wave function. In other words, the electron density $\rho(r)$ is the key quantity in DFT, leading to the following expression of the energy:

$$E[\rho] = T[\rho] + E_{ee}[\rho] + E_{ne}[\rho]$$

(1.2)

where $T$ is the kinetic energy, $E_{ee}$ the electron-electron repulsion, and $E_{ne}$ the attraction between the nuclei and the electrons (the nuclear-nuclear repulsion is a constant in the Born-Oppenheimer approximation and is thus not included). The exact form of the functional, which uniquely maps between the ground state density and the ground state energy is not known, and therefore the corresponding equations cannot be solved.

Kohn and Sham introduced the mathematical framework for the numerical determination of the electronic ground state of many-electron systems \cite{parr}. The Kohn-Sham (KS) equations are based on the one-electron picture, where independent particles move in an effective potential. It means that the real system of interacting electrons is formally described through a fictitious system of non-interacting particles. The total energy can then be rewritten as:

$$E[\rho] = T_s[\rho] + J[\rho] + E_{ne}[\rho] + E_{xc}[\rho]$$

(1.3)

where $T_s[\rho]$ is the kinetic energy of non-interacting particles; $J[\rho]$ and $E_{ne}[\rho]$ are respectively the Coulomb interaction between electrons, and the interaction between nuclei and electrons given by their classical expressions; and finally $E_{xc}[\rho]$ is the exchange-correlation energy containing the corrections to the non-interacting approximation. Similarly to the HF approach, the variational principle together with the normalization constraints leads to the KS equations:
1.3 Using DFT to explore chemical reactivity

\[ \hat{h}_{k\alpha} \chi_i = \xi_i \chi_i \] (1.4)

where \( \hat{h}_{k\alpha} \) includes the potential due to the exchange-correlation energy \( E_{xc} \). The KS approach would strictly provide the exact description of the many-electron system (no approximations have been made so far) if the exact form for \( E_{xc}[\rho] \) was known. It is possible to prove that the exchange-correlation potential is a unique functional, valid for all systems. Approximations in DFT enters in the form of the exchange-correlation potential.

The popularity of DFT functionals for molecules started with the development of gradient-corrected density functionals, which have increased the accuracy of the DFT methods significantly. The introduction by Becke of the exact exchange term given by the HF theory [12, 13] definitely contributed to the success of DFT. In the so-called hybrid functionals, the exchange-correlation energy is expressed in terms of a linear combination of gradient-corrected exchange and correlational energies and the HF exchange. The semiempirical generalization of the extent of mixing of exact exchange led to the formulation of the hybrid functionals, one of this is the B3LYP functional, which can be written as:

\[
E_{xc}^{B3LYP} = (1 - a_1)E_{xc}^{LSD} + a_1 E_x^{HF} + a_2 E_x^{B88} + a_3 E_c^{LYP} + (1 - a_3)E_c^{LSD}
\] (1.5)

The semiempirical coefficient \( a_1 \) determines the extent of replacement of electron-gas exchange (\( E_{xc}^{LSD} \)) with exact exchange (\( E_x^{HF} \)); \( a_2 \) determines the optimum inclusion of gradient-correction for exchange (\( E_x^{B88} \)) [14]; \( a_3 \) defines the inclusion of correlation from the LYP correlation functional [15] and from electron-gas correlation (\( E_c^{LSD} \)). The three coefficients of B3LYP were derived by a linear least-squares fit to thermochemical data with the B3PW91 functional (same as B3LYP but with the PW91 correlation instead of the LYP correlation) [16].

1.3 Using DFT to explore chemical reactivity

DFT methods and in particular hybrid functionals have been widely used to explore chemical reactions due to their good accuracy at a relatively low computational cost. The accuracy of various density functionals, hybrid and non-hybrid, have been probed for a large number of test molecules,
including radicals, non-hydrogen systems, hydrocarbons, substituted hydrocarbons and inorganic hydrides. The comparison of the computed enthalpy of formations with the experimental values for the so-called G2 neutral test set showed that the B3LYP functional gives the lowest mean absolute deviation (3.11 kcal mol$^{-1}$) \cite{17}. The G3/99 test set\textsuperscript{7}, where larger molecules have been added, still shows that the B3LYP functional performs the best among the DFT methods investigated, but the computed mean absolute deviation is larger, 4.27 kcal mol$^{-1}$ \cite{19}. A comparison of the mean absolute deviations for subsets including molecules of different sizes leads to the conclusion that DFT errors tend to accumulate in the larger molecules, and that the B3LYP errors in the enthalpy of formations are proportional to the number of pairs of electrons. One important feature of the DFT methods that has to be noted is the fast convergence of the energy with the basis set. This is not the case for post-HF methods such as MP2 or CCSD(T), where a high accuracy can be obtained only using large basis sets. In contrast, DFT methods provide accurate energies with relatively small basis sets \cite{20}.

Benchmark tests on molecules including transition metals have also been performed. Investigations on first row MR$^+$ complexes (R = H, CH$_3$, CH$_2$, OH) and comparisons with the available experimental data showed that the M–R bond energies computed with the B3LYP method are affected by an error of 3.6–5.5 kcal/mol \cite{21, 22}. In these studies it was further noted that the computed single M–C and M–H bond energies were systematically larger than the experimental ones. The binding energies of carbonyl ligands provided another test set to examine the accuracy of the DFT methods and in particular of the B3LYP functional. The successive M–CO bond energies for [Fe(CO)$_5$]$^+$ and Ni(CO)$_4$ were computed together with the first M–CO bond energies for various metal carbonyl complexes (M(CO)$_6$, M = Cr, Mo, W) and compared with the available experimental values \cite{21, 23, 24}. An average error of 2.6 kcal/mol for the B3LYP functional can be derived from these calculations. All these results together with other available comparisons between theory and ex-

\textsuperscript{6}In the G2 neutral test set, energies were obtained with the 6-311+G(3df,2p) basis set for MP2(full)/6-31G(d) geometries and corrected with scaled HF/6-31G(d) zero-point energies. No significant differences were observed using B3LYP/6-31G(d) geometries \cite{18}.

\textsuperscript{7}In the G3 test set, energies were obtained with the 6-311+G(3df,2p) basis set for B3LYP/6-31G(d) geometries and corrected with scaled B3LYP/6-31G(d) zero-point energies.
1.3 Using DFT to explore chemical reactivity

Table 1.1: Binding energies of O\textsubscript{2} to [Fe\textsuperscript{II}(OH)\textsubscript{2}] and of CO to [Ni\textsuperscript{II}(OH)\textsubscript{2}] calculated with the B3LYP functional and the CCSD(T) method. B3LYP geometries optimized with a standard double-zeta basis set (i.e., the lacv3p basis set) are employed in both methods. The total spin used for each structure is indicated.

<table>
<thead>
<tr>
<th></th>
<th>DFT</th>
<th>CCSD(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lacv3p**</td>
<td>6-311+G(2d,2p)</td>
</tr>
<tr>
<td>Fe\textsuperscript{II}-O\textsubscript{2} (S=3)</td>
<td>11.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Ni\textsuperscript{II}-CO (S=0)</td>
<td>36.7</td>
<td>35.2</td>
</tr>
<tr>
<td>Ni\textsuperscript{II}-CO (S=1)</td>
<td>29.0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

Experiments (e.g., the calculations on binding energies of Mn complexes [20], or of FeO\textsuperscript{+} and [Fe(H\textsubscript{2}O)]\textsuperscript{+} [25, 26]) set, as a rule of thumb, the error of the B3LYP method to 3-5 kcal/mol [27]. Such an accuracy should be enough to discriminate among different reaction mechanisms [28].

Benchmark tests on transition metals are difficult especially because the experimental data to use as a comparison are often not available. However, the performance of B3LYP can be probed by comparing the B3LYP energies with the ones obtained from other accurate methods. Because the present thesis revolves around calculations on dioxygen binding to a transition metal, namely, iron, some CCSD(T) calculations have been done to probe the performance of the B3LYP method in the evaluation of the energy change associated with O\textsubscript{2} binding to Fe\textsuperscript{II}. The computed binding energies derived from calculations with an [Fe\textsuperscript{II}(OH)\textsubscript{2}] model show that the two methods predict almost identical binding energies (table 1.1). In table 1.1 the binding energies of CO to Ni\textsuperscript{II} computed with these two methods are also reported, showing again that in these cases B3LYP and CCSD(T) give very similar results.

It has to be added that the capability of B3LYP to predict the correct spin ground state for metal complexes is often debated. It has been observed that B3LYP may fail to accurately compute spin splittings [8, 29]. Therefore, more comparisons with experiments and with other accurate methods are needed to derive how B3LYP works in describing the spin splittings of transition metal compounds.
1.4 Reaction mechanisms

A chemical reaction is the rearrangement of the pattern of the atomic nuclei relative to each other [30]. Such a transformation occurs through a sequence of elementary chemical steps which defines the reaction mechanism of the reaction. An elementary process joins two subsequent intermediates. An intermediate is any stable geometrical configuration (i.e., that exists at a minimum of the energy) occurring during a reaction and having a lifetime longer than the period of typical molecular vibrations (on the order of $10^{-13} - 10^{-14}$ s) [31].

The equation of an elementary reaction defines its corresponding rate, which at constant temperature is proportional to the products of the concentrations of the reactants\(^8\). Therefore for the elementary reaction:

$$aA + bB \rightarrow \text{Products}$$

the corresponding rate equation is:

$$\frac{-d[A]}{dt} = k[A]^a[B]^b$$  \hspace{1cm} (1.6)

where the coefficient $k$ is called the rate constant and is independent on the concentrations (and dependent on the temperature). A differential rate equation depending on concentrations and microscopic rate constants can be associated with a chemical reaction once its reaction mechanism (i.e., the sequence of elementary reactions) has been defined. To know the reaction mechanism is essential to understand which factors govern the rate of a reaction.

1.5 Transition state theory

When for example two molecules react to form a product, the path which brings the two molecules together and along which they undergo the chemical transformation is called reaction coordinate. The shape of the potential energy surface along the reaction coordinate defines the reaction coordinate diagram, which provides thermodynamic and kinetic information on the reaction mechanism under investigation. Computational chemistry can be a valuable tool to characterize reaction mechanisms by

\(^8\)The rate for an elementary reaction is proportional to the frequency of the collisions bringing the reactant molecules together.
1.5 Transition state theory

deriving energy diagrams. Calculations are able to supply an informative picture of a reaction mechanism by localizing minima and saddle points on the potential energy surface of interest. In particular, the ability to model transition states is crucial for estimating activation barriers.

**What is a transition state?** According to transition state theory, a chemical transformation proceeds through the lowest maximum in the potential energy surface joining the reactants and the products. The rate of passage of molecules over the barrier is influenced by the barrier height itself and the energy distribution, which is in turn determined by the temperature. Transition state theory postulates an equilibrium energy distribution among all possible quantum states along the reaction coordinate. This means that molecules going over a barrier are in equilibrium with all the other reactant molecules, leading thus to the following expression for rate constants:

\[ k = k_B T e^{-\Delta G^\ddagger / RT} \]  

(1.7)

where \( k_B \) is Boltzmann’s constant and \( h \) is Planck’s constant. The value of \( k_B T / h \) at 298.15 K is \( 6.2 \times 10^{12} \) sec\(^{-1}\). \( \Delta G^\ddagger \) corresponds to the free energy of activation and is connected with the probability of reaching the transition state. \( \Delta G^\ddagger \) is calculated at the saddle point (i.e., the transition state) of the Born-Oppenheimer free-energy surface; at this point the transition state is characterized by one imaginary frequency since it is a maximum along the reaction coordinate, and this imaginary frequency is associated with the normal mode corresponding to the chemical transformation of interest. It should be emphasized that the activated complex can be viewed as a mathematical entity rather than a stable or metastable chemical species.

In general the calculation of \( \Delta G \) involves the evaluation of zero-point effects and thermal effects, which must be added to the total electronic energy. The basic equation is:

\[ \Delta G = \Delta H - T \Delta S \]  

(1.8)

\( \Delta G \) is the change in free energy of a system undergoing a transformation at constant pressure and temperature \((T)\). \( \Delta H \) is the change in enthalpy and \( \Delta S \) is the change in entropy.

Classical transition state theory neglects the quantum effect known as tunneling, which allows a small extent of reaction by molecules having
less energy than the one required to pass the barrier. Since tunneling is important for atoms with small mass, it might enter in the description of reactions such as hydrogen atom or hydrogen ion transfers, and it can be safely neglected for most of the other processes \(^9\). A weak point of classical transition state theory resides also in the assumption that the reaction coordinate can be separated from the other molecular motions. Another possible failure has to be mentioned, and it concerns a reaction coordinate involving motion in more than a single dimension. In this case it was proposed that two competing pathways with the same activation energies will not yield equally the two corresponding products. In contrast, the more direct pathway is preferred \(^{38}\).

1.6 Solvent effects

Reliable results on reaction mechanisms occurring in condensed phase are usually achieved when calculations are performed on the isolated gas phase model. However, the final energetics should account for the actual environment, and a correction to the gas-phase energy must be added. The long-range or polarization effects induced by the solvent (e.g., the protein environment in enzymatic catalysis) can be reproduced by modeling the solvent as a macroscopic continuum with dielectric constant \(\varepsilon\), and the solute as contained in a cavity of this continuous medium. In the dielectric cavity methods the solvent acts as a perturbation on the gas-phase behavior of the system since the solute is subjected to the electrostatic potential created by the continuum, which in turn is polarized by the influence of the solute itself. The cavity model is a good approximation, provided that essential hydrogen bonding interaction (i.e., the short-range effects due to individual solvent molecules) are included in the model. When biochemical reactions are investigated, a low dielectric constant \((\varepsilon = 4)\) is appropriate for treating the polarization effects of the protein environment and the dielectric effects are usually small \(^{27}\).

When modeling a reaction occurring in condensed phase, another effect due to the solvent must be considered, the so called 'friction' effect. It is originated by the collisions occurring between the solute and the solv-

\(^9\)Calculations on tunneling effects for various enzymatic reactions give an energy barrier decrease which spans from 0.2 to 3.9 kcal/mol \(^{33-37}\). It should be noted that in the case of B3LYP investigations the error of neglecting the tunneling effects falls within the accuracy of the method.
vent molecules. Kinetic energy is thus dissipated with the consequential effect that the solute is trapped in the wells of the potential energy surface, and within a classical description its motion can be compared with the motion of a ball with high friction. The analogous comparison for the gas phase reaction leads to a ball moving without any friction [39].

1.7 Chemical models

Successful applications of the B3LYP hybrid functional have proved that even transition-metal mediated reactions can be investigated by means of theoretical calculations. Having testified the suitability of hybrid DFT in the scenario of transition-metal chemistry, the remaining problem is the choice of an appropriate model for the investigation of the bioinorganic chemistry of enzymes and enzyme mimics. Since a bare quantum mechanical (QM) approach allows models consisting of a rather limited number of atoms, the real system should be reduced to a minimum-size active site model, where those atoms undergoing chemical changes during catalysis are included. The modeling approach changes when QM/MM (i.e., a hybrid quantum mechanical/molecular mechanical approach) is employed, since a larger system can be afforded within the molecular mechanical part. However, in the following the attention will be paid to the standard approach adopted when a reaction is investigated by means of QM calculations.

When building a model for the investigation of enzymatic catalysis, amino acid residues essential for the chemical transformation of interest can be reduced to smaller molecules. For example, the carboxylate functional group of the glutamic acid or of the aspartic acid can be safely modeled as a formate; the bigger acetate usually does not bring any relevant improvement to the model system. Histidine can be modeled as an imidazole ring. Although it could also be further reduced to the smaller ammonia, artificial hydrogen bonds might affect the theoretical investigation bringing unwanted interactions in the reaction pathway [40]. While modeling enzymatic catalysis, the rigidity of the active site can be reproduced by imposing some structural constraints during the geometry optimizations. It is useful to freeze some atoms according to the available X-ray data especially when the system contains molecules which are not anchored in the first coordination sphere of the metal.
Figure 1.1: Part of the free-energy diagram for the synthesis of prostaglandin $G_2$ by prostaglandin $H$ synthase as calculated by Blomberg et al. [41].

The choice of the model is essential for the correct description of a chemical reaction. The ideal model should be small in order to limit the computational cost but at the same time all the players of the chemical reaction (for example, hydrogen bonding networks which might induce important stabilizations) should be included.\textsuperscript{10}

Having set up a model, computational chemistry allows the exploration of potential energy surfaces (i.e., the dependency of the energy on the nuclear coordinates). Different reaction mechanisms can be probed, and it is possible to associate a reaction coordinate diagram (as the one depicted in figure 1.1) with each mechanism. Important information can

\textsuperscript{10}It is interesting to mention that a theoretical investigation describes the behavior of an abstract system. In principle, the conclusions drawn from the calculations refer to this fictitious system. The goal of computational chemistry is to shape the model and the methodology (method, basis set, dielectric model, etc) in such a way to correctly reproduce the physical system.
be extracted from the energy profile of figure 1.1. Firstly, it defines the sequence of elementary steps describing the overall reaction. Secondly, it shows that in this particular case the whole process is exergonic by 23.5 kcal mol\(^{-1}\). Finally, the relative energies of the transition states indicate that the rate determining step (i.e., the step with the highest energy barrier) for the overall reaction involves \(TS4\), which requires an energy barrier of 14.9 kcal mol\(^{-1}\). The barrier for \(TS4\) is estimated from the intermediate \(3\), which is the lowest point in energy before \(TS4\). This is based on the assumption that any excess of kinetic energy is readily dissipated for a reaction occurring in the enzyme. Therefore, the slowest step of the reaction defines the activation energy of the overall chemical transformation yielding the product \(5\) from the reactant \(1\). Transition state theory then connects the computed barrier with the rate of the reaction.

It should be noted that a computational approach affected by an error of 3–5 kcal mol\(^{-1}\), as the one adopted in the present thesis, cannot lead to accurate calculations of rate constants but it only allows the derivation of energy barriers, which are the major guideline to judge the feasibility of a reaction mechanism. A mechanism is in general dismissed if the computed activation energy barrier is too high, since a very low rate is implied. In general, at room temperature typical energy barriers for enzymatic reactions are in the range 10 to 15 kcal mol\(^{-1}\). Hopefully, a mechanism with a low activation energy and satisfying the available experimental observations is found, and thus proposed to the scientific community\(^{12}\).

1.8 Computational methods

In the present work the reaction pathways of interest are investigated by means of hybrid DFT, using the two quantum chemical programs Gaussian 98/03 and Jaguar 4.0/4.1/4.2 [42, 43]. Initially, the potential energy surfaces are probed by means of constrained optimizations to approximately locate transition states; afterward, the computation of molecular

\(^{11}\)In the specific case of the energy profile depicted in figure 1.1, it is perhaps difficult to identify the rate determining step since a comparable activation energy (14.5 kcal mol\(^{-1}\)) is computed for \(TS1\).

\(^{12}\)In principle, the methodological approach of quantum chemistry cannot provide a certain falsification or verification of a reaction mechanism. In reality, experience taught us that few mechanisms satisfy the strict requirements imposed by the experimental observations and the expected energy barrier.
Hessians (i.e., second derivatives with respect to the nuclear coordinates) helps to fully optimize the transition state structures. Molecular Hessians of reactants, intermediates and transition states are usually calculated in order to derive the zero point effects and the thermal corrections to the total energy, enthalpy and Gibbs free energy. The calculation of molecular Hessians for the transition state geometries is also needed to confirm that the structure is characterized by only one imaginary frequency corresponding to the normal mode associated with the reaction coordinate. When some atoms in the model are constrained according to the available X-ray data, evaluation of the thermal effects turns out not to be sufficiently accurate. In these cases, only the energy changes corrected for the zero-point effects are provided and with good approximation they reflect the enthalpic energy profile.

Standard double zeta basis sets are employed for geometry optimizations and for the evaluations of zero point effects and thermal corrections. However, the final energies for the fully optimized structures are calculated with larger basis sets including polarization functions on all atoms. Sufficiently accurate results are obtained by first optimizing geometries with a double zeta basis set and then by computing final energies with larger basis sets because of the low sensitivity of these final energies to very accurate geometrical structures [40]. Solvent effects are derived by modeling the solvent as a macroscopic continuum with dielectric constant $\epsilon$ and they are usually evaluated at the same level of theory as the geometry optimizations.
2

Enzymes are extraordinary biological machines that Nature developed in order to catalyze biochemical transformations. Some of the chemical reactions are simple, some others very complex and they would not occur at appreciable rate under mild conditions (i.e., at room temperature and in water at neutral pH) in the absence of enzymatic catalysis. Typically, enzymes are proteins\(^1\), but some RNA molecules are also found to function as biological catalysts. Enzymes accelerate the rate of a wide spectrum of reactions (e.g., bond forming, bond breaking, rearrangements, and redox reactions as shown in table 2.1) by decreasing the activation energy with respect to the value of the original process in gas phase or in solution. Theoretical calculations allow the analysis of the elementary chemical transformations occurring in the active site of the enzyme and consequently the reaction can be microscopically followed, highlighting which factors govern catalysis.

2.1 Basics of enzyme kinetics

Traditionally, the rates of enzymatic reactions are measured during a steady-state period, when the reaction rate changes very slowly with time. In steady-state kinetics, the concentrations of enzyme-bound intermediates are considered constant. When an enzyme (typical enzyme

\(^1\)From the Greek *proteios*, 'primary, of the first rank', the name protein was first suggested by Jöns J. Berzelius in 1838.
2 Enzyme catalysis and non-heme iron proteins

Table 2.1: As recommended by the International Union of Biochemistry and Molecular Biology, enzymes are currently grouped in six subclasses based on the reaction type.

<table>
<thead>
<tr>
<th>Enzyme Family</th>
<th>Reaction Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidoreductase</td>
<td>Redox reactions</td>
</tr>
<tr>
<td>Transferase</td>
<td>Transfer of a functional group (e.g., a methyl group or a glycosyl group)</td>
</tr>
<tr>
<td>Hydrolase</td>
<td>Hydrolysis reactions (e.g., C–O, C–N, C–C bonds)</td>
</tr>
<tr>
<td>Lyase</td>
<td>Cleavage of bonds by elimination (e.g., C–O, C–N, C–C bonds)</td>
</tr>
<tr>
<td>Isomerase</td>
<td>Geometrical rearrangements</td>
</tr>
<tr>
<td>Ligase</td>
<td>Formation of a bond between molecules</td>
</tr>
</tbody>
</table>

Concentrations are in the range of $10^{-8}$–$10^{-10}$ M) is mixed with a large excess of substrate (with a concentration usually greater than $10^{-6}$ M), the concentrations of the intermediates reach a steady level after a very short induction period (pre-steady state). Under steady-state kinetics, experiments show that for many enzymes $v$, the initial rate of formation of product (or depletion of substrate)\(^2\), is proportional to the total enzyme concentration $[E]_0$, but it does not increase indefinitely with the substrate concentration $[S]$. On the contrary, $v$ generally exhibits saturation kinetics with respect to substrate concentration. The Michaelis-Menten equation, a purely empirical law, describes this behavior:

$$v = \frac{k_{cat}[E]_0[S]}{K_M + [S]}$$

(2.1)

For high values of $[S]$, $v$ reaches a limiting value, $V_{max}$, proportional to the enzyme concentration:

$$V_{max} = k_{cat}[E]_0$$

(2.2)

\(^2\)The initial velocity is measured before more than 10% of the substrate has reacted.
Therefore, $k_{\text{cat}}$ defines the rate at saturation conditions and is a measure of the enzyme efficiency; $k_{\text{cat}}$ is also known as the turnover number because it is the number of substrate molecules converted into product by an enzyme molecule in a unit time per active site. $K_M$ (the Michaelis constant) is defined as the substrate concentration at which the initial rate reaches half of the maximum value.

The simplest model accounting for the Michaelis-Menten equation is a reaction divided into two steps (the Michaelis-Menten model):

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} P + E$$

where E, S, ES, and P refer to the enzyme, substrate, enzyme-substrate complex (a non-covalently bound complex), and products, respectively. In the first step, in which no chemical changes take place, E and S are considered in rapid and reversible equilibrium with the enzyme-substrate complex ES. Within the steady-state approximation, which assumes that [ES] remains constant until the substrate is nearly consumed, it can be derived that $k_{\text{cat}}$ and the Michaelis constant $K_M$ correspond to:

$$K_M = \frac{k_2}{k_1} + \frac{k_{-1}}{k_1}; \quad k_{\text{cat}} = k_2$$

(2.3)

It should be noted that, when $k_{-1} \gg k_2$, $K_M$ corresponds to the dissociation constant of the enzyme-substrate complex. In the Michaelis-Menten model, $k_{\text{cat}}$ is simply the first order rate constant for the chemical conversion of ES to the product, which means that $V_{\text{max}}$ (the initial velocity at high concentration of S) is:

$$V_{\text{max}} = k_2[E]_0$$

(2.4)

$k_{\text{cat}}$ will depend on all the first-order rate constants of the elementary steps if catalysis of an enzyme cannot be reduced to the simple two-step Michaelis-Menten mechanism.

When $[S]$ is low, a few enzyme-substrate complexes are formed and therefore the enzyme will be mainly free. Consequently, the total concentration of enzyme $[E]_0$ can be reasonably approximated to the concentration of free enzyme $[E]$. Under these conditions, the initial velocity $v$ (equation 2.1) becomes:

$$v = \frac{k_{\text{cat}}[E][S]}{K_M}$$

(2.5)
It follows that \( \frac{k_{\text{cat}}}{K_M} \) is an apparent second order rate-constant, which is also called the specificity constant. For \( k_1 \ll k_2 \), it corresponds to \( k_1 \) since:

\[
\frac{k_{\text{cat}}}{K_M} = \frac{k_2}{K_M} = \frac{k_1 k_2}{k_2 + k_1} \approx k_1
\]  

Therefore, \( k_1 \) sets the upper limit of the specificity constant and of course it cannot be greater than the frequency of collisions between substrate and enzyme, that is the diffusion-controlled limit, in which case \( k_1 \) corresponds to \( 10^8 \text{ to } 10^9 \text{M}^{-1} \text{s}^{-1} \). Very efficient enzymes exhibit such values of \( k_{\text{cat}}/K_M \), meaning that they catalyze the reaction every time they collide with the substrate.

The steady-state kinetics provide a phenomenological description of enzymatic catalysis and do not give any information on the number or the nature of the intermediates. This is not surprising because under steady state, only the product and the reactant are observed\(^3\). Nevertheless, a proposed mechanism has to satisfy the kinetic data measured under steady state. Insight into enzymatic multi-step reaction mechanisms can be gained with various experimental techniques, such as spectroscopic analysis or pre-steady-state measurements, which may provide information on the identity of the transient intermediates. Isotope substitution has also proved to be a useful means to elucidate reaction mechanisms by monitoring kinetic isotope effects.

In the present thesis, energy diagrams for enzymatic catalysis are constructed by 'isolating' transition states and intermediates by means of theoretical methods, and energy barriers of the elementary processes can then be provided. A mechanism derived from the theoretical investigation has to be compatible with the available experimental data, which means that the synergism between theory and experiments is essential to shed light on enzymatic catalysis.

\(^3\)Interestingly, by imaging the enzyme as a complicated network of water pipes, steady-state kinetics measures the relation between the input pressure and the output flow when all the pipes are filled with water. No detailed information can be gained on the water flow throughout the pipes [44].
2.2 Enzymatic catalysis

The two striking features of enzymes are selectivity and catalytic power. Enzymes not only specifically bind the reactant (i.e., substrate), but they also selectively yield one product avoiding side-reactions, which are instead typical of uncatalyzed processes. The selectivity of the enzymes was first recognized by Emil Fischer in 1894, who proposed the lock-and-key analogy for the enzyme-substrate interactions [45]. Concerning the catalytic power, Linus Pauling proposed that enzymes lower the activation energy because they mainly stabilize the structure of the transition state [46]. Enzymatic catalysis can be thought to work so efficiently because of a pre-organized environment that effectively stabilizes the transition state.

The exact strategy adopted by enzymes to stabilize transition states and thus to accelerate reaction rates by a factor of as large as $10^{19}$ [48], is an interesting issue. Many proposals have been put forward to explain the great catalytic efficiency of enzymes and some of these proposals are listed below: a) desolvation hypothesis (the enzyme provides a non-polar environment that destabilizes highly charged ground states); b) entropic guidance (enzymatic reactions take place in the confines of enzyme-substrate complex and there is no loss of translational and rotational entropy; the advantage of the entropy is paid by the enzyme-substrate binding energy); c) high effective concentration (the binding of substrates in the active site results in an effective concentration of the participating groups as high as 55 M); d) orbital steering (orbital alignment of reacting atoms); e) electrostatic effects (enzymes can stabilize ion pairs and charge distributions more effectively than water because dipoles are appropriately preoriented in the active site); f) dynamic effects (the enzyme induces transient conformational fluctuations yielding deviations of the transmission coefficient from unity); g) tunneling. Discussions on the validity of the view points mentioned above are still on-going in the scientific community.

Both Emil Fischer and Linus Pauling were awarded with the Nobel Prize in Chemistry in 1902 and 1954, respectively. A thorough historical perspective of mechanistic enzymology can be found in Ref. [47].

The reader can find more comprehensive discussions about the different proposals in, for example, Ref. [35, 37, 49–53].
2 Enzyme catalysis and non-heme iron proteins

![Diagram](image)

**Figure 2.1:** Some possible roles of metal ions (generically indicated as $M^{2+}$) in enzymatic catalysis: **a**) electrophilic catalysis where developing negative charges are stabilized; **b**) activation of the leaving group; **c**) metal-bound hydroxyl are potent nucleophiles; **d**) the metal can act as transient reducing agent reaching high-oxidation states.

### 2.3 Metal ion catalysis

Many enzymes are entirely peptidic in nature, but others employ prosthetic groups to assist in their catalytic function. These groups may be complex organic molecules or metal ions, which directly participate in catalysis or act to perturb geometries. Some possible roles of the metals in enzymatic catalysis are sketched in figure 2.1. Catalysis of metal ions is usually associated with their capability to stabilize or shield negative charges that are formed (electrophilic catalysis). Moreover, metals can increase the leaving ability of a group. Activation of water through the formation of hydroxyl ions, which are powerful nucleophiles, is another common role of metal ions in enzymatic catalysis. Importantly, transition metal ions can also act as reducing/oxidizing agents during catalysis. Transition metals can catalyze spin forbidden transitions due to the large spin-orbit coupling of the metal.

### 2.4 Non-heme iron proteins

Iron plays a central role in biological systems. It is a versatile metal, which can uniquely tune its properties by means of different coordination environments. Iron proteins are usually classified as: **a**) heme proteins,

---

6In general, metalloenzymes are those enzymes where the metal (iron, copper, zinc, manganese, cobalt, nickel, molybdenum) is tightly bound to the protein backbone. Metal-activated enzymes instead contain metals such as $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, which are loosely bound.
2.4 Non-heme iron proteins

b) iron-sulfur proteins, c) non-heme proteins (see figure 2.2). In the heme proteins, iron is coordinated by a porphyrin ligand, while the coordination environment of the non-heme iron proteins is dominated by oxygen and nitrogen ligands, such as the side-chains of tyrosine, histidine and aspartate or glutamate. The majority of the non-heme iron proteins exploits the oxidative power of dioxygen, whose uncatalyzed reactions with organic substrates are thermodynamically favorable, albeit kinetically too slow. The reason is that dioxygen has a triplet ground state and therefore any reaction between $^3\text{O}_2$ and singlet organic molecules is spin-forbidden. On the contrary, singlet dioxygen is very reactive but it is not energetically accessible because it lies too high in energy ($22 \text{ kcal mol}^{-1}$) with respect to $^3\text{O}_2$.

In Nature, mono- and bi-nuclear non-heme iron proteins use $^3\text{O}_2$ to

Figure 2.2: Examples of metal sites in iron proteins: a) the heme iron complex in cytochrome $c$ oxidase; b) the $[2\text{Fe}-2\text{S}]$ Rieske cluster in naphthalene dioxygenase; c) the mononuclear non-heme iron(II) active site in naphthalene dioxygenase.
Figure 2.3: The four different families of non-heme iron enzymes with the 2-His-1-carboxylate motif [54, 55].
catalyze a broad spectrum of different oxidative reactions such as desaturation, oxidative cyclizations, mono-oxygenations and dioxygenations, hydroperoxidations and epoxidations [56–59]. Some mononuclear non-heme iron proteins (e.g., lipoxygenase and intradiol dioxygenases) uses an Fe$^{II}$ active site to activate dioxygen, whereas others carry out their catalytic activity employing Fe$^{II}$.

2.5 The 2-His-1-carboxylate motif

The increasing number of available crystal structures of mononuclear non-heme iron proteins reveals that the ferrous ion in the catalytic site of several unrelated metalloenzymes is coordinated by a common structural motif, the so-called 2-His-1-carboxylate facial triad (see for example figure 2.2c) [54–56, 60]. The coordination of iron is usually completed by water ligands. The aspartate or glutamate residues binding in a bi- or monodentate fashion provide the carboxylate functional group, which stabilizes the divalent iron through the negative charge. This type of coordination environment creates a weak ligand field, which stabilizes the high-spin state of the metal; in the case of Fe$^{II}$, the ground state is characterized by a total spin $S=2$, and therefore a multiplicity $M=5$ [57].

The mononuclear non-heme iron(II) enzymes with the 2-His-1-carboxylate motif are commonly classified in four families as depicted in figure 2.3: extradiol dioxygenases, pterin-dependent hydroxylases, Rieske dioxygenases, $\alpha$-keto-acid-dependent enzymes [54, 55]. They all employ dioxygen to perform various oxidative reactions. Extradiol-cleaving catechol dioxygenases are isolated from soil bacteria and they catalyze the degradation of different aromatic rings through the oxidative cleavage of the catecholic compounds formed during the biodegradation pathway. Tetrahydrobiopterin-dependent hydroxylases use a pterin cofactor (BH$_4$) to carry out hydroxylation of the ring in the aromatic amino acids. In the mammals, they play a fundamental role in the catabolism of amino acids (phenylalanine hydroxylase) and in the synthesis of neurotransmitters/hormones (tyrosine and tryptophan hydroxylases). The Rieske dioxygenases, the third family of oxygenases shown in figure 2.3, are enzymes found in soil bacteria and they start the catabolism of aromatic compounds yielding the corresponding cis-dihydrodiols, which are subsequently degraded by the intradiol and extradiol oxygenases. The Rieske dioxygenases are multicomponent enzymes containing various iron-sulfur clusters, which
mediate the transfer of electrons during catalysis. The fourth family, the α-keto-acid-dependent enzymes, participates in the synthesis of primary and secondary metabolites. In microorganisms, these enzymes are involved in the biosynthesis of β-lactam antibiotics. Isopenicillin N synthase (IPNS) does not use any cosubstrate, but the other members of the family generally require the cosubstrate 2-oxoglutarate (α-ketoglutarate), as for example clavaminate synthase (CAS) or prolyl hydroxylases. CAS is involved in the biosynthesis of clavulanic acid, a β-lactamase inhibitor, which is clinically used against bacteria resistant to β-lactam antibiotics. The prolyl hydroxylases are involved in cellular oxygen sensing.

It has been observed that the enzymes belonging to the same family often shares high structural and sequential similarities, but enzymes of different families are completely unrelated although all of them anchor iron(II) in the active site employing two histidines and one carboxylate as ligands. This observation suggests that the 2-His-1-carboxylate coordination environment might be classified as a new recurring motif among metalloproteins, like the iron-sulfur clusters and the porphyrin ligand of the heme proteins.

The striking feature of the mononuclear non-heme iron(II) enzymes is the flexibility of the metal coordination environment. In contrast to heme enzymes, where the metal is coordinated by the sterically hindered porphyrin, non-heme iron enzymes may liberate the water ligands offering some free coordination sites to exogenous ligands like the cofactor or dioxygen. In the heme enzymes there is instead only one available site for the accommodation of dioxygen or its analogues or derivatives.

On the basis of different experimental observations collected from
mechanistic and spectroscopic studies, a common strategy for dioxygen activation by non-heme iron(II) enzymes is proposed and it is illustrated in figure 2.4 [57]. In the resting state of the enzyme, the iron complex is usually 6-coordinate, but becomes 5-coordinate upon binding of the substrate and/or the cofactor. In this state the metal center is sensitive to dioxygen and is able to produce a highly reactive iron-oxygen intermediate. In the case of tetrahydrobiopterin-dependent hydroxylases it is, for instance, observed that catalysis starts when all the reactants (the aromatic amino acid, the pterin cofactor and dioxygen) are bound in the active site.

What is the identity of the activated oxygen species? Proposed intermediates in dioxygen activation by iron(II) complexes are, for example: iron(III)-superoxo, iron(IV/III/II)-peroxo, and iron(IV/V)-oxo species. The iron(III)-superoxo species requires that iron carries out the one-electron reduction of dioxygen. An iron(IV)-peroxo species originates from the two-electron reduction of O$_2$ provided that all the reducing equivalents are supplied by iron; if, however, the metal donates only one electron, an iron(III)-peroxo species can be obtained when another electron donor completes the two-electron reduction of dioxygen. Finally, a peroxide binding to iron(II) may be formed if both electrons do not come from the metal. In analogy to the generally accepted mechanism for heme oxygenases, where a high-valent iron-oxo species is involved, non-heme iron oxygenases may also employ the Fe$^{III}$/Fe$^{IV}$ redox couple. In the specific case of Rieske dioxygenases an even higher oxidation state is proposed for iron, namely, Fe$^{V}$, associated with the Fe$^{III}$/Fe$^{V}$ redox couple. Interestingly, also cytochrome P450 formally exploits the same Fe$^{III}$/Fe$^{V}$ redox couple, although the corresponding high-valent iron-oxo species (Compound I) is in this case better described as iron(IV)-oxo coupled to a porphyrin radical cation.

Whether high-valent iron-oxo species could be obtained for a non-heme environment has been long debated, but lately, experimental evidence is accumulating to support the existence of non-heme high-valent iron-oxo complexes. Particularly, Fe$^{IV}$=O complexes could be characterized and also isolated in the case of model complexes with ligands mimicking the non-heme environment of the enzymes [61–63]. Moreover, experimental studies on taurine/$\alpha$-ketoglutarate dioxygenase showed that the enzymatic mechanism involves a high-spin iron(IV) species capable of hydrogen atom abstraction [64, 65]; strong evidence that this species
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corresponds to an Fe$^{IV}$=O intermediate comes from resonance Raman spectroscopy [66]. Direct experimental proofs or support from calculations are instead lacking for the involvement of an iron(V)-oxo species as intermediate in the catalytic cycle of Rieske-type dioxygenases. On the other hand, an Fe$^{V}$=O species is likely to be involved in the catalysis by biomimetic complexes [67-69].

2.6 Functional mimics

In the past few years bio-inspired catalysts have been successfully employed to gain understanding on the role of the iron center in the non-heme iron enzymes. The advantage of functional analogues is that their reactivity might be finely tuned and controlled, providing insight into the various steric and electronic factors governing the reaction of interest. Furthermore, biomimetic catalysts could represent valuable alternatives to the industrial heavy metal reagents. As an example, some popular ligands are shown in figure 2.5

Families of non-heme iron complexes derived from tetradeutate pyridine/amine ligands (e.g., TPA = tris(2-pyridylmethyl)amine and BPMEN = N,N'-dimethyl-N,N'-bis(2-pyridylmethyl)-1,2-diaminoethane) brought deep insight on the enzymatic mechanisms of non-heme iron proteins [55, 70]. Of great interest is the experimental evidence that high-valent iron-oxo species can be accessed in these complexes typically by the reaction of a precursor iron compound with a proper oxidant (e.g., PhIO or CH$_3$CO$_3$H or alkyl peroxide). Evidence for high-valent iron-oxo intermediates are collected directly from spectroscopic investigations or indirectly from mechanistic studies. Very recently Que and coworkers reported the characterization of stable iron(IV)-oxo complexes with tetradeutate and pentadeutate ligands, such as TMC, TPA, Bn-tpen and N4Py [61–63]. It was also observed that the TMC complex is capable of hydrogen atom transfer to PPh$_3$ while the TPA complex performs epoxidation of cyclooctene. The Bn-tpen and N4Py complexes can react with various hydrocarbons at room temperature yielding the corresponding alcohols. Indirect evidence of Fe$^{IV}$=O was previously obtained from the observation that the O–O bond homolysis occurring in alkylperoxoiron(III)-TPA complexes resulted in intramolecular hydroxylation of a phenyl ring [71]. This was taken as evidence of a non-heme Fe$^{IV}$=O intermediate capable

\footnote{A high-resolution crystal structure of the TMC complex is also available.}
of aromatic hydroxylation. All these observations lend support to the possibility that some mononuclear non-heme iron enzymes may activate dioxygen through the formation of Fe$^{IV}$=O intermediates.
3

Aromatic amino acid hydroxylases

Phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TyrH) and tryptophan hydroxylase (TrpH), also known as tetrahydrobiopterin-dependent amino acid hydroxylases, are non-heme iron enzymes that employ molecular oxygen and the pterin cofactor (BH$_4$) to carry out hydroxylation of the side chains of the aromatic amino acids (see figure 3.1) [56, 57, 72–74]. The catalytic cycle can be viewed as occurring in two steps: dioxygen activation associated with hydroxylation of the cofactor followed by the hydroxylation of the aromatic ring$^1$. The cofactor supplies two of the four electrons required to fully reduce O$_2$, while the other two electrons are supplied by the aromatic amino acid. PAH, TyrH and TrpH are closely related in structure and mechanism, and they all catalyze the coupled hydroxylations of the substrate and the cofactor in the active site hosting iron coordinated by the 2-His-1-carboxylate motif. The enzymatic activity requires iron in the ferrous oxidation state.

3.1 Introduction: structure

The three aromatic amino acid hydroxylases host iron in the active site as derived from the crystal structures solved for PAH, TyrH and TrpH [75–79]. The cofactor is found to bind in the proximity of the metal

$^1$Our computational investigations of catalysis of aromatic amino acid hydroxylases analyze both the cofactor (Paper I) and the substrate (Paper II) hydroxylation mechanisms.
3 Aromatic amino acid hydroxylases

Figure 3.1: The coupled hydroxylations of the pterin cofactor and the side chain of aromatic amino acids catalyzed by aromatic amino acid hydroxylases PAH, TyrH and TrpH.

complex and, to date there is no evidence that the pterin enters the first coordination sphere of the metal.

Recently, the structure of PAH binding both the cofactor and a substrate analogue (e.g., 3-(2-thienyl)-L-alanine) was solved, highlighting some differences with respect to the previous binary complexes including only BH$_4$. Figure 3.2 compares the structural arrangement of the active site in the binary [75, 77] and the ternary complexes [76, 80]. In the binary complex, iron is six-coordinate with the glutamate ligand (Glu330) binding in a monodentate fashion$^2$. In the ternary complex, there are some major modifications induced by the binding of the substrate analogue. First of all there are fewer water molecules in the proximity of iron; more specifically, the X-ray data indicate that probably only one water molecule (not shown in figure 3.2b) is ligated to the metal. This structural change fits the strategy for dioxygen activation illustrated in figure 2.4, where the substrate binding provokes a structural change at the metal site. The absence of more water ligands positions the cofactor

$^2$The structure of the binary complex reported in figure 3.2a is taken from Ref [75] (1.5 Å resolution), while the X-ray structure used in Paper I refers to Ref [77] (2.0 Å resolution); the two structures are very similar.
3.2 Introduction: proposed mechanisms

The high sequential and structural homology of PAH, TyrH, and TrpH suggests that the three aromatic amino acid hydroxylases share a similar...
catalytic mechanism, which has to account for the experimental observation that the oxygen atom of the hydroxylated cofactor and the oxygen atom of the hydroxylated amino acid originate from molecular oxygen. Figure 3.3 illustrates two mechanisms proposed for dioxygen activation in these enzymes\(^3\). The two pathways differ in the initial role played by iron in catalysis. In one case (pathway a), which will be referred to as the metal-free mechanism, dioxygen activation is initiated by the one-electron transfer from the pterin cofactor and this transformation does not involve any metal activity. In the other suggested mechanism (pathway b), which will be called the metal-catalyzed pathway, reduction of molecular oxygen by the cofactor requires the activity of iron, for example, through a dioxygen-bound iron complex. Both pathways depicted in figure 3.3 lead to the formation of an iron(II)-peroxy-pterin intermediate, which might itself be responsible for aromatic hydroxylation of the substrate. Alternatively, the peroxide decomposes through O–O bond heterolysis producing the hydroxylated cofactor and an iron(IV)-oxo intermediate, namely, the activated oxygen species, which subsequently carries out substrate hydroxylation. The metal-free pathway resembles the mechanism proposed for the autoxidation of tetrahydrobiopterins in solution, where the super-

\(^3\)A more comprehensive discussion about the mechanisms can be found in the recent review by Fitzpatrick [74].
oxide and the pterin cationic radical are first formed. The two species then combine yielding a pterin-peroxide, whose decomposition generates the observed products: the oxidized pterin and hydrogen peroxide. The passive role of iron in the first steps also resembles the catalytic mechanism of flavoproteins. These metal-free enzymes employ flavin cofactors and O$_2$ to catalyze aromatic hydroxylations involving the 4a-peroxyflavin intermediate [81], which would be very similar to the corresponding 4a-peroxypterin species:

![Diagram of 4a-peroxyflavin and 4a-peroxypterin]

The conclusion that O$_2$ may be reduced by the pterin cofactor without any catalytic activity of iron is also proposed on the basis of recent spectroscopic and kinetic studies on PAH mutants (Arg158Gln and Glu280Lys) [82].

Despite the many mechanistic proposals, no direct experimental evidence is available which strongly supports either one of the two pathways depicted in figure 3.3 [74]. For example, experiments with the apo-enzyme indicate that pterin is oxidized by O$_2$ even in the absence of iron, albeit with lower rate, but it was argued that this activity might be due to the presence of other metals, nickel and copper, present as contaminants [83]. Furthermore some mutants characterized by low affinity for iron do not show any enzymatic activity [84]. As mentioned above, the peroxy-pterin species resulting from the two-electron reduction of dioxygen by the cofactor may carry out aromatic hydroxylation, paralleling the mechanism of the flavoproteins. Critics to this pathway arise from the observation that flavoproteins only hydroxylate activated rings containing hydroxyl, amino or thiol substituents [74]. In contrast, tetrahydrobiopterin-dependent hydroxylases not only catalyze unactivated aromatic rings, but they are also found to carry out benzylic hydroxylation [85]. This suggests that a more potent oxidant, such as a high-valent iron-oxo species like Fe$^{IV}=O$, is required for PAH, TyrH and TrpH.
3.3 Dioxygen activation

The theoretical modeling of pterin hydroxylation in the active site of aromatic amino acid hydroxylases led us to propose a reaction mechanism for dioxygen activation that resembles pathway b of figure 3.3, namely, the metal-catalyzed mechanism (Paper I). As summarized in figure 3.4, we found that an iron(II)-peroxy-pterin intermediate is first generated through the formation of a new C–O bond, which implicates the two-electron reduction of dioxygen by the pterin. In this step iron does not carry out any redox activity but it only stabilizes the developing charge on the peroxy group. It should be mentioned that the cofactor hydroxylation was found to involve the quintet potential energy surface, which is computed to be an excited state of the Fe$^{II}$-O$_2$ reactant. In the calculations it is assumed that the presence of iron makes the first spin transition fast because of its large spin-orbit coupling constant. Once the peroxide is formed, the subsequent step is the O–O bond heterolysis promoted by one of the water ligands, which donates a proton to the distal oxygen leading to the formation of HO-Fe$^{IV}$=O and the hydroxylated cofactor. The overall process is computed exergonic by 6.3 kcal mol$^{-1}$ with respect to the initial Fe$^{II}$-O$_2$ complex and it is found to require an activation barrier of 16.6 kcal mol$^{-1}$ associated with the C–O bond formation. While scanning the potential energy surface joining the reactant and the first stable intermediate (i.e., the pterin-peroxide) a metastable species interpreted as a cationic pterin radical coupled to an iron(II)-superoxo complex was optimized and found to lie 7-8 kcal mol$^{-1}$ higher in energy than the reactant. This structure was highly unstable and could be easily converted

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*Figure 3.4:* The computed free energy changes for the formation of the high-valent iron(IV)-oxo species (i.e., the hydroxylating intermediate) in aromatic amino acid hydroxylases.

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More details concerning the energetics for the binding of O$_2$ to iron(II) are given in the next paragraph.
3.3 Dioxygen activation

into the reactant with small geometrical perturbations. It can be concluded that our DFT calculations suggest that the C–O bond formation is rate-limiting in the cofactor hydroxylation process and therefore the slow step arises from the two-electron reduction of O$_2$ forming a peroxide and not with the one-electron reduction forming the superoxide.

In order to probe whether O$_2$ might react with BH$_4$ in a metal-free fashion, dioxygen was placed in the metal second coordination sphere, but the wave function corresponding to the pterin-radical-cation/superoxide couple was never obtained. A smaller model was tested, namely, a cluster including only triplet dioxygen and the pterin. Despite the great efforts to force reduction of dioxygen to superoxide by the pterin, the Mulliken spin population analysis yet indicated little electron transfer. A model with dioxygen, pterin and a water molecule was also considered to ascertain if the charge separation could be stabilized by the direct inclusion of hydrogen bonds. Again, no significant electron transfer was detected.

From the evaluation of the ionization potential (IP) of the pterin and the electron affinity (EA) of dioxygen, it was clear that the state with the superoxide/radical-cation couple lied too high in energy (approximately 30 kcal mol$^{-1}$) with respect to the dioxygen-pterin reactant, justifying why the proper wave function corresponding to the one-electron transfer could never be obtained.

Since the oxygen reaction with pterin in PAH was estimated $10^7$ faster than the corresponding reaction in solution, and since the rate limiting step of the reaction in solution is proposed to be the one-electron transfer from the pterin to dioxygen [86], in a simplistic way it can be concluded that in the metal-free mechanism of figure 3.3 the enzyme should stabilize the developing pterin-radical-cation/superoxide couple with respect to the solution case. This can be achieved with, for example, charged residues. However, the active site of aromatic amino acid hydroxylases is located in a rather hydrophobic pocket, which does not provide positively charged groups (except for iron) capable of activating O$_2$ for the

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5 In order to stabilize the charge separation that the electron transfer would imply, geometries were optimized in solution. The effects of a larger basis set for geometry optimization were tested. The open shell singlet was also considered. With $S=1$, the lowest spin population on O$_2$ was found to be 1.7-1.8.

6 Evaluation of the IP and the EA was performed using a large basis set including diffuse functions necessary to model the superoxide anion. Dielectric effects, which were obtained for a low dielectric constant ($\varepsilon = 4$), influenced substantially the final IP and EA values.
one-electron transfer. In contrast, the flavin cofactor in glucose oxidase can readily reduce dioxygen to superoxide, which is, however, bound in the proximity of a protonated histidine [87]. Taking a closer look at the active site, it is clear that Glu286, positioned in the second coordination sphere of iron, might instead serve to stabilize any pterin cationic intermediate. The experimental observation that the substrate binding removes one water molecule and causes the direct interaction between Glu286 and the cofactor, lends further support to the formation of this type of cationic intermediate. Nevertheless, the calculations indicate that the carboxylate group alone is not sufficient to stabilize the charge separation corresponding to the radical-cation/superoxide couple.

In summary, our studies favor the metal-catalyzed pathway because the ferrous ion provides the required electrostatic stabilization for the developing negative charge on dioxygen. We were not able to locate a positively charged residue in the active site of these enzymes that could perform an analogous role. Furthermore, the computed free-energy profile indicated that the rate-determining step is associated with the two-electron reduction of \( \text{O}_2 \).

### 3.4 Thermodynamics of \( \text{O}_2 \) binding to iron

When dioxygen binds to \( \text{Fe}^{II} \), the resulting complex can be described by two extremes: \( \text{Fe}^{II}-\text{O}_2 \) or \( \text{Fe}^{III}-\text{O}^{-} \). The metal coordination environment, which tunes the redox potential of iron, will determine which of the two formula better describes the dioxygen-bound iron complex. The metal ligands will also determine which multiplicity characterizes the ground state of the complex. In the case of iron complexes with the 2-His-1-carboxylate motif, the ferrous ion is usually in the quintet ground state (\( S=2 \)), and, when triplet dioxygen binds, different spin states, such as the triplet (\( S=1 \)), the quintet (\( S=2 \)) and the septet (\( S=3 \)) states, arise from the different spin coupling between the d-electrons of iron and the unpaired spin density on bound dioxygen. In the particular case of aromatic amino acid hydroxylases, the ground state of \( [\text{Fe-O}_2]^{2+} \) is found to be the septet state with the model taken from figure 3.2a. The triplet state does not lie too high in energy and, for example, it is computed the ground state in the case of the \( \alpha \)-ketoglutarate-dependent enzymes (Paper V). An important spin state is the quintet, which, in the case of the aromatic amino acid hydroxylases and the \( \alpha \)-ketoglutarate-dependent
3.4 Thermodynamics of O₂ binding to iron

Figure 3.5: Dioxygen-bound iron(II) complexes investigated in this thesis.

enzymes defines the reactive potential energy surface. Importantly, the dioxygen-bound iron(II) complex in the quintet state is better described as an iron(III)-superoxo species (vide infra).

We have investigated various dioxygen-bound iron(II) species, which are shown in figure 3.5. Table 3.1 gathers the corresponding relevant geometrical details and the computed spin distributions for these complexes, most of them ligated by the 2-His-1-carboxylate facial triad. In order to interpret the spin populations reported in table 3.1, it is useful to recall that for high spin iron complexes the computed spin population on the metal does not strictly correspond to the number of d-electrons as expected from the oxidation state. With the coordination environment dominated by oxygen and nitrogen ligands the computed spin population on high-spin Fe^{II} (S=2) is usually 3.7-3.8; it is about 4.0-4.1 for high-spin Fe^{III} (S=5/2). The spin populations on iron reported in table 3.1 reflect
3 Aromatic amino acid hydroxylases

**Table 3.1**: Relevant bond distances and computed spin distributions for some dioxygen-bound non-heme iron(II) complexes (see figure 3.5) characterized by different total spins S. Binding of dioxygen in the end-on fashion is usually considered, but in some cases the side-on binding mode (so) is reported. Relative free energies within each group are shown.

<table>
<thead>
<tr>
<th></th>
<th>Distance (Å)</th>
<th>Spin</th>
<th>ΔG (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe-O</td>
<td>O1-O2</td>
<td>Fe</td>
</tr>
<tr>
<td>AA S=3</td>
<td>2.24</td>
<td>1.31</td>
<td>4.07</td>
</tr>
<tr>
<td>AA S=2</td>
<td>2.09</td>
<td>1.36</td>
<td>4.21</td>
</tr>
<tr>
<td>α-KG S=3</td>
<td>2.28</td>
<td>1.31</td>
<td>4.06</td>
</tr>
<tr>
<td>α-KG S=2</td>
<td>2.09</td>
<td>1.34</td>
<td>4.17</td>
</tr>
<tr>
<td>α-KG S=1</td>
<td>2.30</td>
<td>1.26</td>
<td>3.71</td>
</tr>
<tr>
<td>NDO⁺ S=3</td>
<td>2.55</td>
<td>1.25</td>
<td>3.73</td>
</tr>
<tr>
<td>NDO⁺ S=3 so</td>
<td>2.20-2.32</td>
<td>1.32</td>
<td>3.87</td>
</tr>
<tr>
<td>NDO⁺ S=2</td>
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<tr>
<td>NDO⁺ S=1</td>
<td>2.26</td>
<td>1.26</td>
<td>3.66</td>
</tr>
<tr>
<td>NDO⁺ S=3</td>
<td>2.28</td>
<td>1.33</td>
<td>3.98</td>
</tr>
<tr>
<td>Biomim S=3</td>
<td>2.57</td>
<td>1.26</td>
<td>3.83</td>
</tr>
<tr>
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<td>2.24-2.33</td>
<td>1.32</td>
<td>3.90</td>
</tr>
<tr>
<td>Biomim S=2</td>
<td>2.04</td>
<td>1.29</td>
<td>3.80</td>
</tr>
<tr>
<td>Biomim S=2 so</td>
<td>2.16-2.26</td>
<td>1.34</td>
<td>3.99</td>
</tr>
<tr>
<td>Biomim S=1</td>
<td>2.28</td>
<td>1.26</td>
<td>3.65</td>
</tr>
</tbody>
</table>

*No water ligated to iron(II). †One water ligated to iron(II) and a carboxylate group located in the metal second coordination sphere.

Concerning the energetics of the following process:

\[
\text{Fe}^{2+} + \text{O}_2 = [\text{Fe-O}_2]^{2+}
\]

we find that molecular oxygen is not strongly bound to iron(II). The process is computed near-thermoneutral in the ferrous complexes that we have investigated. Table 3.2 summarizes the corresponding thermodynamic values and shows that \(\text{O}_2\) binding to iron(II) becomes endergonic by about 10 kcal mol⁻¹ mainly because of the high entropic contribution \((-T\Delta S)\), which is not balanced by the enthalpic binding energy between iron and molecular oxygen. The computed loss of entropy can be mainly associated with the loss of translational entropy when free \(\text{O}_2\) is trapped to form the adduct. The thermodynamic values of table 3.2 indicate that...
3.4 Thermodynamics of $O_2$ binding to iron

Table 3.2: Thermodynamics (in kcal mol$^{-1}$) for binding of $O_2$ to iron(II) complexes with end-on binding mode (see figure 3.5). The gas-phase energy change ($\Delta E$), $\Delta H$ and $\Delta G$ are reported, together with the entropic contribution ($-T\Delta S$) and the correction due to the solvent effects ($\delta_s$).

<table>
<thead>
<tr>
<th></th>
<th>$\Delta E$</th>
<th>$\delta_s$</th>
<th>$\Delta H$</th>
<th>$-T\Delta S$</th>
<th>$\Delta G$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA S=3</td>
<td>0.7</td>
<td>2.3</td>
<td>3.1</td>
<td>9.5</td>
<td>12.6</td>
</tr>
<tr>
<td>AA S=2</td>
<td>5.0</td>
<td>1.2</td>
<td>5.8</td>
<td>11.9</td>
<td>17.7</td>
</tr>
<tr>
<td>$\alpha$-KG S=3</td>
<td>1.6</td>
<td>-0.02</td>
<td>2.4</td>
<td>10.6</td>
<td>13.0</td>
</tr>
<tr>
<td>$\alpha$-KG S=2</td>
<td>3.5</td>
<td>-1.2</td>
<td>3.3</td>
<td>13.3</td>
<td>16.5</td>
</tr>
<tr>
<td>$\alpha$-KG S=1</td>
<td>-0.9</td>
<td>1.5</td>
<td>1.5</td>
<td>9.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Biomim S=3</td>
<td>2.8</td>
<td>0.3</td>
<td>2.9</td>
<td>9.9</td>
<td>12.8</td>
</tr>
<tr>
<td>Biomim S=2</td>
<td>8.4</td>
<td>-1.4</td>
<td>7.9</td>
<td>10.4</td>
<td>18.3</td>
</tr>
<tr>
<td>Biomim S=1</td>
<td>2.3</td>
<td>0.05</td>
<td>3.3</td>
<td>9.0</td>
<td>12.3</td>
</tr>
</tbody>
</table>

A dioxygen-bound non-heme iron(II) complex is not a stable species$^7$. We also tested different cluster models for the same iron complex in order to probe if there were any chemical effects stabilizing bound $O_2$. More specifically, water molecules hydrogen-bonding to the iron complex were added, but the energetics were not significantly affected.

Wirstam et al. presented a QM/MM study on the reversible binding of $O_2$ to hemerythrin (a binuclear non-heme iron enzyme) showing that a significant stabilization (-9.8 kcal mol$^{-1}$) of the oxy form arises from the Van der Waals and electrostatic interactions between bound $O_2$ and the protein environment [88]. It should be noted that dioxygen binding to iron in hemerythrin entails a two-electron reduction of dioxygen coupled to a proton transfer:

$$\text{Fe}^{II} \text{H} \text{Fe}^{II} \xrightarrow{O_2} \text{Fe}^{III} \text{O} \text{Fe}^{III}$$

$^7$Initially we could not exclude that a substantial error in the evaluation of the binding energy of $O_2$ to iron(II) arose from the B3LYP method. Therefore we performed some calculations at the CCSD(T) level of theory with a small model. As shown in table 1.1, the two methods predict very similar binding energies. Moreover, the investigation on the TP$^{Ph2}$ complex (Paper VI) was undertaken to compare experimental and theoretical thermodynamic values; indeed in this case the calculations show good agreement with the available experimental enthalpy and entropy changes.
3 Aromatic amino acid hydroxylases

Figure 3.6: a) Optimized geometry of the 5-coordinate iron(II) complex with the pterin cofactor in the second-coordination sphere (S=2). Asterisks indicate those atoms frozen during the geometry optimizations according to the X-ray crystal structure [76]. Relevant distances (in Å) are reported. b) Comparison between the corresponding optimized and experimental geometries of the 5-coordinate complex.

The stabilization due to the protein environment found in the hemerythrin case might also stabilize the dioxygen-bound iron complex in the mononuclear enzymes. This stabilization would cancel out the loss of entropy, making the free energy for the binding of molecular oxygen almost zero. Nevertheless, this effect remains to be verified for mononuclear non-heme iron(II) enzymes with a proper QM/MM approach.

3.5 Refinement of the model

The present QM modeling of pterin hydroxylation predicts a barrier of 16.6 kcal mol\(^{-1}\) with respect to the dioxygen-bound iron complex, and this barrier increases roughly by 10 kcal mol\(^{-1}\) when the entropic contribution associated with binding of \(\text{O}_2\) is included. Unfortunately, this is too high a barrier for an enzymatic reaction. Similarly to the hemerythrin case discussed above, Van der Waals and electrostatic interactions might
contribute to decrease the computed activation energy, balancing the entropic loss. Alternatively, the model we employed is not appropriate to accurately describe the enzymatic reaction and in light of the more recent X-ray data for the ternary complex, we have performed some preliminary calculations with a model derived from the crystal structure sketched in figure 3.2b. In the new model including approximately 90 atoms (figure 3.6a), Glu286 is hydrogen bonding to N3 of the pterin and only one water molecule is coordinated to iron instead of two water molecules as in the previous study presented in Paper I. Furthermore the model has been extended and the hydrogen bonding interaction of the cofactor with the backbone is added together with the backbone connecting Glu286 to His285. In this investigation, some coordinates have been frozen according to the X-ray crystal structure as shown in figure 3.6, which also highlights a comparison between the optimized and the experimental geometries.

While the thermodynamics for dioxygen binding is not significantly affected by the new structural arrangement, the approximate transition state corresponding to the C–O bond formation leading to the iron(II)-peroxy-pterin intermediate (figure 3.7), requires a computed energy barrier of 9.9 kcal mol$^{-1}$, which is about 5 kcal mol$^{-1}$ lower than the corresponding value obtained in our first study. The lower activation barrier is likely to be associated with the stabilization of the positive charge developing on the pterin during the C–O bond formation. Notably, at the transition state, the two electron reduction of dioxygen by the cofactor is almost completed as inferred by various factors: the rather long O–O bond distance of 1.44 Å, the absence of unpaired spin in the pterin and in dioxygen, and finally a spin population of 3.72 on iron indicating a

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Note that zero-point effects and thermal effects have not been computed for the new model yet. However, the entropy effects due to O$_2$ binding, which gives the most significant correction, can be derived from the smaller models.

Another structure with the cofactor coordinated to iron could be obtained and this geometry is 9.2 kcal mol$^{-1}$ lower in energy than the minimum shown in figure 3.6. Since there is not any X-ray crystal structure detecting the cofactor ligated to iron, for the time being it is assumed that the pterin is held in the second coordination sphere by the protein environment, which is not reproduced in the present modeling (for example, all crystal structures show that the cofactor is involved in a π-stacking interaction).

The barrier, which includes neither the entropy nor the zero-point effects, is expressed with respect to unbound dioxygen and the 5-coordinate complex of figure 3.6. The C–O bond formation yields a charge separation on the substrate: a peroxide and a positive charge stabilized by resonance in the pyrimidyl ring.
ferrous oxidation state for the metal. The subsequent intermediate is also stabilized by the new structural arrangement and it lies -1.9 kcal mol$^{-1}$ with respect to the initial reactant. The details of the O–O bond cleavage yielding the iron(IV)-oxo species have not been yet investigated with the larger model.

The new model indicates that the activation barrier for the two-electron reduction of dioxygen is affected by a different structural organization around the cofactor, and more specifically by the glutamate, which is likely to stabilize the positive charge on the peroxy-pterin species. Interestingly, the new structure does not lead to any particular stabilization of the dioxygen-bound complex, and the binding of O$_2$ to iron(II) is still found to be an endergonic process. In contrast, the transition state is lowered by a slightly different arrangement highlighting not only the importance of the chosen model but also the crucial importance of the experimental information. On the basis of the new geometrical insights we are also exploring if Tyr138, which was found to enter the active site upon substrate binding (figure 3.2), might play any role in dioxygen activation.
3.6 Mechanism of aromatic hydroxylation

Pterin hydroxylation is coupled to the formation of activated oxygen, namely, Fe\(^{IV}\)=O. Using two different models for the metal complex, in Paper II we have investigated whether this species is able to carry out aromatic hydroxylation. In one model, the high-valent iron-oxo species was ligated by the hydroxo ligand formed during the preceding O–O bond cleavage. In the other model the hydroxide was simply replaced by a water ligand\(^{12}\). We then studied hydroxylations of phenylalanine and tryptophan using benzene and indole, respectively. The carboxylic group of the amino acids is engaged in hydrogen bonding interactions with residues which are not included in the present modeling; by neglecting this part of the substrate we simply assumed that these chemical interactions remain constant during the reaction and do not affect the computed energetics.

Our DFT calculations indicate that substrate hydroxylation occurs along the quintet potential energy surface (i.e., the ground state for Fe\(^{IV}\)=O) and starts with an electrophilic attack by Fe\(^{IV}\)=O on the aromatic ring. Figure 3.8 summarizes the computed energy profiles for the two models and the two substrates. The subsequent step is a 1,2-hydride shift, known as NIH shift, yielding the keto-form of the actual products, the hydroxylated benzene and indole. Keto-enol tautomerization finally completes the catalytic cycle of aromatic amino acid hydroxylases.

We found that the rate determining step for substrate hydroxylation is the initial C–O bond formation, which requires a lower activation energy in the case of H\(_2\)O-Fe\(^{IV}\)=O than the corresponding energy computed for HO-Fe\(^{IV}\)=O (figure 3.8). The difference can be explained simply by noting that the negative hydroxo ligand makes the high-valent iron-oxo species less electrophilic. It is useful to recall that substrate hydroxylation implies a two-electron reduction of the aromatic ring. For H\(_2\)O-Fe\(^{IV}\)=O, this redox process is achieved in the first step, generating iron in the initial ferrous oxidation state. In contrast, the C–O bond formation following the attack by HO-Fe\(^{IV}\)=O leads to a ferric ion and a radical on the substrate, indicative of a one-electron redox process.

Which is the model that better describes the enzyme active site at this stage of the reaction? The following aspects of catalysis need to be

\(^{12}\)Since cofactor hydroxylation yields a protonated pterin, we tried to model the possible proton transfer from the cofactor to the hydroxo ligand generating water and the unprotonated 4a-carbinolamine. A suitable pathway associated with this transformation was not located.
Figure 3.8: Computed free energy changes in kcal mol$^{-1}$ for phenylalanine hydroxylation and tryptophan hydroxylation (in parenthesis) by an iron(IV)-oxo intermediate as derived in Paper II. The results from two possible models for the iron complex are reported, showing the formation of a ferric-radical intermediate in one case and a ferrous-cationic species in the other model.

considered. Kinetic isotope effects indicated that the formation of the hydroxylating intermediate is rate-limiting in PAH (and TyrH) turnovers. TrpH exhibits a different kinetic behavior consistent with the aromatic hydroxylation being slower than cofactor hydroxylation. Assuming that the cofactor hydroxylation involves a very similar activation energy for both PAH and TrpH, the energy barrier for tryptophan hydroxylation by Fe$^{IV}$=O has to be higher than the one for phenylalanine hydroxylation and also higher than the one for pterin hydroxylation (16.6 kcal mol$^{-1}$). The computed activation energies for substrate hydroxylation by H$_2$O-Fe$^{IV}$=O are considerably lower than 16.6 kcal mol$^{-1}$ for both benzene (10.0 kcal mol$^{-1}$) and indole (6.8 kcal mol$^{-1}$). Furthermore, these values predict that tryptophan undergoes faster hydroxylation than phenylalanine. Consequently the model with the water ligand fails to reproduce the experimental evidence and we therefore favor the HO-Fe$^{IV}$=O oxidant. The latter pathway gives activation energies similar to the ones associated with the pterin hydroxylation for both substrates.
4

α-ketoglutarate-dependent enzymes

The active site of α-ketoglutarate-dependent enzymes contain a non-heme iron(II) complex, where the ferrous ion is coordinated by the 2-His-1-carboxylate motif. In these enzymes, molecular oxygen is employed to oxidize the substrate concomitantly with oxidative decarboxylation of the cosubstrate, 2-oxoglutarate (i.e., α-ketoglutarate, hereafter shortened to α-KG), which is converted to succinate (see figure 4.1). Substrate oxidation generally consists of hydroxylation of unactivated carbon atoms, but other oxidative processes are observed such as cyclization, ring expansion, and desaturation reaction.

4.1 Introduction

α-KG-dependent enzymes and tetrahydrobiopterin-dependent hydroxylases share a similar strategy to carry out full reduction (i.e., four-electron reduction) of molecular oxygen: one of the two oxygen atoms of O₂ is reduced by the substrate, while the other oxygen atom is reduced by an external organic compound [55, 57, 59]. For both families of enzymes, isotope labeling indicates that one oxygen atom originating from molecular oxygen is incorporated in the hydroxylated substrate while the other oxygen atom of O₂ ends up in the oxidized cosubstrate, that is, the succinate (figure 4.1) or the 4a-carbinolamine (figure 3.1).

In the case of α-KG-dependent enzymes, the cosubstrate binds in the metal first coordination shell after displacing two water ligands; it is pro-
posed that a third water is displaced when the substrate binds in the proximity of the metal complex. A free metal site becomes then available for dioxygen activation, which, as in the case of aromatic amino acid hydroxylases, may involve a dioxygen-bound iron(II) complex. According to the mechanisms suggested, the α-keto acid ligated to iron is then subjected to nucleophilic attack by bound dioxygen, and finally succinate, carbon dioxide and an activated oxygen species are formed as illustrated in figure 4.1. Subsequently the latter can carry out oxidation of the substrate.

α-KG-dependent enzymes are providing crucial information concerning the strategy for dioxygen activation. Recent experiments strongly support the involvement of an iron(IV)-oxo intermediate along the reaction pathway [64–66]. It means that the activated oxygen intermediate is likely to be a high-valent iron-oxo species capable of functionalization of unactivated C–H bonds. In order to probe if Fe$^{IV}O$ is a feasible intermediate in the catalytic cycle of α-KG-dependent enzymes, a computational model was constructed from the available structural information for clavaminic acid synthase (figure 4.2), whose structure was solved with the α-KG bound to the metal [89].

4.2 Dioxygen activation

The quantum chemical studies of dioxygen activation in α-KG-dependent enzymes allow us to propose a mechanism which involves the following steps (Paper V): a) binding of dioxygen to iron(II); b) two-electron reduction of dioxygen by the cosubstrate yielding an Fe$^{II}$-OO-R intermediate; c) O=O bond heterolysis generating the high-valent iron-oxo species. Remarkably, this sequence highly resembles the one already discussed for aromatic amino acid hydroxylases. It is found that the attack of Fe-bound dioxygen at the carbonyl carbon of α-KG (i.e., in the 2-position as shown in figure 4.1) implicates the C–O bond formation coupled to the C1–C2 bond cleavage. An iron(II)-peracid species is thus generated while carbon dioxide is liberated. The oxidative decarboxylation makes up the rate-determining step of dioxygen activation and it is an irreversible process due to its high exergonicity. Because the subsequent O=O bond cleavage requires lower energy barriers, the rate of formation of the iron(IV)-oxo intermediate is thus controlled by the initial two-electron reduction of
4.2 Dioxygen activation

![Proposed mechanism for oxidative decarboxylation of the cosubstrate and oxidation of the substrate in α-KG-dependent enzymes.](image)

Figure 4.1: Proposed mechanism for oxidative decarboxylation of the cosubstrate and oxidation of the substrate in α-KG-dependent enzymes.

![X-ray crystal structure of the active of clavaminic acid synthase.](image)

Figure 4.2: X-ray crystal structure of the active of clavaminic acid synthase.

dioxygen (the computed barrier for dioxygen activation with respect to the dioxygen-bound complex is 16.1 kcal mol\(^{-1}\)).

The electronic configuration of the various species involved in the elementary processes of catalysis suggests that iron plays the essential role to activate molecular oxygen for the nucleophilic attack at the carbonyl group of the α-keto-acid. The electronic configuration of the dioxygen complex corresponding to an iron(III)-peroxo species is required to carry out the initial attack on α-KG with a low activation energy. This explains why dioxygen activation in the α-KG-dependent enzymes involves the quintet potential energy surface, for which the computed spin population corresponding to the dioxygen-bound complex is better interpreted.
as an iron(III)-superoxo species (see table 3.1).

Only the dioxygen activation step leading to the high-valent iron-oxo species in the α-KG-dependent enzymes has been investigated. As demonstrated in the study of aromatic amino acid hydroxylases discussed in Paper II, a non-heme Fe$^{IV}$=O species is a potent oxidant capable of, for example, benzylic hydroxylation. Also a more recent theoretical investigation on 4-hydroxyphenylpyruvate dioxygenase, which is closely related to the α-KG-dependent enzymes, showed that Fe$^{IV}$=O can carry out benzylic-type hydroxylation [90].

### 4.3 A general strategy for dioxygen activation

A comparison between the proposed mechanisms describing the oxidation of α-KG (in the α-KG-dependent enzymes) and the oxidation of pterin (in the aromatic amino acid hydroxylases) highlights that in the two enzymes the dioxygen activation pathway generates an Fe$^{IV}$=O intermediate. Moreover, in both cases, the rate determining step for the formation of the high-valent iron-oxo species is not the O–O bond heterolysis, but the two-electron reduction of O$_2$ yielding an Fe$^{II}$-OOR species (the peracid for α-KG-dependent enzymes and the peroxide in the tetrahydrobiopterin-dependent enzymes). Interestingly, the two computed barriers associated with the rate-limiting step in the two enzymes are very similar (about 16 kcal mol$^{-1}$ with respect to the dioxygen-bound iron complex), although the thermodynamics of the two corresponding steps is very different. Formation of the pterin-peroxide is found endergonic by 8.0 kcal mol$^{-1}$, while formation of the peracid, which is coupled to decarboxylation of the cosubstrate, is highly exergonic (-41.1 kcal mol$^{-1}$). Furthermore, the full process of dioxygen activation generating Fe$^{IV}$=O is only 6.3 kcal mol$^{-1}$ exergonic in the hydroxylases compared with the 70.6 kcal mol$^{-1}$ exergonicity calculated in the α-KG-dependent enzymes.

A common aspect of the two mechanisms is the ‘reactive’ potential energy surface, which in both cases is characterized by a total spin S=2. It should be recalled that the quintet state is not the ground state for the reactant (i.e., the dioxygen-bound iron complex) as discussed in paragraph 3.4 and illustrated in table 3.1. However, the quintet is the ground state for the product, the high-valent iron-oxo species. A few words about the electronic structure of Fe$^{IV}$=O are compulsory at this stage of the discussion. It was observed that the Fe=O moiety parallels the electronic
structure of triplet dioxygen with two unpaired electrons occupying two antibonding orbitals of π* symmetry [91, 92], as illustrated in the qualitative orbital picture sketched in figure 4.3. When the other ligands of iron creates a weak ligand field, two more unpaired electrons are localized on the metal non-bonding orbital of δ symmetry yielding a spin population of about 3 on iron and about 1 on the oxo-ligand and a total spin S=2.

Both the energy profiles presented for dioxygen activation in the tetrahydrobiopterin-dependent hydroxylases and the α-KG-dependent enzymes are expressed with respect to the dioxygen-bound iron(II) complex. As amply discussed in paragraph 3.4, such a treatment neglects the entropic contribution due to the loss of translational and partially rotational entropy connected with the formation of the iron-O₂ adduct. When TΔS with respect to free O₂ is included to get the free energy change, too high an activation barrier for dioxygen activation is predicted. Nevertheless, energy pathways implicating lower barriers could not be located with the chosen model system. In the specific case of tetrahydrobiopterin-dependent enzymes, it was shown, for example, that a different structural arrangement of the carboxylate group of Glu286 might contribute to decrease the energy barrier of dioxygen activation.

4.4 A functional analogue

The reaction between O₂ and the synthetic non-heme iron complex [Fe-(Tp\text{Ph₂})BF] shown in figure 4.4 resembles the activity of α-KG-dependent enzymes, where the α-ketoacid (BF in the case of the synthetic complex) undergoes oxidative decarboxylation and the substrate (one of the aromatic rings of the Tp\text{Ph₂} ligand) is hydroxylated by activated oxygen.

\footnote{Tp\text{Ph₂} = hydrotris(3,5-diphenylpyrazol-1-yl)borate; BF = benzoylformate.}
Kinetic experiments by Que and coworkers allowed the measurement of the entropy and enthalpy of activation for this reaction\(^2\), providing the opportunity to compare the experimental data with the computed ones [93].

The calculations (Paper VI) support a reaction mechanism between O\(_2\) and [Fe(Tp\(^{Ph2}\))BF] which is similar to the one proposed for the \(\alpha\)-KG-dependent enzymes, where dioxygen activation implicates an Fe\(^{IV} = \text{O}\) species (figure 4.1). Similarly to the aromatic hydroxylation mechanism summarized in figure 3.8, the biomimetic iron(IV)-oxo intermediate is then found capable of performing an electrophilic attack at one of the aromatic rings of the Tp\(^{Ph2}\) ligand. The identification of the sequence of elementary processes shows that the rate-determining step of the reaction is associated with the reduction of dioxygen by BF (i.e., the keto-acid) coupled to oxidative decarboxylation and O–O bond cleavage. The computed energy barriers could then be compared to the available kinetic data, showing a remarkable agreement between the DFT calculations and the experiments (experiments: \(\Delta H = 6.0\) kcal mol\(^{-1}\), \(-T\Delta S = 12.8\) kcal mol\(^{-1}\); calculations: \(\Delta H = 10.0\) kcal mol\(^{-1}\), \(-T\Delta S = 11.3\) kcal mol\(^{-1}\)).

The investigation of the reaction between [Fe(Tp\(^{Ph2}\))BF] and dioxygen provided a direct and useful comparison between theory and experiments. The reactivity of BF was finely tuned by adding appropriate substituents in the phenyl ring as it was also done experimentally. In agreement with the experimental observations, the calculations established that electron-withdrawing groups (such as NO\(_2\)) lower the activation energy of the slow step (i.e., C–O bond formation coupled to O–O bond cleavage and decarboxylation).

\(^2\)Importantly, it was proposed that the collected experimental data of the reaction of the biomimetic complex refer to the oxidative decarboxylation of BF. It is useful to emphasize that the reaction between dioxygen and Tp\(^{Ph2}\) is not a catalytic cycle
Rieske non-heme iron dioxygenases are bacterial enzymes that initiate the degradation of aromatic hydrocarbons. The best studied Rieske dioxygenase is naphthalene 1,2-dioxygenase, which catalyzes cis-dihydroxylation of naphthalene to cis-dihydrodiol using molecular oxygen (figure 5.1). Full reduction of $\text{O}_2$ is achieved employing two more electrons supplied by an external source, namely, nicotinamide adenine nucleotide (NADH). Naphthalene 1,2-dioxygenase is a three-component enzyme, which accommodates a mononuclear non-heme iron(II) center in the catalytic domain located in the oxygenase component (hereafter the oxygenase component will be denoted NDO). The other two components of the enzyme, the reductase and the ferredoxin components, provide the electron transfer pathway, through which the two external electrons reach the active site. The ferrous ion in the catalytic domain is coordinated by the 2-His-1-carboxylate facial triad and a water molecule completes the metal first coordination sphere in the resting state of the enzyme.

### 5.1 Introduction

Dioxygenation of naphthalene occurs in the proximity of the mononuclear iron(II) center, which receives the two external electrons, required to complete catalysis, from the Rieske $[2\text{Fe-2S}]$ cluster hosted in the adjacent Rieske domain [94–96]. As shown in figure 5.1, the Rieske cluster is a binuclear iron site with one metal ion coordinated by two cysteine
residues, and one ion ligated by two histidines. The mononuclear iron(II) center and the Rieske cluster are connected via the carboxylate group of Asp205, which hydrogen bonds with one histidine ligand (His208) of the mononuclear complex and with one histidine ligand (His104) of the Rieske site. The Rieske cluster, where the two iron atoms are initially in the formal ferric oxidation state, controls the electron transfer process exploiting the Fe$^{III}$-Fe$^{III}$/Fe$^{III}$-Fe$^{II}$ redox couple.

The mechanism accounting for the remarkable chemical transformation catalyzed by naphthalene 1,2-dioxygenase is still under debate, but several mechanistic and structural studies lead to the proposal of the sequence of elementary processes depicted in figure 5.2. Substrate binding triggers dioxygen activation through a one-electron transfer from the reduced Rieske cluster to the mononuclear iron center, yielding an iron(II)-superoxo (i.e., iron(III)-peroxo) species. Subsequent protonation leads to an iron(III)-hydroperoxo intermediate, where the peroxo ligand binds in a side-on fashion to the metal. Only after dioxygenation is completed, the second external electron reaches the mononuclear complex with bound diol, regenerating the ferrous oxidation state of iron and facilitating release of the product. It should be noted that, if the second external elec-
5.1 Introduction

Figure 5.2: Proposed mechanism for dioxygen activation and naphthalene cis-dihydroxylation by NDO.

electron is supplied before substrate dioxygenation, an iron(II)-hydroperoxo intermediate will be formed. It is useful to mention some key experiments that helped to assemble this mechanistic picture. The involvement of only one external electron for dioxygen activation is suggested on the basis of single turnover studies showing that fully reduced NDO (i.e., the enzyme deprived of the other two components) is still capable of naphthalene dioxygenation [97]. During single turnover both the Rieske cluster and the mononuclear iron(II) site were oxidized, indicating that only one external electron (i.e., the electron located in the reduced iron-sulfur cluster) was sufficient to yield activated oxygen since the mononuclear iron(II) complex could provide the second reducing equivalent. Another important observation is derived from recent X-ray data that showed a dioxygen ligand binding in a side-on fashion to iron after displacement of a water ligand [94, 98].

In addition to cis-dihydroxylation, naphthalene 1,2-dioxygenase catalyzes a number of diverse reactions: monooxygenation and oxygen-dependent desaturation of benzocyclic and alkyl-substituted aromatic compounds, O- and N-dealkylation, stereospecific sulfoxidation of aryl alkyl sulfides [99]. This broad spectrum of reactions resembles the activity of cytochrome P450, which, however, catalyzes arene epoxidation instead of cis-dihydroxylation.

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1 In fully reduced NDO the Rieske cluster is in the FeIII-FeII form and the mononuclear iron complex contains FeII.
5.2 Dioxygen activation

Our investigation (Paper IV) of dioxygen activation by fully reduced NDO focuses on the electron transfer step from the reduced Rieske cluster to the mononuclear iron complex according to the following scheme:

\[
\begin{align*}
\text{Fe}^{III} + \text{Fe}^{II} + \text{O}_2 &\rightarrow \text{Fe}^{III} + \text{Fe}^{II} + \text{O}_2^- \\
\end{align*}
\]

A distance of only \(\sim 12 \, \text{Å}\) separates the mononuclear iron complex from the neighboring Rieske cluster, and therefore we could rather easily build a model of approximately 95 atoms including both metal centers (figure 5.3). We also performed calculations with smaller structures where either one of the two metal sites was present. These smaller models turned out to be useful to gain insight into the different effects influencing the energetics of the chemical transformation under investigation.

The calculations with the model depicted in figure 5.3 show that binding of molecular oxygen to iron(II) coupled with the one-electron transfer from the reduced Rieske cluster is exothermic by 9.9 kcal mol\(^{-1}\). If the reaction is decomposed into two subsequent steps (i.e., the dioxygen binding and the electron transfer steps) the computed exothermicity can be partitioned into two energy contributions. The first contribution is associated with the enthalpic energy change for O\(_2\) binding to iron(II), which, similarly to the tetrahydrobiopterin- and \(\alpha\)-KG-dependent enzymes (see table 3.2), is close to zero (\(\Delta E = -0.5\) kcal mol\(^{-1}\)). The second contribution arises from the difference between the ionization potential (IP) of the reduced Rieske cluster and the electron affinity (EA) of the dioxygen-bound iron(II) complex; this difference was calculated to be -9.4 kcal mol\(^{-1}\) as summarized in table 5.1.

A free energy change for the dioxygen activation process was obtained assuming that the only significant contribution to the entropic term originates from the binding of O\(_2\) to Fe\(^{II}\). The overall process leading to an iron(II)-superoxo species then becomes slightly endergonic (\(\Delta G = 1.1\) kcal mol\(^{-1}\), \(-T\Delta S = 11.0\) kcal mol\(^{-1}\))\(^2\). The computed free energy change of only 1 kcal mol\(^{-1}\) together with the estimate of a low activation barrier for the electron transfer process seems to indicate that the first step of

\(^2\)The entropy was obtained from a small cluster including only the mononuclear center.
5.3 Substrate cis-dihydroxylation

According to the proposed mechanism depicted in figure 5.2, hydroxylation of the substrate involves an iron(III)-hydroperoxo species, which
can be readily formed from the iron(II)-superoxo species when a proton is supplied by an external source. We did not derive the energetics associated with the protonation step because a detailed structural information on the proton source is not available. At this stage it should be noted that our investigation on the electron transfer process described above is based on a structural model where the water ligand was maintained in the first coordination sphere of the mononuclear complex during binding of O₂. However, on the basis of the crystal structure with bound dioxygen, the water was not included in the metal first coordination sphere of the ferric-hydroperoxo complex in the investigation of the substrate oxidation mechanism.

As suggested by the available X-ray data, a side-on hydroperoxo species might be formed and the possible fates of this intermediate are sketched in figure 5.4. It may undergo O–O bond cleavage leading to HOFeV=O, which then carries out substrate dihydroxylation. Alternatively, the ferric-hydroperoxide directly attacks the substrate yielding the dihydrodiol. A third possible route implicates a second electron transfer before substrate...
5.4 Binding of NO to iron(II)

Nitric oxide (NO) is often used as surrogate for O₂ to gain structural insights into the enzyme active site in the absence of turnovers. Therefore, it is interesting to compare the properties of the Fe^{II}-O₂ and the Fe^{II}-NO complexes. In the case of naphthalene 1,2-dioxygenenase, signals of a nitrosyl adduct arise in the EPR spectra of resting NDO upon exposure...
Figure 5.5: Energetics (ΔE) for NO and O₂ binding to Fe^{II} as derived from three different models of NDO (entropy contribution is not included). Electron affinities (EA) of the corresponding adducts are also shown.

to NO [97]. Under exposure of O₂ no corresponding dioxygen-bound complexes are detected. In analogy to the investigation performed for [Fe^{II}O₂]^{2+}, the binding energy of NO to Fe^{2+} is computed using different models: the large model shown in figure 5.3 and two smaller models including only the mononuclear iron(II) site. The iron(II)-NO adduct is considered in the quartet spin state (S=3/2) and the water ligand is maintained in the first coordination sphere. Figure 5.5 compares the computed energetics for the binding of the two small molecules as derived from the three different models. In all the three cases nitric oxide tends to bind stronger to Fe^{II} than dioxygen. The carboxylate group of Asp205 placed in the metal second coordination sphere favors the binding of NO or O₂ as also observed for hemerythrin [88]. If the entropic term TΔS is not included, the large model predicts that NO is bound to iron(II) by 6.5 kcal mol⁻¹ compared with a binding energy of only 0.5 kcal mol⁻¹ for O₂.

Employing the large model depicted in figure 5.3, we also probed whether the NO-adduct is able to trigger the one-electron transfer from the reduced Rieske cluster, as it was previously proposed for the dioxygen

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5 Resting NDO contains the diferric Rieske cluster and the ferrous mononuclear iron complex.
6 The quartet spin state corresponds to the four unpaired d-electrons on Fe^{II} anti-ferromagnetically coupled to the unpaired electron on NO.
activation process. Addition of one electron to the iron(II)-NO adduct leads to a triplet ground state (S=1) for the mononuclear center, where the metal can be considered in the ferrous oxidation state and two unpaired electrons are located in the NO-ligand (the corresponding spin distribution is: Spin(Fe)=3.59 and Spin(NO)=1.89). The electron transfer coupled to NO binding is computed exothermic by 3.8 kcal mol$^{-1}$, which should be compared to the exothermicity of 9.9 kcal mol$^{-1}$ calculated for the analogous process with molecular oxygen. As shown in the energy profiles of figure 5.6, the iron(II)-NO adduct is more stable than its corresponding reduced form by 2.7 kcal mol$^{-1}$, while the dioxygen-bound complex is instead stabilized by the one-electron transfer. The difference simply arises from the computed electron affinities of the mononuclear iron complex with bound NO or O$_2$. With dioxygen bound to Fe$^{II}$, the EA of the mononuclear complex is higher than the IP of the reduced Rieske cluster (81.9 kcal mol$^{-1}$), in contrast to the nitrosyl adduct which has a lower EA as reported in figure 5.5.

The energetics discussed above indicate that binding of NO can occur despite the oxidation state of the Rieske cluster. It might be argued that the computed binding energy for NO is not sufficient to balance the entropy loss due to the formation of the adduct. With one small model having all the coordinates released, the entropy term was in fact evaluated to be -$T\Delta S = 11.6$ kcal mol$^{-1}$ with respect to free NO. Stabilization effects due to the protein environment might justify the experimental evidence from EPR spectroscopy and X-ray crystallography that a nitrosyl
adduct is formed. The computed binding energy of molecular oxygen is about 6 kcal mol\(^{-1}\) lower, which should account for the observation that a dioxygen-bound iron(II) complex is not detected when the Rieske cluster is in the oxidized state\(^7\).

5.5 Monooxygenation and sulfoxidation

Naphthalene dioxygenase is found capable of catalyzing other reactions than cis-dihydroxylation (e.g., monohydroxylation and sulfoxidation), raising the question on the mechanism of dioxygen activation with alternative substrates. Similarly to the three pathways summarized in figure 5.4, the activated oxygen species that oxidizes the alternative substrates might be HO-Fe\(^V\), or HO-Fe\(^{IV}\), or the ferric-hydroperoxide. Since we proposed that the iron(V)-oxo route is unlikely due to the high activation barrier associated with the O–O bond cleavage, the iron(IV)-oxo route or the direct attack of the ferric-(hydro)peroxo species remains to account for the diverse reactions catalyzed by naphthalene 1,2-dioxygenase. The investigation on tetrahydrobiopterin-dependent hydroxylases showed that benzylic-type hydroxylation by an Fe\(^{IV}=O\) core is energetically feasible through a rebound mechanism. Consequently, if the second external electron is available in NDO, the iron(II)-hydroperoxo species may be formed and undergo O–O bond lysis yielding the iron(IV)-oxo intermediate, which would account at least for the observed monooxygenation reactions. Would the (hydro)peroxo ligand be able to attack directly the substrate leading to the oxidized products (e.g., alcohol or sulfoxide)? In order to answer this question, we investigated the reaction mechanism of monooxygenation and sulfoxidation by the ferric-(hydro)peroxo complex\(^8\).

**Monooxygenation by a ferric-peroxo species.** We studied whether a ferric-peroxo (i.e., ferrous-superoxo) complex may hydroxylate benzocyclic and alkyl-substituted aromatic compounds using toluene as probe. The mechanism was investigated following a rebound-like pattern, which

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\(^7\)According to EPR spectra of NDO, formation of a nitrosyl adduct is not observed when the Rieske cluster is in the reduced state and the substrate is not present in the active site [97]. This type of behavior is justified if in these conditions geometrical constraints prevent the access of NO to the active site.

\(^8\)The following results refer to preliminary calculations that need further refinements. Enthalpic energy changes are provided.
5.5 Monooxygenation and sulfoxidation

Figure 5.7: a) Computed energetics (in kcal mol\(^{-1}\)) for the first step of toluene monohydroxylation by \([\text{Fe-O}_2]^+\). b) The transition state corresponding to the hydrogen atom abstraction step; computed spin populations and relevant geometrical parameters (in Å) are shown.

starts with a hydrogen atom abstraction by the superoxo ligand from the methyl group of the substrate as illustrated in figure 5.7a. The corresponding transition state shown in figure 5.7b lies quite high in energy (\(\Delta E^\dagger = 25.3\) kcal mol\(^{-1}\)) with respect to the reactant\(^9\). The computed energy change for the hydrogen atom abstraction step of 15.5 kcal mol\(^{-1}\) corresponds to the difference between the C–H and O–H bond strengths of the methyl group in toluene and of the ferric-hydroperoxide, respectively. The computed bond dissociation energies (BDE) are: BDE = 70.8 kcal mol\(^{-1}\) for \([\text{Fe-OOH}]^+\) and BDE = 85.6 kcal mol\(^{-1}\) for the substrate. The subsequent step following the hydrogen atom abstraction is the formation of the C–O bond step coupled to the O–O bond cleavage yielding the alcoholate bound to Fe\(^{III}\). Since the computed activation energy of the first step is already rather high for the enzymatic reaction, the details of the final step have not yet been investigated.

Monooxygenation by a ferric-hydroperoxo species. The hydroxylation reaction was studied with the ferric-hydroperoxo species and toluene as reactant. The reaction mechanism investigated for this type of transformation involves an initial hydrogen atom abstraction by the hydroperoxo ligand occurring concomitantly with O–O bond cleavage. The hydrogen atom abstraction may be promoted by the protonated oxygen of the per-

\(^{9}\) The reactant comprises the iron complex (S=5/2) and the substrate in the second coordination sphere
Figure 5.8: Two possible mechanisms for toluene monohydroxylation by [Fe-OOH]^{2+} (a and c) and the corresponding transition states for the hydrogen atom abstraction coupled to O–O bond cleavage (b and d). Computed spin populations and relevant geometrical parameters (in Å) are reported together with enthalpic energy changes (in kcal mol$^{-1}$).

oxide as shown in figure 5.8a, yielding a water molecule$^{10}$. Alternatively, the unprotonated oxygen of the hydroperoxide abstracts the hydrogen from the substrate during the O–O bond cleavage (figure 5.8c). The computed energies of the corresponding transition states (figure 5.8) suggest that the latter route is favored by about 7 kcal mol$^{-1}$ ($\Delta E^1 = 20.7$ kcal mol$^{-1}$). For both pathways the subsequent intermediate corresponding to a benzylic radical and an iron(IV) moiety is not stable and the two

$^{10}$A similar mechanism was proposed for the hydrogen atom abstraction by a ferric complex in bleomicyn [57].
transition states directly lead to the monohydroxylated substrate bound to the metal complex.

**Sulfoxidation by a ferric-hydroperoxo species.** Paralleling the investigation of the monohydroxylation reaction, we studied the sulfoxidation mechanism of methyl phenyl sulfide by the ferric-hydroperoxo species. As in the monohydroxylation pathway, the O–O bond cleavage occurs concomitantly with the formation of another bond, in this case the S–O bond as illustrated in figure 5.9. This step leads directly to the sulfide and requires a relatively low activation barrier (ΔE‡ = 9.2 kcal mol⁻¹).

**Fe^{III}OOH or Fe^{IV}=O?** The reaction pathway for monohydroxylation and sulfoxidation involving the direct attack of Fe^{III}-OOH on the substrate cannot be excluded on the basis of the computed energy barriers, which are not found prohibitively high. It can be observed that the lowest energy barrier computed for the monohydroxylation reaction of toluene (ΔE‡ = 20.7 kcal mol⁻¹) is on the limit for regarding the enzymatic reaction energetically feasible. This observation poses the question whether the iron(IV)-oxo species is instead responsible for the monohydroxylation reaction of toluene and perhaps also for the sulfoxidation reaction. Interestingly, α-KG-dependent enzymes not only catalyze alkyl hydroxylation but also sulfoxidation. It was already discussed how for these

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11The sulfoxidation reaction by Fe^{IV}=O has not yet been investigated.
enzymes the involvement of an iron(IV)-oxo intermediate is supported by experiments and calculations\textsuperscript{12}.

\textsuperscript{12}It would be interesting to test experimentally if fully reduced NDO is able to carry out monohydroxylation of toluene or sulfoxidation of methyl phenyl sulfide in single turnovers as it was observed for dioxygenation of naphthalene. The calculations predict that sulfoxidation is feasible but some uncertainties remain on monohydroxylation.
Biomimetic non-heme iron catalysts

Biomimetic studies aim at identifying catalysts able to resemble the efficiency and selectivity of enzymes. Among functional analogues mimicking the chemistry of mononuclear non-heme iron enzymes, the TPA (tris(2-pyridylmethyl)amine) family showed interesting catalytic activity, being able to carry out stereospecific alkane hydroxylation as well as olefin cis-dihydroxylation and epoxidation. The catalytic properties of the biomimetic complexes are finely tuned by the ligand topology as clearly exemplified by two representatives of the TPA family, which will be discussed below: $[\text{Fe}^{II}(\text{TPA})(\text{CH}_3\text{CN})_2]^{2+}$ and $[\text{Fe}^{II}(6-\text{Me}_3\text{-TPA})-(\text{CH}_3\text{CN})_2]^{2+}$ (figure 6.1).

6.1 Introduction: the experiments

In contrast to enzymes which are capable of dioxygen activation, the TPA family employs hydrogen peroxide as oxidant. In the particular case of the TPA ligand, a ferric-hydroperoxo species has been trapped, suggesting that $\text{H}_2\text{O}_2$ initially oxidizes the ferrous complex generating the actual ferric catalyst. With the 6-Me$_3$-TPA ligand, only the corresponding fer-

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1 Our theoretical investigations on the catalytic activity of synthetic non-heme iron complexes are related to the experimental work of Que and coworkers at the University of Minnesota.

2 Initial consumption of $\text{H}_2\text{O}_2$ without product formation is observed with the TPA complex.
ric alkyl peroxide has been characterized, but, by analogy to the TPA complex, it is proposed that a 6-Me₃-TPA-ferric-hydroperoxo species is formed prior to catalysis. From table 6.1, which reports a summary of some relevant experimental observations describing the behavior of TPA and 6-Me₃-TPA, the following aspects should be emphasized:

a) The two catalysts generate ferric intermediates, namely, [Fe⁵⁺(TPA)(OOH)]²⁺ or [Fe⁵⁺(TPA)(OOtBu)]²⁺ and [Fe⁵⁺(6-Me₃-TPA)(OOtBu)]²⁺, which have a low-spin ground state with TPA and a high-spin ground state with 6-Me₃-TPA.

b) 6-Me₃-TPA is likely to involve long-lived alkyl radicals in the alkane hydroxylation pathway. A more selective metal-based oxidant instead explains the stereospecificity observed with TPA for the same reaction.

c) The cis-diol obtained from olefin oxidation by TPA contains one oxygen atom originating from solvent water and one oxygen atom from hydrogen peroxide. In contrast, the 6-Me₃-TPA catalyst yields a cis-diol with both oxygen atoms originating from H₂O₂. Notably, the latter pattern resembles the type of labeling observed in the enzymatic cis-dihydroxylation by naphthalene 1,2-dioxygenase discussed in the previous chapter.

The mechanistic scheme sketched in figure 6.2a was proposed by Que and coworkers to account for the catalytic behavior of the TPA ligand. Initially [Fe²⁺(TPA)(CH₃CN)]²⁺ is oxidized to a low-spin ferric precursor, where the two cis-labile sites are occupied by the hydroperoxo ligand and a water molecule (figure 6.1a). Subsequent O–O bond heterolysis yields the electrophilic HO-Fe⁵⁺=O species, which may carry

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3More details concerning the experiments can be found in Ref. [67] (alkane hydroxylation), Ref. [68] (olefin oxidation) and Ref. [69] (short review).
6.1 Introduction: the experiments

**Table 6.1: Relevant experimental observations concerning catalysis by [Fe^{II}(TPA)(CH3CN)2]^{2+} and [Fe^{II}(6-Me3-TPA)(CH3CN)2]^{2+}**.

<table>
<thead>
<tr>
<th>Low spin state</th>
<th>High spin state</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA</td>
<td>6-Me3-TPA</td>
</tr>
<tr>
<td>- [Fe^{II}(TPA)(CH3CN)2]^{2+}, [Fe^{II}-(OH)]^{2+} and [Fe^{II}(OO'Bu)]^{2+} prefer a low-spin ground state.</td>
<td>- [Fe^{II}(6-Me3-TPA)(CH3CN)2]^{2+} and [Fe^{II}(OOG'Bu)]^{2+} prefer a high-spin ground state.</td>
</tr>
</tbody>
</table>

**Stereospecific alkane hydroxylation**
- Oxidation of cyclohexane gives ~30% of solvent water incorporation in the product (i.e., cyclohexanol).
- Short-lived alkyl radicals (if any) are involved.

**Alkane hydroxylation**
- Oxygen originating from O2 is incorporated into the alcohol product.
- Long-lived alkyl radicals are involved.

**Olefin oxidation**
- The cis-diol contains one oxygen atom originating from solvent H2O and one oxygen coming from H2O2.
- The epoxide contains a small percentage of oxygen originating from H2O.
- Diol:Epoxide ratio from cyclooctene oxidation is 1.2:1.
- Oxidation of electron-deficient olefins results in cis-dihydroxylation, but no epoxidation.
- Competition experiments show a preference for oxidation of electron-rich olefins over oxidation of electron-deficient olefins.

**Alkaline hydroxylation**
- The cis-diol contains both oxygen atoms originating from H2O.
- The epoxide contains a significant amount of oxygen originating from O2.
- Diol:Epoxide ratio from cyclooctene oxidation is 7:1.
- Oxidation of electron-deficient olefins results in cis-dihydroxylation, but no epoxidation.
- Competition experiments show a preference for oxidation of electron-deficient olefins over oxidation of electron-rich olefins.

*A significant amount of incorporation of molecular oxygen is symptomatic of the presence of long-lived alkyl radicals. Recent experiments seem to indicate that the diole is more favored than initially reported.*

out stereospecific alkane hydroxylation as well as epoxidation and cis-dihydroxylation of olefins. Importantly, the hydroxo ligand of the high-valent iron-oxo species is originating from water, and therefore it provides the possible source of water incorporation in the products. In scheme 6.2a, the o xo-hydroxo tautomerization was for example suggested to explain the observations from the labeling experiments of alkane hydroxylation and olefin epoxidation. The chemical pattern explaining the chemistry of the high-spin 6-Me3-TPA is still rather elusive. As mentioned above, by analogy to the TPA case, the formation of a ferric-hydroperoxo precursor is suggested, but an analogous iron(V)-oxo route proposed for TPA cannot explain the experimental outcomes. It was already mentioned that the 6-Me3-TPA complex resembles the mononuclear iron site of naphthalene 1,2-dioxygenase in the nature of catalysis, yielding iden-
6 Biomimetic non-heme iron catalysts

Figure 6.2: Proposed mechanisms describing the catalysis of $\text{Fe}^{III}(\text{TPA})-(\text{CH}_3\text{CN})_2]^2+ (a)$ and $[\text{Fe}^{III}(6\text{-Me}_3\text{-TPA})(\text{CH}_3\text{CN})_2]^2+ (b)$.  

It has been proposed that the reactivity of the TPA and 6-Me$_3$-TPA complexes is controlled by the different spin states of the two ferric-hydroperoxo precursors. While spectroscopic techniques indicated that the TPA-Fe$^{III}$-OOH species has a low-spin ground state, the 6-Me$_3$-TPA ligand should favor the high-spin state as inferred from the characterization of $[\text{Fe}^{II}(6\text{-Me}_3\text{-TPA})(\text{CH}_3\text{CN})_2]^2+$ and $[(6\text{-Me}_3\text{-TPA})-\text{Fe}^{III}-\text{OO}^t\text{Bu}]$. We have optimized the corresponding geometries of the two ferric-hydroperoxo precursors (figure 6.1) exploring three relevant spin
6.3 The O–O bond cleavage

states: $S=5/2$, $S=3/2$ and $S=1/2$. Our DFT calculations, described in Paper IX, predict that in the case of the methylated ligand the ground state is a sextet, corresponding to a weak ligand field. The low-spin state lies about 10 kcal mol$^{-1}$ higher in energy. In contrast, the TPA ligand stabilizes the low-spin state, making the sextet and the doublet near-degenerate. Interestingly, the thermal effects play a significant role in determining the actual ground-state of TPA-Fe$^{III}$-OOH, and at a very low-temperature (e.g., the temperature at which this species has been experimentally characterized) the doublet state is favored by about 3 kcal mol$^{-1}$. In light of the experimental observations concerning the spin states of these complexes, it can be concluded that the chosen hybrid DFT functional is able to qualitatively predict the stability of the different spin states. Similar conclusions were previously drawn from an analogous investigation on parental biomimetic iron catalysts, where the spin splittings were evaluated employing different computational methods [100]. On the basis of the observation that fairly accurate spin splittings for transition metal compounds are derived from an optimum exact exchange of ca. 15%, we also tested a modified B3LYP functional, confirming the stability of the sextet for the 6-Me$_3$-TPA complex and of the doublet for the TPA species.

6.3 The O–O bond cleavage

The stereospecificity of the reactions catalyzed by the TPA complex suggests that a metal-based oxidant is produced along the catalytic cycle. An attractive candidate is the iron(V)-oxo species generated by the O–O bond heterolytic cleavage of the peroxo ligand as shown in scheme 6.2a. We probed whether the O–O bond cleavage was a viable transformation and whether the high-valent iron-oxo species was a feasible intermediate (Paper VII). A free activation energy of about 19 kcal mol$^{-1}$ was computed for the heterolysis, while from the thermodynamic point of view the reaction was found to be endergonic by 5 kcal mol$^{-1}$. These energetics demonstrate that the iron(V)-route is possibly describing catalysis by TPA. Interestingly, the corresponding transition state for the methylated

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$^4$In these studies by Morokuma and coworkers the energies of the low-spin and the high-spin states were extrapolated from CCSD(T) and MP2 calculations [100].

$^5$As already mentioned in the introductory chapter, an accurate prediction of spin splittings for transition metal compounds is not an easy task.
ligand requires an energy barrier of about 29 kcal mol\(^{-1}\) with respect to the corresponding ferric reactant shown in figure 6.1b. Given that the O–O bond cleavage involves the doublet state for both ligands, the higher energy barrier found for the 6-Me\(_3\)-TPA ligand can be related to the destabilization of the low-spin state by the methyl substituents\(^6\).

Concerning the product of the cleavage, it should be noted that the molecular orbital scheme reported in figure 4.3 can still be employed to describe the Fe\(^V\)=O moiety, which is found to have a quartet ground state (S=3/2). Compared to Fe\(^IV\)=O, Fe\(^V\)=O is simply deprived of one electron from one of the \(\delta\)-orbitals and it is therefore not surprising that the computed Fe–O bond distances in the iron(IV)- and iron(V)-oxo species are very similar (1.65-1.66 Å). The three unpaired electrons occupying two \(\pi^*\) orbitals and one \(\delta\) orbital account for the quartet ground state.

### 6.4 Alkane hydroxylation

The capability of the (TPA)(OH)Fe\(^V\)=O intermediate of catalyzing alkane hydroxylation\(^7\) is analyzed following the rebound paradigm as exemplified in the following scheme with methane as substrate (Paper VIII)\(^8\):

![Alkane hydroxylation reaction](image)

Alkane hydroxylation starts with a hydrogen atom abstraction by the oxo group yielding an alkyl radical, which is subsequently trapped by the metal complex giving the alcohol. In the case of methane hydroxylation, the hydrogen atom abstraction generates the HO-Fe\(^IV\)-OH species and the methyl radical, which can then form a new C–O bond with one of the two hydroxo groups. The rebound step is found to involve a very low barrier, thus leading to retention of configuration at the carbon. When the secondary C–H bond of propane is abstracted, the calculations indicate

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\(^6\)Notice that the increase of the barrier by 10 kcal mol\(^{-1}\) upon methylation parallels the destabilization of the doublet state in the corresponding ferric reactants as explained in the previous paragraph.

\(^7\)We have not explored alkane hydroxylation by the 6-Me\(_3\)-TPA catalyst.

\(^8\)Notice that methane is not a substrate used in the experiments; however, due to the numerous studies of methane hydroxylation by, for example, cytochrome P450 or methane monooxygenase (MMO), its study was worthwhile.
that the propyl radical is not a stable intermediate, since the nascent propyl radical immediately reacts with one of the two hydroxo ligands. Again, the rebound process is computed to be fast, thus accounting for stereospecific alkane hydroxylation, as observed in the experiments\textsuperscript{9}. It is useful to note that both the hydroxo ligands of the nascent HO-Fe\textsuperscript{IV}-OH species are found to be involved in the rebound process, explaining the incorporation of oxygen from water in the alcohol.

### 6.5 Acetonitrile hydroxylation

The computed energy profiles for methane and propane hydroxylation indicate that the hydrogen atom abstraction from methane requires a higher activation energy than the one for the abstraction of the weaker secondary C–H bond of propane. If the C–H bond strength alone drives the abstraction step by the iron(V)-oxo species, it would be expected that acetonitrile (i.e., the solvent used in the experiments) is hydroxylated by the biomimetic species. The C–H bond strength of acetonitrile is indeed very similar to the computed bond dissociation energy (BDE) of the secondary C–H bond of propane as reported in table 6.2. In order to shed light on the possible interaction between the iron(V)-oxo intermediate and the solvent, the rebound mechanism is probed with acetonitrile as substrate. A slightly larger model than the one employed with propane and methane had to be used since acetonitrile tends to hydrogen bond with the hydroxo group of HO-Fe\textsuperscript{V}=O. Two molecules of CH\textsubscript{3}CN are therefore included in the model: one representing the substrate and one hydrogen bonding to the hydroxo ligand of HO-Fe\textsuperscript{V}=O. This type of model prevents the hydrogen bond between the substrate and the oxidant to affect the energetics of the rebound process. Therefore, the computed activation energies for the hydrogen atom abstraction and for the rebound step can be directly compared with the corresponding energies for methane and propane hydroxylation.

Although the C–H bond strengths of propane and acetonitrile are very similar, the computed activation energies for the hydrogen atom abstraction by the oxo-group differ by more than 10 kcal/mol: 7.0 kcal/mol is the energy barrier computed for propane and 18.3 kcal/mol the one for acetonitrile (table 6.2). The transition state associated with the hydrogen

\textsuperscript{9}Hydroxylation of propane to 2-propanol is used in the calculations to mimic the hydroxylation of, for example, cyclohexane.
Table 6.2: The computed C–H bond dissociation energies (BDE) of methane, propane (secondary C–H bond) and acetonitrile. Activation and reaction energies corresponding to the hydrogen atom abstraction step by HO–Fe$^{V}=\text{O}$ are reported.

<table>
<thead>
<tr>
<th></th>
<th>CH$_4$</th>
<th>H$_3$C-CH$_2$-CH$_3$</th>
<th>H$_3$C-CN</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE$^a$ (kcal/mol)</td>
<td>101.6</td>
<td>92.7</td>
<td>92.4</td>
</tr>
<tr>
<td>$\Delta G^\ddagger$ (kcal/mol)</td>
<td>17.0</td>
<td>7.0</td>
<td>18.3</td>
</tr>
<tr>
<td>$\Delta G$ (kcal/mol)</td>
<td>5.2</td>
<td>-</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

$^a$ BDE are corrected for the solvent effects ($\epsilon = 36.64$).

Figure 6.3: a) The transition state for the hydrogen atom abstraction from acetonitrile (S=3/2). Relevant bond distances (in Å) and spin populations are reported. b) The energy diagram for acetonitrile hydroxylation by HO–Fe$^{V}=\text{O}$ (S=3/2).

atom abstraction from CH$_3$CN (S=3/2) is shown in figure 6.3a, where relevant bond distances and spin populations are reported. This structure subsequently decays to a stable radical, where the electron is delocalized over the cyano group as already inferred from the computed spin population of the transition state. The computed energetics for the entire rebound process are displayed in figure 6.3b.

It should be noted that the hydrogen atom abstraction from CH$_3$CN requires an activation energy comparable to the one obtained for methane. Also the C–H and O–H bond distances of the transition state shown in fig-
Figure 6.4: Energetics for a probe hydrogen atom abstraction step with three different substrates. B3LYP (bold) and G2MS (italic) activation energies are compared.

Figure 6.3a resemble the corresponding ones of methane rather than the ones of propane. However, the energy change of the hydrogen atom abstraction step yielding a radical on the substrate and the iron(IV)-hydroxo species parallels the C–H BDE as derived from the computed energies gathered in table 6.2. A comparison with the propyl radical cannot be made since the corresponding intermediate following the hydrogen atom abstraction from propane is not stable and evolves directly to the hydroxylated product. The calculations show that the activation energies of the hydrogen atom abstraction from alkanes correlate with the corresponding C–H bond strengths; the energy barrier of the same step with acetonitrile as substrate instead does not depend entirely on the C–H bond strength but it is strongly affected by the electron withdrawing cyano group.

The three substrates, methane, propane and acetonitrile, give rise to an equivalent energy pattern when a simpler hydrogen atom abstraction reaction is explored. More specifically, the abstraction by the hydroperoxyl radical was investigated and the corresponding energetics are summarized in figure 6.4. Again, the activation energy for the hydrogen atom abstraction from acetonitrile is more similar to the corresponding energy for methane than that for propane. On the other hand, the free energy changes of the reaction match the trend of the computed C–H BDE of the three substrates. The computed energy difference for acetonitrile is somewhat lower than the one for propane because the product (i.e., the radical and hydrogen peroxide) is characterized by a new hydrogen bond.
between the cyano group and \( \text{H}_2\text{O}_2 \).

This small model system is used to compare the performance of B3LYP with a different computational method, and the G2MS extrapolation scheme [101] is employed to derive the activation energies for the hydrogen atom abstraction from methane, propane and acetonitrile. These calculations confirm the B3LYP energy pattern showing again that the energy barrier for the hydrogen atom abstraction for \( \text{CH}_3\text{CN} \) is comparable to the one for \( \text{CH}_4 \). Because the difference between the two methods is somewhat larger for acetonitrile (figure 6.4), the more accurate G2 calculation [102] is performed for this substrate, yielding an activation energy of 22.9 kcal/mol, which should be compared to 20.3 kcal/mol obtained from the B3LYP method. This simple reaction corroborates the conclusions obtained from the study of the hydrogen atom abstraction by the high-valent iron-oxo species, showing that the thermodynamic and kinetic profiles of the hydrogen atom abstraction for methane and propane correlate with the corresponding BDE. The electron withdrawing group of acetonitrile instead makes the corresponding activation energy resemble the one computed for methane, although its C–H bond strength is similar to the one of the secondary C–H bond of propane. This result confirms the available experimental data suggesting that hydroxylation of acetonitrile is not a competing reaction during the hydroxylation of secondary alkanes.

### 6.6 Olefin oxidation

Our DFT calculations show that the HO-Fe\(^V\)=O species generated with the TPA ligand might react with an olefin (we have used \textit{cis}-2-butene and 1-propene as test molecules) through two different channels: olefin

**Figure 6.5:** The two channels describing the reactivity of HO-Fe\(^V\)=O toward olefins.
oxidation can start with the attack of the oxo group or alternatively with the attack of the OH ligand as illustrated in figure 6.5 (Paper VIII). The route which is initiated by the electrophilic attack of the oxo-group on the olefin yields the epoxide (pathway a of figure 6.5). The second possible route involving the hydroxo group instead leads to the diol (pathway b of figure 6.5). For both channels, the first step, which implicates the formation of a new C-O bond, is found rate-limiting for the oxidation process of the substrate. The energy barrier associated with the second C-O bond leading to the epoxide or the diol is estimated to be rather low. The radical intermediate is short-lived since it is readily quenched by one of the two oxygens. Consequently, the two pathways would lead to olefin oxidation stereospecifically. It should also be noted that the diol produced through the hydroxo channel properly accounts for the incorporation of oxygen from H$_2$O and H$_2$O$_2$.

Analogous reaction pathways cannot provide an explanation of the labeling pattern observed in the cis-dihydroxylation reaction with 6-Me$_3$TPA. Furthermore, it was already pointed out that the O-O bond heterolysis with the methylated ligand is likely to be precluded by a rather high activation energy. On the basis of the mechanism proposed for the enzymatic cis-dihydroxylation by naphthalene 1,2-dioxygenase, we investigated an equivalent reaction mechanism, which implies the attack of the side-on hydroperoxo at the olefin double bond concertedly with the O-O bond cleavage, yielding an epoxide. In principle, this pathway is possible.
for both ligands, TPA and 6-Me$_3$-TPA, since the corresponding side-on hydroperoxo species do not lie prohibitively high in energy compared to the corresponding end-on structures of figure 6.1. The computed energetics indicate that the one-step epoxidation resembling the enzymatic reaction mechanism is a possible pathway also in the catalytic cycle of the two synthetic catalysts. However, in the case of TPA the epoxidation through the iron(V)-oxo route is energetically favored.

While the epoxide formed in the enzyme may evolve toward the diol through a carbocation intermediate, the epoxidation by the synthetic catalysts is followed by the release of the epoxide because the dissociation of the product in the biomimetic case is energetically preferable over the epoxide-to-diol conversion. Figure 6.6, where a comparison between the epoxide-to-diol conversion in the enzyme and in the functional analogue is shown, highlights the different energetics and the different mechanisms describing the formation of the diol from the bound epoxide$^{10}$. The main difference between the two systems is the carbocation intermediate (i.e., a ferric-intermediate with the bound carbocation), which is easily formed in the enzyme, but which is absent with TPA. In the biomimetic case, only a species corresponding to a radical bound to Fe$^{IV}$ could be obtained, but it lies rather high in energy and it is not involved in the epoxide-to-diol conversion. One reason behind the different chemical behavior of the two systems may be simply connected with the substrate used as probe molecule in the calculations: butene is employed in the studies of the synthetic complexes, while naphthalene is used for NDO. The latter substrate provides a resonant stabilization of the carbocation, justifying the low-energy pathways found for the epoxide-to-diol conversion in the enzyme$^{11}$.

Finally, it should be recalled that with 6-Me$_3$-TPA the epoxide is not the main product of olefin oxidation. How the cis-diol with the biomimetic high-spin catalyst is formed remains unclear.

$^{10}$Figure 6.6 shows the free energy changes for the synthetic catalyst; the entropy effects are not included in the energy profile of the enzyme. Despite that, the direct comparison between the diagrams is still meaningful since the thermal effects do not significantly contribute to determine the overall shape of the profile. Furthermore, the diagram reports the epoxide-to-diol conversion as determined from the study of the TPA catalyst. Preliminary calculations indicate that a similar behavior arises for 6-Me$_3$-TPA.

$^{11}$It would be interesting to investigate experimentally and theoretically the reactivity of TPA with naphthalene. The substrates used in the experiments are, for example, cyclooctene, 1-octene and styrene.
Concluding remarks and summary

Max Planck formulated quantum theory one hundred years ago, but only very recently quantum mechanical calculations have acquired real importance in chemistry\(^1\). In general the use of computational methods in the investigation of chemically interesting problems has grown exponentially thanks to the development of efficient computational algorithms and to the tremendous technological progress, which is providing fast computers at relatively low cost. Nowadays, computational chemistry, which is playing a vivacious role in the development of modern chemistry, is currently employed as an efficient tool to model, interpret, rationalize, and possibly predict chemical behaviors. In Nature most of the chemical transformations of interest involve systems of large size, as in the bioinorganic chemistry of enzymes and synthetic catalysts discussed in this thesis. Fortunately, the modern computational facilities permit quantum mechanical (QM) models counting on the average 70-80 atoms, which should provide a sufficient description of the chemical problem under investigation\(^2\).

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\(^1\)In the XIX century, when chemistry started to settle its foundations (it is in the XIX century, for example, that Faraday formulated the laws of electrolysis, Mendeleev arranged the known elements in the periodic table and Pasteur resolved stereoisomeric mixtures), the French philosopher Auguste Comte wrote that "every attempt to refer chemical questions to mathematical doctrines must be considered, now and always, profoundly irrational, as being contrary to the nature of the phenomena" [103].

\(^2\)Modern computational facilities include not only the facilities offered by national supercomputer centers but also the low-cost desktop computers. The Swedish national
The quantum chemical calculations presented in this thesis attempt to elucidate the elementary processes accounting for dioxygen activation and the subsequent oxidative reactions in the mononuclear non-heme iron oxygenases. The identification of the reaction mechanism is indispensable to provide the description of the enzymatic reactions from the thermodynamic and kinetic point of view. The free energy change of a chemical process indicates whether it is energetically favored, while the activation free energy obtained from the energy of the corresponding transition states determines the rate of the reaction. Sometimes a chemical transformation might be thermodynamically favored, but the high activation barrier prevents it to occur. We have explored how enzymes activate dioxygen and carry out the oxidative reactions by calculating the thermodynamic and the kinetic quantities for different possible mechanisms, and on the basis of these key quantities the mechanisms were discarded or suggested as possible viable pathways. It should be noted that the theoretical investigations undertaken to shed light on the catalytic activity of mononuclear non-heme iron enzymes try to account for the numerous experimental observations collected by means of various techniques (e.g., X-ray crystallography, EPR, NMR, isotope labeling).

The studies on tetrahydrobiopterin-dependent hydroxylases and α-ketoglutarate-dependent enzymes highlight a common strategy for the dioxygen activation step, which in both cases results in the formation of a high-valent iron-oxo species (Fe$^{IV}$=O). The process implies that one oxygen atom of O$_2$ is temporarily reduced by iron(II), while the other oxygen atom is reduced by the cofactor/cosubstrate. The involvement of a high-valent iron-oxo species and more specifically an iron(V)-oxo intermediate is instead excluded on the basis of the computed energetics for naphthalene 1,2-dioxygenase. For this enzyme we propose an alternative reaction pathway where the O–O bond cleavage is concerted with the oxidation of the substrate. On the other hand, we find that the iron(V)-oxo species is still a possible intermediate with a low-spin biomimetic complex, where iron is coordinated by the TPA ligand.

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3As already pointed out in the first introductory chapter, experience taught us that there are few possible mechanisms satisfying not only all the requirements imposed by the experimental observations but also the prerequisite to be thermodynamically favored and to occur with a feasible activation barrier.

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supercomputer center provided CPU time in the shared memory supercomputer SGI Origin 3800 with 128 processors and in the linux cluster with 200 PC computers.
References


REFERENCES


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REFERENCES

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[103] In the "Cours de Philosophie Positive" (1830) August Comte wrote that "every attempt to refer chemical questions to mathematical doctrines must be considered, now and always, profoundly irrational, as being contrary to the nature of the phenomena. .... if the employment of mathematical analysis should ever become so preponderant in chemistry (an aberration which is happily almost impossible) it would occasion vast and rapid retrogradation, by substituting vague conceptions for positive ideas, and easy algebraic verbiage for a laborious investigation of facts". The English text is taken from "The Positive Philosophy" freely translated and condensed by Harriet Martineau (1896) pp 289–290 (Batoche Books, Kitchener, 2000).
I owe deep thanks to everyone who has encouraged, challenged, taught, and inspired me throughout the intellectual adventure that this thesis has represented to me.

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It is impossible to thank one by one all the other people who in all sorts of ways have contributed to this thesis. Huge thanks go to my past and present colleagues, to my friends in Stockholm, to my friends in Italy and last but not least to my family (alla mia mamma, al mio papà e ad Elena un enorme grazie per essermi sempre stati vicini anche da 2000 km di distanza!).

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THANK YOU ALL!