Computational Modeling of Enzyme Reactions Involved in Aromatic Compound Metabolism

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Martin Culka

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Amtierender Dekan: Prof. Dr. Stefan Schuster

Prüfungsausschuss:
Prof. Dr. G. Matthias Ullmann (Erstgutachter)
Dr. Lubomír Rulíšek (Zweitgutachter)
Prof. Dr. Jürgen Senker (Vorsitz)
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Contents

Summary

In biological sciences, the computers are nowadays exploited for a large variety of data processing tasks. Furthermore, computer models of biological systems can be built to connect the experimental data from different sources and deepen our understanding of the phenomenon of interest. This thesis deals with computer modeling of enzyme catalysis, in particular with the enzymes involved in the little-understood steps of aromatic compound metabolism.

According to the transition state theory, the kinetics of a chemical reaction can be related to its energy profile. The relation between conformation and energy in computer models is often imagined as a landscape with valleys and saddle points. In order to find the reaction mechanism of a chemical reaction, the goal is to connect two valleys, which represent initial and final state of the reaction.The catalytic power of an enzyme lies in its ability to guide the reaction through a low-energetic pathway, which is more feasible than a mechanistically different reaction in solvent. Especially finding the right transition states along this pathway is a challenging task.

A large number of reaction path search methods has been described in literature. Conjugate peak refinement (CPR) is one of the chain-of-states methods that aim at connecting two stable states by a discretized chain of structures. Based on energy and gradient along the discretized path, CPR is able to progressively approach and identify all transition states (saddle points) on the reaction pathway and thus deliver the energy profile of the reaction in question. Herein presented PyCPR variant of CPR was developed to find catalytic paths within hybrid QM/MM models of enzyme-substrate complexes using versatile python-based framework pDynamo. PyCPR was able to correct the previously reported transition state of 4-hydroxyphenylacetate enzymatic radical decarboxylation. Furthermore, PyCPR identified the most probable mechanism of key steps in aromatic phenazine biosynthesis and anaerobic aromatic compound degradation.

Phenazines are aromatic secondary metabolites that play important role in bioenergetics as well as in survival and virulence of the bacteria that produce them. The tricyclic phenazine skeleton is formed in a branch of aromatic acid biosynthesis by condensation of two cyclohexene rings with adjacent amino and keto groups. The last step before this condensation is catalyzed by isomerase PhzF, which performs two subsequent side-specific proton transfers. QM/MM calculations presented here identified the first step as a quantitative transfer of a proton between two carbons via a glutamate residue. The intermediate state with a proton on the glutamate is high-energetic, which explains why a pericyclic mechanism was originally proposed. The second enol-keto isomerization step also involves the glutamate residue together with an active-site water molecule. The theoretical results are in line with kinetic measurements. The involvement of the glutamate explains stereospecific product formation suggested by NMR experiments.

Degradation of aromatic compounds is, especially at anoxic conditions, a difficult task for bacteria. The BamB-I complex was postulated to use electron bifurcation scheme to drive the benzoyl-CoA reduction at a very low redox potential. The structurally characterized catalytic subunit BamB contains tungsten in the catalytic site. The complete ligand sphere of the tungsten, as well as the detailed catalytic mechanism was so far unclear. I tested a large number of BamB mechanism variants using continuum-electrostatic and QM/MM methods. In the end, the most probable mechanism can be proposed. In addition, based on the calculations presented in this thesis, the previously uncertain small inorganic ligand was identified as a water molecule. The mechanism involves a hydrogen atom transfer from the proposed aqua ligand of the tungsten to the aromatic ring forming a radical intermediate. Second electron transfer directly follows and is accompanied by protonation from nearby histidine residue. Although unusual among the tungsten enzymes, the proposed mechanism is in line with the expected two-electron chemistry. Together, this thesis establishes a methodology to study mechanisms of enzyme catalysis and concurrently brings new light into mechanistic details of key reactions in aromatic compound metabolism.

Zusammenfassung

In den Biowissenschaften werden Computer heute für eine Vielzahl von Datenverarbeitungsaufgaben genutzt. Außerdem können Computermodelle von biologischen Systemen erstellt werden, um die experimentellen Daten aus verschiedenen Quellen zu verbinden und unser Verständnis des Phänomens von Interesse zu vertiefen. Die vorliegende Arbeit beschäftigt sich mit der Computermodellierung von Enzymkatalyse, insbesondere mit den Enzymen, die an den wenig verstandenen Schritten des Stoffwechsels von Aromaten beteiligt sind.

Aufgrund der Übergangszustandstheorie kann die Kinetik einer chemischen Reaktion mit ihrem Energieprofil verbunden werden. Die Beziehung zwischen Konformation und Energie in Computermodellen wird oft als eine Landschaft mit Tälern und Sattelpunkten berchrieben. Um den Reaktionsmechanismus einer chemischen Reaktion zu finden, müssen zwei Täler verbunden werden, die den Anfangs- und Endzustand der Reaktion darstellen. Die katalytische Kraft eines Enzyms liegt in seiner Fähigkeit eine Reaktion durch einen niederenergetischen Weg zu führen, der günstiger ist als eine mechanistisch unterschiedliche Reaktion im Lösungsmittel. Übergangszustände auf diesem Weg zu finden ist eine besonders anspruchsvolle Aufgabe.

Eine große Anzahl von Methoden für Reaktionspfadsuche ist in der Literatur beschrieben. Conjugate Peak Refinement (CPR) ist eine der chain-of-states-Methoden, die darauf zielen, zwei stabile Zustände durch eine diskretisierte Kette von Strukturen zu verbinden. Aufgrund von Energie und Gradienten entlang des diskretisierten Weges kann CPR schrittweise alle Übergangszustände (Sattelpunkte) auf dem Reaktionsweg identifizieren und dabei das Energieprofil der Reaktion liefern. Die PyCPR-Variante der CPR, die in diese Arbeit präsentiert wird, nutzt das pythonbasierte Framework pDynamo. Es wurde entwickelt, um katalytische Wege innerhalb hybrider QM/MM-Modelle von Enzym-Substrat-Komplexen zu finden. PyCPR konnte den Übergangszustand in der enzymatischen Radikaldecarboxylierung von 4-Hydroxyphenylacetat korrigieren sowie den wahrschein-

lichsten Mechanismus der wichtigsten Schritte in der aromatischen Phenazin-Biosynthese und in dem anaeroben Aromatenabbau identifizieren.

Phenazine sind aromatische Sekundärmetaboliten, die sowohl eine wichtige Rolle in Bioenergetik als auch in Überleben und Virulenz den Bakterien spielen. Das tricyclische Phenazinskelett wird über einen Zweig der Synthese von aromatischen Aminosäuren durch Kondensation von zwei Cyclohexenringen mit benachbarten Amino- und Ketogruppen gebildet. Der letzte Schritt vor dieser Kondensation wird durch die Isomerase PhzF katalysiert, die zwei aufeinanderfolgende seitenspezifische Protonentransfers durchführt. Die QM/MM-Berechnungen identifizierten den ersten Schritt als quantitative Übertragung eines Protons zwischen zwei Kohlenstoffen über einen Glutamatrest. Der Zwischenzustand mit einem Proton auf dem Glutamat ist hochenergetisch, was erklärt, warum ein pericyclischer Mechanismus ursprünglich vorgeschlagen wurde. Der zweite Enol-Keto-Isomerisierungsschritt beinhaltet auch den Glutamatrest zusammen mit einem Wassermolekül. Die theoretische Ergebnise stehen im Einklang mit kinetischen Messungen und die Beteiligung des Glutamats erklärt die stereospezifische Produktbildung, die auf Grund von NMR-Experimente vorgeschlagen wird.

Der Abbau von Aromaten ist, besonders unter anoxischen Bedingungen, eine schwierige Aufgabe für Bakterien. Der BamB-I-Komplex verwendet vermutlich ein Bifurkationschema, um die Benzoyl-CoA-Reduktion bei niedrigem Redoxpotential zu treiben. Die katalytische Untereinheit BamB enthält Wolfram im katalytischen Zentrum. Der katalytischer Mechanismus von BamB, sowie die vollständige Ligandensphäre des Wolframs, waren bisher unklar. Eine große Anzahl von Mechanismusvarianten wurde mit kontinuumelektrostatischen und QM/MM Methoden getestet. Darüber hinaus kann vorgeschlagen werden, dass der bisher ungewisse kleine anorganische Ligand ein Wassermolekül ist. Der wahrscheinlichste Mechanismus beinhaltet einen Wasserstoffatomtransfer aus dem vorgeschlagenen Aqua-Liganden des Wolframs zu dem aromatischen Ring, wobei ein Radikalzwischenprodukt gebildet wird. Der zweite Elektronentransfer folgt direkt und wird von einer Protonierung aus dem nahe gelegenen Histidinrest begleitet. Obwohl untypisch, ist der Mechanismus in Übereinstimmung mit der erwarteten Zwei-Elektronen-Chemie. Gemeinsam beschreibt diese Arbeit eine Methodik zur Untersuchung von Mechanismen der Enzymkatalyse und bringt neues Licht in mechanistische Details von Schlüsselreaktionen im Stoffwechsel von Aromaten.

List of Abbreviations

AH	Acetylene hydratase
AOCHC	6-amino-5-oxo-2-cyclohexene-1-carboxylate
AOR	Aldehyde oxydoreductase
Bam	Benzoic acid metabolism
BCR	Benzoyl-CoA reductase
BTEX	Benzene, toluene, ethylbenzene and xylenes
CoA	Coenzyme A
DHHA	2,3-dihydro-3-hydroxyanthranilate
FDH	Formate dehydrogenase
MM	Molecular mechanics
NEB	Nudged elastic band
NMR	Nuclear magnetic resonance
PAH	Polycyclic aromatic hydrocarbon
PES	Potential energy surface
PyCPR	Python-based conjugate peak refinement
QM	Quantum mechanics
ROS	Reactive oxygen species
TST	Transition state theory
WPT	Tungstopterin cofactor

1. Computers in Modern Bioscience

For billions of people on Earth, computers have become part of their everyday life. Although many of the today's computer users consider them merely as means of entertainment or communication, computers were originally developed by scientists to make the complex mathematical calculations possible. It would be surprising if the power of the modern computers was not utilized for complex calculations in wide variety of scientific disciplines till now. The major theme of this thesis is the application of the computer power to solve complex problems of biochemistry and molecular biology, in particular the mechanistic details of key catalytic steps in aromatic compound mechanism in bacteria.

The computers are employed for two general, partly overlapping, purposes in bioscience:

(i) Data processing – harvesting new information from experimental data

(ii) Modeling – explaining experimental observations with a computer model

In the following paragraphs, I provide a brief overview of applications of computers within these two categories. The goal here is not to give an exhaustive list of computer applications in bioscience, but rather to place the topic of this thesis into a broader context.

Data Processing Apart from rather trivial applications such as arithmetics and statistics in spreadsheet calculator or data point plotting and fitting, the computers are utilized for many difficult data processing tasks in bioscience. Probably the most well known and cited[1] data processing application of the computers in life science is in the gene sequence alignments. Since the amount of gene sequences stored in public databases is enormous and it is still growing, this field becomes more important and challenging. Biologists usually want to extract information about genetics and evolution from the genetic sequence data. On

the other hand, for a biochemist, the most valuable information is the relation between protein or nucleic acid sequence and its structure and function. Several approaches attempt to combine sequence and structural data in order to assign function to uncharacterized proteins.[2]

A complementary field to the genetics is proteomics, which seeks information about proteins present in various circumstances in the cell or its compartments. The protein content of desired compartment is isolated, proteolytically cleaved and analyzed by mass spectrometry. Computers play an irreplaceable role not only in peak detection in raw data, but more importantly in the subsequent data mining process where proteins present in the sample are identified from the set of fragments detected by the spectrometer.[3] Furthermore, the data from proteomic studies are made publicly available and can be further processed in computational studies.[4]

Computers also find progressively more application in scientific image processing. They can be used not only for improving the image quality, but also for extracting quantitative information out of large image sets such as cell plate photographs or DNA chips.[5] Specific category of images are those obtained from high-resolution cryo-electron microscopy (cryo-EM). The images are processed to obtain better resolution in the first stage. 3D structure can be reconstructed from a series of images in the second stage.[6]

Modeling Computer models try to explain experimental observations by a model that can capture experimentally inaccessible features of the phenomenon in study. Most of the experimental approaches in biochemistry can be viewed as a black box (Figure 1.1). We know what are the inputs and outputs of the experiment, however, our knowledge of what is happening in detail inside the black box (i.e. our sample) is limited. Even without computers involved, the researchers often build a working model based on combination of experimental findings. A model is an idealized representation of the real system that covers its certain aspects in order to help us understand the macroscopic behavior of the real system. Computers are employed to build models of certain aspects of biological systems with intention to explain macroscopic experimental observations, such as change in absorption spectra upon reduction, loss of enzyme activity caused by a mutation, or substrate specificity on an enzyme.

Cryo-electron microscopy discussed above represents together with X-ray crys-

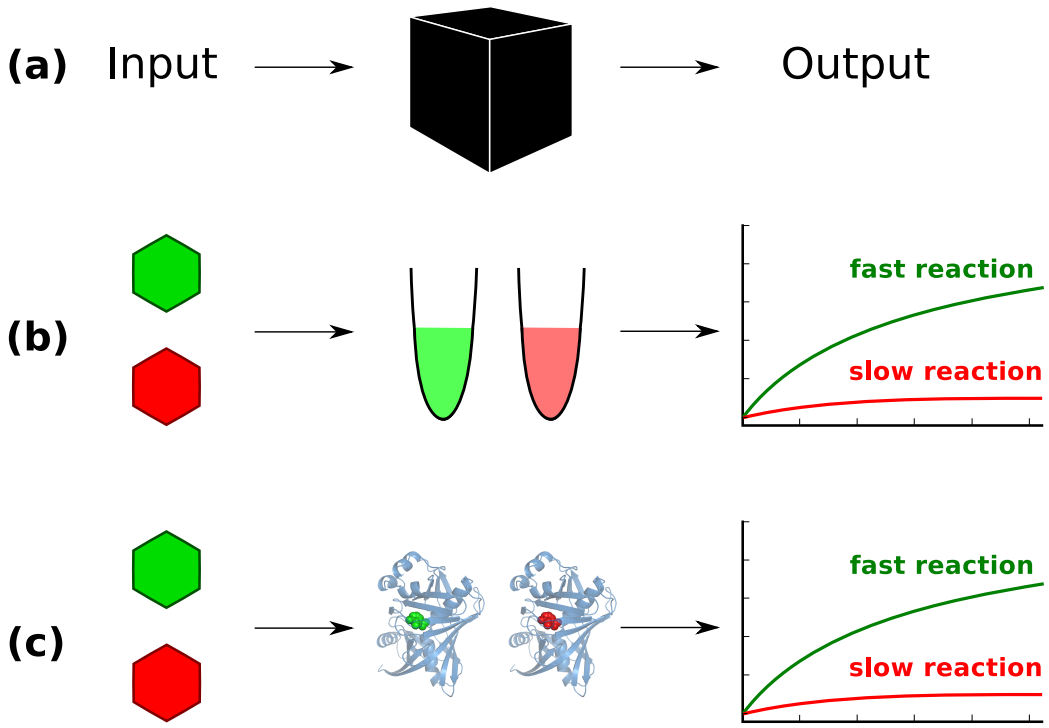

Figure 1.1. **(a)** The black box problem – the inputs are known, the outputs can be observed, but the microscopic details of the process are hidden. **(b)** In experimental biochemistry, for instance, when two different substrates are mixed with the same enzyme in the reaction tube, two different rate curves are measured. **(c)** A computational model built based on the structure of the enzyme can explain microscopic details that cause the macroscopic change in the reaction rate curves.

tallography and nuclear magnetic resonance (NMR) structure determination a bridge from pure experimental data processing by computers to computer modeling based upon experimental data. For instance, atomistic protein models can be built based on the 3D images by fitting known crystal structures of individual elements into a 3D image of a protein complex.[6] If the resolution is high enough, an atomistic protein model can be built *de novo* from the cryo-EM structure based on the protein sequence and general structure data for individual aminoacids.[6] The most popular structure-determination method, X-ray crystallography, also employs computer modeling principles to interpret the experimental data.[7] An atomistic model is built based on the electron density map, which is constructed from the experimental X-ray diffraction data after solving the phase problem. The structural model and the electron density map are then recursively refined to fit the diffraction data. The NMR-based structures are in fact also structural

computer models built upon the NMR-derived distance restraints.[7]

The protein and nucleic acid structures derived from X-ray crystallography, NMR, or cryo-EM can be used to build atomistic computer models where the connection to the experimental data is more indirect. Typically, such a computer structural model consists of an energy function, which assigns energy to a given 3D coordinates of the atoms based on physical principles. Investigation of enzyme catalysis using structural computer models relies largely on transition state theory (TST). According to TST, the reaction rate of an elementary reaction step can be related to a free energy difference between the stable state and the transition state on the reaction path towards the next stable state ($\Delta G^{\ddagger}$). Commonly, this relation between the temperature-dependent reaction rate constant $k(T)$ and the free energy needed to reach the transition state $\Delta G^{\ddagger}$ is expressed by the Eyring-Polanyi equation:

$$k(T) = \zeta \frac{k_B T}{h} exp\left(\frac{\Delta G^{\ddagger}}{k_B T}\right) \tag{1.1}$$

where T is the absolute temperature, k_B is the Boltzmann constant, h is the Planck constant and ζ in the transmission coefficient, which corresponds to the probability of being reactive once the system reaches the transition state. When dealing with a complex, often multistep, reaction pathway of an enzyme, the computer-model-derived free energy difference between the lowest stable state and the highest transition state can be taken as $\Delta G^{\ddagger}$ and related to the experimentally determined reaction rate through the equation (1.1).

Movements in biomolecule structures can be modeled, for example, based on classical physics within molecular-mechanical (MM) force fields. Simply put, in the MM models, the atoms are represented as charged spheres connected by springs. Especially popular field of use of MM models are the molecular dynamics (MD) approaches where dynamic behavior at finite temperature is studied at atomistic detail in relation to time. MD techniques using MM models were used to study conformational changes, membrane transport, protein folding or ligand binding, to name just a few.[8]

For studying chemical reactions, in particular enzyme catalysis interesting for biochemists, the MM approaches are insufficient, since the classical representation of atoms cannot properly describe chemical transitions. Quantum-mechanical (QM) methods explicitly describe the quantum-physical nature of the electrons and thus model the chemical bonding more realistically than the classical springs

in MM. Although approaches that can treat whole proteins on QM level are emerging thanks to the development of high-performance computer clusters and highly-parallelized algorithms,[9] big QM models are still far from routine use. Furthermore, it is often handy to restrict the degrees of freedom in the model in order to concentrate on one particular aspect of the problem. Therefore, hybrid QM/MM models are usually used to model the reactions in biochemistry.[10] In QM/MM, a reasonably chosen reaction site is treated on QM level, while the rest of the biomolecule (usually enzyme) is treated on much computationally cheaper MM level. A large variety of techniques can be used to find reaction pathways within QM and QM/MM models of enzymes. These techniques together with more detailed description of various enzyme models are reviewed in detail in Manuscript A.

It is not always necessary to model the movements of the biomolecules in order to understand their properties. For instance, proton or electron transfer can be studied using continuum-electrostatic methods combined with Monte Carlo approaches.[11] In continuum electrostatics, the surroundings of a biomolecule is modeled as high-dielectricity continuum, while the atoms of the biomolecule are modeled as point charges in low-dielectricity continuum. Outcome of continuum-electrostatic methods can be also used to build kinetic models of electron transfer in large protein complexes.[12]

In this thesis, I use the continuum electrostatics to determine protonation behavior of the enzyme active sites prior to employing QM/MM techniques. The techniques to model enzyme catalysis are thoroughly reviewed in Manuscript A. The Python-based conjugate peak refinement (PyCPR) method for simulating enzyme catalysis within QM/MM framework together with its implementation and application examples is described in Manuscript B. Manuscripts C and D describe applications of continuum-electrostatic and QM/MM methods (including PyCPR) to elucidate detailed catalytic mechanism of two bacterial enzymes involved in aromatic compound metabolism, which is reviewed in the next chapter.

2. Aromatic Compound Metabolism

The aromatic compounds are very stable cyclic chemical substances, many of them of a particular smell, hence the name. Their stability is caused by a delocalized π-electron system above and below the aromatic ring plane. Homocyclic aromatic compounds contain one or more planar six-carbon benzene rings (C_6H_6). Heterocyclic aromates contain nitrogen, oxygen or sulfur atoms in addition to the carbons in their planar aromatic rings.

Aromatic compounds are crucial components in the chemistry of living organisms.[13] Four protein aminoacids (phenylalanine, tyrosine, tryptophan and histidine) are aromatic. All five nucleic acid bases (adenine, guanine, cytosine, thymine and uracil) are heterocyclic aromates. Many essential enzyme cofactors, such as flavines, porphyrines, quinols or folic acid have aromatic rings in their structure. Furthermore, various aromatic secondary metabolites, such as lignins, flavonoids or phenazines, are secreted into the environment by bacteria, fungi and plants.[14–16] The anabolism of aromatic compounds will be reviewed below.

Although many derivatized aromatic compounds are biogenic, others, often originating from human activity, are classified as dangerous environmental pollutants. The aromatic hydrocarbons have tendency to accumulate in soil, water and atmosphere.[17] Accumulation of aromates in the environment poses serious threat to human health, since mono- and polycyclic aromatic hydrocarbons often have toxic, mutagenic and carcinogenic properties caused by their interaction with DNA, RNA and proteins.[18] The major monoaromatic pollutants are benzene, toluene, ethylbenzene and xylenes (often jointly abbreviated as BTEX). The sources of BTEX in environment are traffic emissions, spills during the petrol transport and tobacco smoke.[19] BTEX either accumulate in the atmosphere, or are dissolved in the groundwater and thus pose danger to human health.[20] The more complex polycyclic aromatic hydrocarbons (PAHs) are formed from organic substances at high temperature, *e.g.* during forest fires or fuel combustion, but

also during meat barbecuing or tobacco smoking.[21] The PAHs are usually unsoluble due to their hydrophobicity and adsorption to the sediments and thus their bioavailibility is limited. In spite of the chemical stability of the aromatic hydrocarbons, bacteria (and to lesser extend archea and fungi)[18] developed strategies to break down the aromatic rings and degrade the aromates in soil or groundwater into simple metabolites that can be used as carbon and energy sources, such as acetyl-coenzyme A. The catabolic strategies towards aromate degradation will be reviewed further below.

2.1 Biosynthesis of Aromatic Compounds

Although humans and other animals can synthesize aromatic heterocycles of nucleobases *de novo*,[22] they are unable to assemble the homocyclic benzene rings.[23] Aromatic aminoacids and various aromatic enzyme cofactor precursors have to thus be ingested in the animal diet, although they can be further derivatized in the animal metabolism.[13] Humans are, for example, able to synthesize tyrosine by phenylalanine hydroxylation and further transform the tyrosine into thyroid hormones, adrenalin or dopamine. Plants, fungi and bacteria, on the other hand, possess metabolic pathways to synthesize the homocyclic biogenic aromates.[23] Bacteria can in addition be genetically engineered to overproduce the aromatic compounds, which are further processed in pharmaceutical, food and chemical industry.[24] I mainly focus on bacterial anabolism of aromatic compounds in this section.

The synthesis of nucleic acid bases is highly conserved in evolution from bacteria to humans.[25] Heterocyclic purine nucleobases (adenine and guanine) are assembled in one or few atom steps from simple compounds.[22] Pyrimidine bases (cytosine, thymine, uracil) are synthesized starting with condensation of aspartate and carbomoyl phosphate. Heterocyclic aromatic aminoacid histidine is synthesized from ATP and phosphoribose pyrophosphate.[13] The majority of benzene-ring-containing aromates is derived from erythrose and phospohoenolpyruvate through the shikimic acid pathway in bacteria.[14] Bacterial secondary metabolites aromatic polyketines are made by malonyl-CoA polymerization through polyketine pathway.[26] Furthermore, the benzene ring of the tricyclic isoalloxazine in flavines is derived from condensation of two guanine-derived dimethyllumazines.[27]

In the shikimic acid pathway[23] (Figure 2.1), a hexacarbon ring is formed from

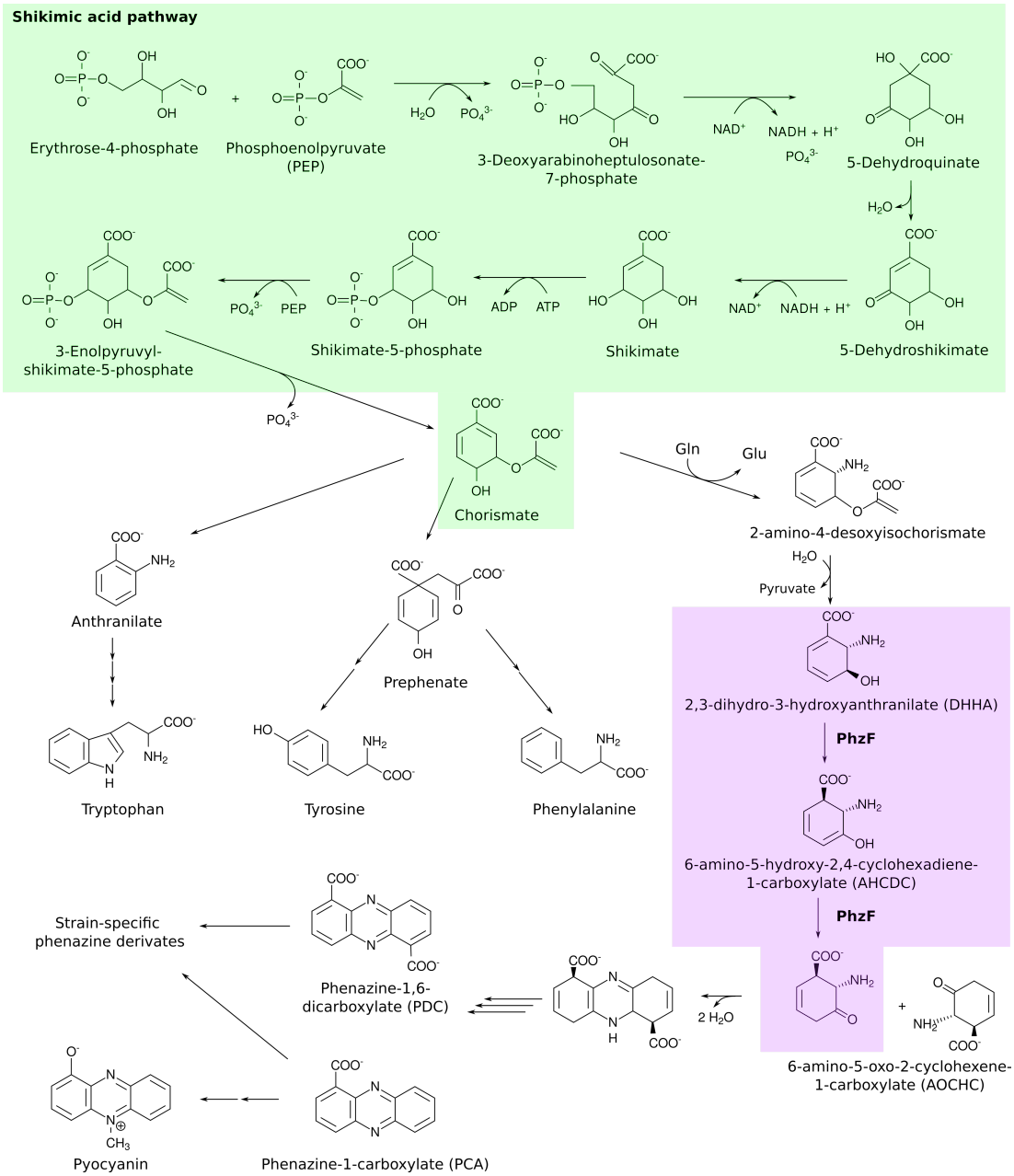

Figure 2.1. Shikimic acid pathway (highlighted by a green background) and consecutive biosynthesis pathways of aromatic aminoacids and phenazines in bacteria. Reactions catalyzed by PhzF studied in Manuscript C are highlighted by a violet background.

erythrose and phospohoenolpyruvate and another phospohoenolpyruvate is attached to a *meta* hydroxy group of the shikimate to form chorismate. Chorismate is a central intermediate on the way to aromatic aminoacids phenylalanine, tyrosine and tryptophan. Chorismate can be isomerized to prephenate, which is subsequently decarboxylated 4-hydroxyphenylpyruvate or decarboxylated and dehydroxylated to phenylpyruvate. These oxo acids are transformed to tyrosin and phenylalanine by transamination. Chorismate can be transformed to 2-aminobenzoate (anthranilate), which is then ribosilated on the nitrogen. In next steps, the five-membered pyrrole ring is closed forming indole side chain of tryptophan.

2.1.1 Phenazines

Another pathway towards a group of aromatic secondary metabolites – phenazines – begins from the chorismate as well (Figure 2.1).[28] Phenazines are important bacterial secondary metabolites with antibiotic, virulence and oxidation-reduction properties.[29] Over 100 phenazine derivatives are produced by a wide variety of bacterial species, although the phenazine metabolism of *Pseudomonas* species is probably the most well studied one.[16] In general, the phenazines secreted into the environment have primarily an antibiotic role that should promote competitive survival of the phenazine-producing bacteria. For instance, the oldest known phenazine pyocyanin was identified as a virulence factor of the opportunistic pathogen *Pseudomonas aeruginosa* in lungs of cystic fibrosis patients.[30] The pyocyanin levels during the lung infection are correlated with lower incidence of yeast infections.[31,32] Other members of the *Pseudomonas* species living in soil also produce high amounts of phenazine derivatives.[33,34] These pseudomonads are forming biofilms on the roots of wheat and might be responsible for the wheat survival in arid environments.

The essence of phenazine function lies in their properties as electron carriers. In *Pseudomonas aeruginosa*, the phenazines may serve as terminal electron acceptors in fermentation metabolism and carry the electrons to the extracellular space, thereby supporting anaerobic survival of the bacterium.[35] In hand with this electron carrier function of phenazines goes the proposal that the antibiotic and virulence properties of phenazines are associated with formation of reactive oxygen species (ROS).[16] The reduced phenazines secreted to extracellular space

might get oxidized by molecular oxygen and thereby the ROS can emerge.

The phenazine biosynthetic pathway (Figure 2.1), which is catalyzed by a series of phzBDEFG enzymes in *Pseudomonas*,[28] begins with an introduction of amino group to the chorismate and the ester group hydrolysis to a hydroxy group catalyzed by PhzE and PhzD, respectively. The resulting 2,3-dihydro-3-hydroxyanthranilate (DHHA) is isomerized to 6-amino-5-oxo-2-cyclohexene-1-carboxylate (AOCHC) by the PhzF.[36] In principle, the two AOCHCs might condense to a tricyclic product in the interdomain space of the PhzF dimer. In reality, the condensation is most likely catalyzed by another enzyme PhzB.[37] The tricyclic precursor is further oxidized to form first aromatic phenazines. Phenazine-dicarboxylic acid – PDC and phenazine-1-carboxylic acid – PCA are considered to be central precursors of further phenazine derivates, such as above mentioned pyocyanin.[28] The mechanistic details of many steps of phenazine biosynthesis are still to be unreveled. In Manuscript C, the detailed mechanism of the PhzF enzyme is revealed.

2.2 Biodegradation of Aromatic Compounds

Although some fungi and algae are also able to degrade the aromatic compounds,[18] I focus here on the bacterial metabolism. The bacterial aromatic compound degradation strategies can be in general divided into aerobic and anaerobic (Figure 2.2). When molecular oxygen is present, it is used not only as a terminal electron acceptor, but also as a co-substrate in the process of aromatic compound degradation. In anaerobic conditions, in contrast, the aromatic ring is reduced in the central dearomatization step. In both cases, the initial aromatic compounds taken-up from the environment are derivatized to central intermediates, which are then dearomatized and subsequently further degraded to simple metabolites in oxidative processes.[17] Similar degradation principles are applied to degradation of simple monocyclic aromates, as well as complex PAHs in the aerobic environment.[18] The anaerobic degradation pathways have so far been studied far less then their aerobic counterparts. Although it has been shown that anaerobic bacteria can degrade heavier PAHs, only the degradation of monoaromatic compounds and diaromatic naphthalene has been described in more detail.[38]

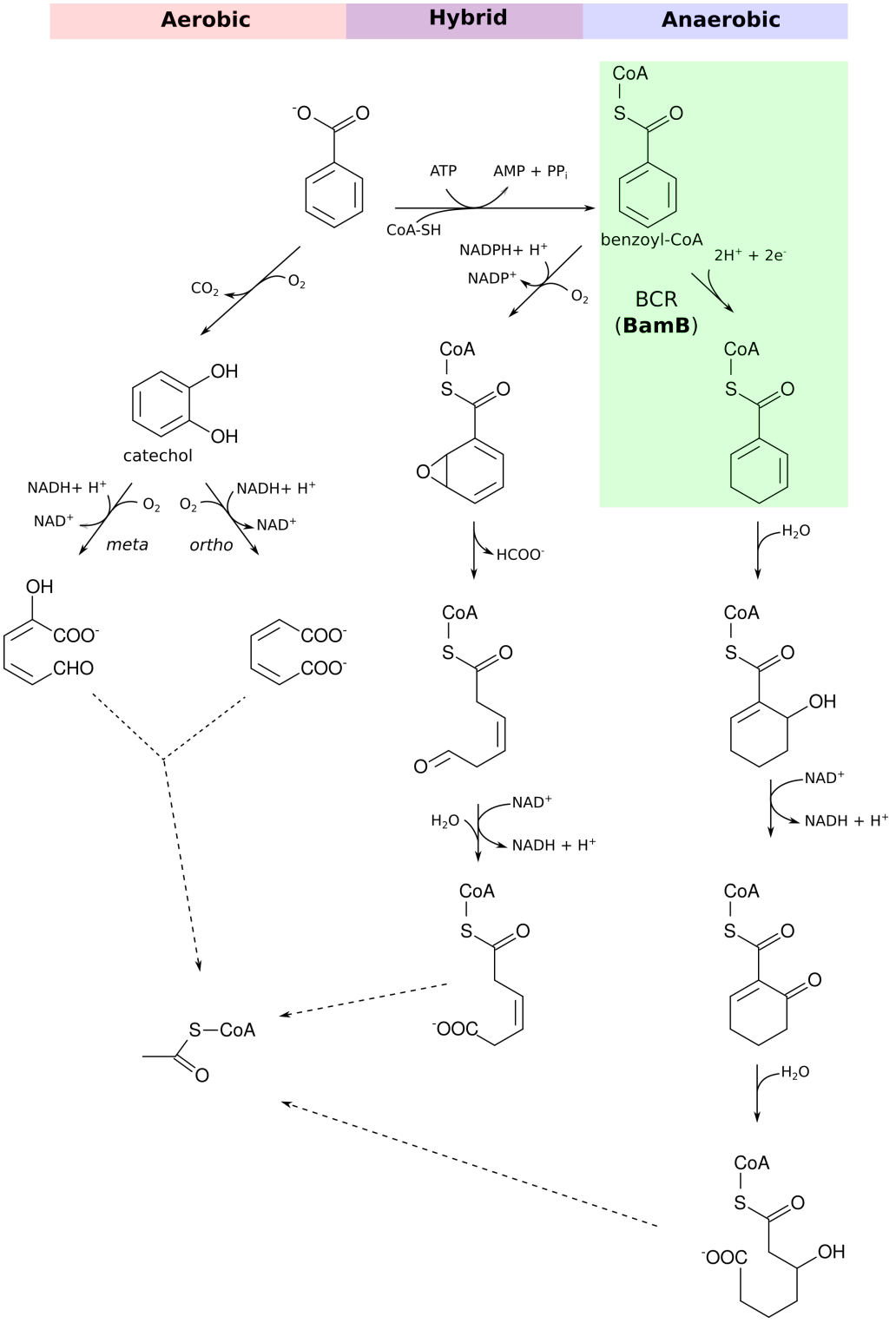

Figure 2.2. Degradation fates of benzoate under aerobic and anaerobic conditions. So-called hybrid pathway shares the activation step with anaerobic pathways, yet uses molecular oxygen instead of reduction for the key dearomatization step. The reaction catalyzed by BamB discussed in Manuscript D is highlighted by green background.

2.2.1 Aerobic Degradation Pathways

In oxidative environment, the aromatic compounds are first hydroxylated by monooxygenases or dioxygenases. The monooxygenases cleave the O_2 molecule by reducing one of the oxygens to H_2O and insert the second oxygen atom into the substrate.[39] Usually, at least two monooxygenation steps are needed before the aromatic ring can be cleaved. The dioxygenases are able to attach both oxygen atoms in one step to the aromatic ring in a reductive process that leads to cis-dihydrodiols.[40] The resulting central intermediates are catechols or hydroxy-substituted aromatic acids. The activated central intermediates are dearomatized in the process of *ortho* or *meta* cleavage catalyzed by dioxygenases.[41] The linearized hydrocarbon chain is further degraded to produce energy and/or precursors for anabolic pathways. In case of polyaromates, the aromatic rings are usually cleaved and degraded one by one.[21]

2.2.2 Anaerobic Degradation Pathways

Due to the rapid oxygen depletion in the soil, the aerobic degradation of the aromatic compounds is often not possible.[20] The anaerobic bacteria living in the anoxic soil layers, nevertheless, developed strategies how to break the aromatic rings in anaerobic conditions. These bacterial cultures have to use alternative terminal electron acceptors to the molecular oxygen such as sulfate, nitrate, iron(III) or carbon dioxide.[42] As in the aerobic case, the aromatic ring is first derivatized to a central intermediate before the key dearomatization step. Although the aromatic ring can be hydroxylated as well in the anaerobic environment in some cases,[43] the hydroxy groups are removed prior to the central reductive dearomatization the majority of the anaerobic aromate degradation pathways.[44] The aromatic substrate is initially activated by carboxylation, fumarate insertion or phosphorylation.[17] Subsequently, a carboxy group adjacent to the aromatic ring is thioesterified by a coenzyme A (CoA) and the central intermediate such as benzoyl-CoA is thus formed. The aromatic ring of the central intermediate is reduced and subsequently cleaved into acetyl-CoA pieces in a repetitive sequence of hydrolysis and oxidation similar to fatty acid β-oxidation (Figure 2.2 right column).[45] Alternatively, in a so-called hybrid pathway, the typical anaerobic central intermediates such as benzoyl-CoA or phenylacetyl-CoA can be monooxygenated to ring epoxides under aerobic conditions (see Figure 2.2 in the middle).[46,47] The

destabilized ring is subsequently hydrolytically cleaved and degraded in the β-oxidation fashion as in the other cases. Is has been proposed that bacteria might apply this hybrid strategy under sufficient oxygen levels, while they switch to the reductive dearomatization in cases of oxygen depletion.[48]

The dearomatization step of the anaerobic aromate degradation has mainly been studied on benzoyl-CoA as a model central intermediate. Two distinct classes of benzoyl-CoA reductases (BCRs) have been described in literature. The BCR-I class enzymes from facultative anaerobes contain iron-sulfur clusters and require two molecules of ATP for the two-electron reduction step.[49] The BCR-I reduces the benzoyl-CoA by a stepwise radical mechanism.[50] In contrast, the BCR-II class from strict anaerobes catalyzes ATP-independend two-electron reduction of the aromatic ring.[45] The source of energy of this endergonic step is so far not clear. It has been proposed that either membrane potential or flavin-based electron bifurcation can be the driving force for the dearomatization.[51] In the bifurcation scheme, the two electrons from a single source (such as $FADH_2$) are conducted in two directions through chains of single electron transporters such as iron-sulfur centers or hemes.[52] On one side, exergonic reduction of a standard electron acceptor such as NAD^+ takes place, while on the second site the endergonic reduction of the substrate (here benzoyl-CoA) occurs. The unfavorable reaction of interest is thus driven by the favorable side reaction.

The best described BCR-II from iron-reducing bacterium *Geobacter metallireducens* is part of a large eight-subunit complex BamBCDEFGHI (Bam stands for benzoic acid metabolism).[51] The $BamB_2C_2$ heterotetramer that contains the catalytic BamB subunit has been structurally characterized.[53] BamB contains a tungstopterin (WPT) cofactor in the active site. An iron-sulfur center in WPT proximity connects the active site to the electron conduit network of the whole protein complex. The detailed mechanism of benzoyl-CoA reduction was unclear till now, also because of uncertainties in the tungsten ligand sphere. In Manuscript D, I performed extensive computational investigation of mechanistic possibilities of benzoyl-CoA reduction by BamB. In the next chapter, I put BamB into the context of other tungsten enzymes known so far.

3. Tungsten Enzymes

Tungsten, a third row transition metal with atomic number 74, is the heaviest element with a biogenic role. The living organisms started to utilize tungsten as a redox-active element in the enzyme active sites already in an early stage of evolution.[54] The reason for adaptation of such a rare element might be that the tungsten ion can cycle between three oxidation states (IV, V and VI) under the physiological conditions and thus represents a bridge between one-electron and two-electron redox biochemistry. On the dawn of life on Earth, soluble tungsten sulfides were available in the predominantly reductive environment.[55] In later stages of evolution, when molecular oxygen levels in the environment begun to rise, the oxygen-sensitive tungsten enzymes could have been exchanged by more stable molybdenum equivalents.The aerobic organisms including humans employ molybdenum enzymes, while the strictly anaerobic bacteria and archea retained the tungsten enzymes. Although many tungsten enzymes seem to be evolutionary older homologues of molybdenum enzymes with a similar role in the metabolism, some of them have unique function, mainly in extremophilic organisms. Among the unique tungsten enzymes are acetylene hydratase[56] from *Pelobacter acetylenicus* and also benzoyl-CoA reductase BamB from *Geobacter metallireducens*, which was studied in detail in Manuscript D. Both above mentioned tungsten enzymes play a key role in the carbon metabolism of the strictly anaerobic organisms that express them, since acetylene hydratase enables *Pelobacter acetylenicus* to grow solely on acetylene[57] and BamB enables *Geobacter metallireducens* to grow solely on benzoate.[45]

3.1 Tungstopterin Cofactor

In all the tungsten enzymes known so far, the tungsten ion is coordinated by two pyranopterin cofactors (Figure 3.1), which are synthesized from GTP and cystein sulfur.[58] The tricyclic pyranopterin cofactor (pyranopterin-dithiolate) is

composed of bicyclic pterin moiety fused with a pyran ring, which binds two thiol groups on neighboring double-bonded carbons. The thiolates serve as ligands of the tungsten ion. The tricyclic pyranopterin moiety is either linked to a methylphosphate group, or it forms a larger dinucleotide-like structure with methyldiphosphate and guanosine (R group in Figure 3.1). Furthermore, the tungsten is coordinated by one or two additional ligands that differ among the enzymes (L1 and L2 and inset table in Figure 3.1). The tungsten ion together with all its ligands forms a tungstopterin cofactor (WPT).

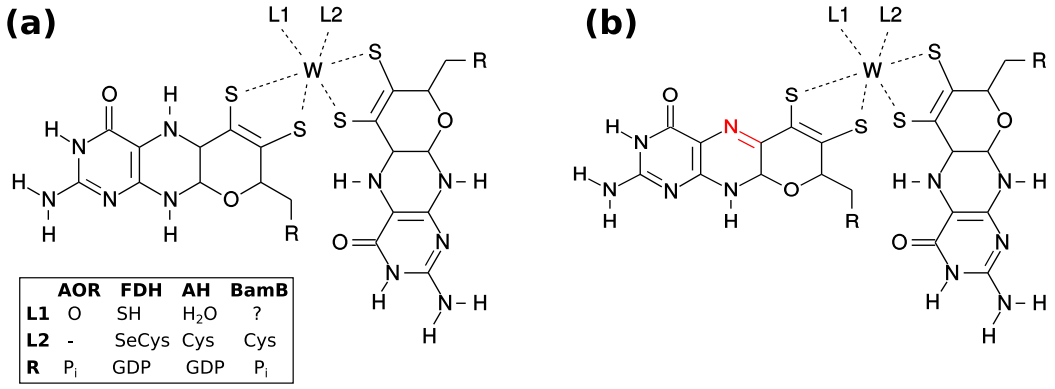

Figure 3.1. The tungstopterin cofactor is consisted of the central tungsten ion, two pyranopterins and one or two additional ligands (L1 and L2), where one of them is a small inorganic ligand and the other can be a protein residue. In the inset table, the nature of ligands L1 and L2 in the reduced form of different tungsten enzymes is summarized (AOR – aldehyde oxydoreductase, FDH – formate dehydrogenase, AH – acetylene hydratase, BamB – benzoyl-CoA reductase). In addition, the pyranopterins in some tungsten enzymes are bound to a guanosine side chain, while in other tungsten enzymes, they have just an inorganic phosphate at the same position (R). The pyranopterins can either both occur in the reduced tetrahydropterin form **(a)**, or one of them, which is distal to the iron sulfur cluster, can be oxidized to the dihydropterin form **(b)** in some tungsten enzymes.[59]

Two parts of the pyranopterin are potentially redox active – the dithiolate part and the pterin part. It has been shown that the pterin part of the two pyranopterins can occur in different oxidation states is some crystal structures[59] (see the difference between Figure 3.1 **a** and **b**) and sometimes the pyran ring can be even oxidatively opened by cleavage of the bond between pyran oxygen and the middle ring carbon.[60,61] Moreover, pyranopterin radical has been detected by EPR spectroscopy.[62] Despite some researchers designate the pyranopterin as

a non-innocent ligand due to these hints,[63] little is known about its actual role in catalysis. The redox activity of the pyranopterins can presumably facilitate the electron transfer from iron-sulfur centers, which commonly accompany the tungstopterin cofactor, to the tungsten ion.[64] My calculations on tungstoenzyme BamB (Manuscript D) show that the pyranopterin cofactor might even serve as an electron buffer and reduce the tungsten forming a radical cation in case of electron shortage.

3.2 Mechanisms of Tungsten Enzymes

The so-far characterized tungsten enzymes catalyze a diverse variety of redox reactions and even one nonredox hydratation reaction. First structurally characterized tungsten enzyme belonged to the aldehyde oxidoreductase (AOR) family.[65] AORs catalyze oxidation of aldehydes to the corresponding carboxylic acids.[66] In the resting state, the tungsten ion in the AOR active site is coordinated, in addition to the two pyranopterins, to an oxo ligand. In course of catalysis, the aldehyde is bound to the tungsten through its oxygen as a sixth ligand and the other oxygen is bound to the carbonyl carbon forming a carboxylate.[67] Glyceraldehyde-3-prosphate:ferredoxin oxidoreductase is the only AOR with known specific metabolic function – it is a part of an alternative Embden-Meyerhof glycolysis pathway.[68] Other AORs were reported to have a broad substrate spectrum of small[69] and larger[70] aldehydes and their metabolic role remains unclear.

The tungsten-containing formate dehydrogenase (FDH) from *Desulfovibrio gigas* is oxidizing formate to carbon dioxide.[71] The tungsten ion is coordinated by a selenocysteine residue and a hydrogensulfido ligand in the resting state. In the so-called "sulfur-shift" mechanism of FDH,[72] the selenocysteine is first displaced from the tungsten by the formate, which is ligated to the metal through one of its oxygens. The selenocysteine forms a disulfide-bridge-like structure with the inorganic sulfido ligand of the tungsten ion. The fumarate is oxidized by the tungsten and deprotonated by the selenocysteine and a carbon dioxide is released.

N-formylmethanofuran dehydrogenase plays a key role in CO_2 reduction in methanogenes and in general catalyses a reverse process to the reaction of FDH.[73] A molecule of carbon dioxide is fused with methanofuran in a reductive process forming N-formylmethanofuran. Very recently solved crystal structure[74] of N-

formylmethanofuran dehydrogenase from *Methanothermobacter wolfeii* revealed that the enzyme contains two active sites connected by a 35 Å long channel. The first active site contains tungstopterin with similar ligand composition as the one of FDH, only the selenocysteine ligand of the tungsten ion is replaced by a cysteine. It was proposed that CO_2 is reduced to formate at the tungsten site and then channeled to a Zn-Zn site where it is fused to the methanofuran.[74] The detailed mechanism of this remarkable reaction is, however, still to be unraveled.

Acetylene hydratase (AH) from *Pelobacter acetylenicus* is unique among tungsten and molybdenum enzymes, since it does not catalyze a net redox reaction. Instead, it catalyzes hydratation of acetylene to acetaldehyde. Although the AH is not redox active, a fully reduced state of the enzyme is required for the catalysis.[56] The active site tungsten ion is coordinated by two pyranopterins, a cysteine residue and a water molecule.[75] A second shell mechanism, where the water molecule performs a nucleophilic attack on the triple bond of the acetylene with assistance of a nearby protonated aspartate, was proposed.[75] Other researchers proposed a first shell mechanism instead, where the aqua ligand is displaced by the acetylene, which is coordinated to the tungsten ion through the π-system of its triple bond.[76] The carbons are then susceptible to an attack by a hydroxy ion formed through water deprotonation by nearby aspartate. Which of the mechanisms is the right one still remains to be resolved.

The tungsten-containing benzoyl-CoA reductase BamB described in Manuscript D is also unusual among the tungsten and molybdenum enzymes. It does not catalyze any oxygen atom transfer, nor it is probable that the bulky substrate can directly be ligated to the tungsten ion. The active site is similar to FDH or AH – the tungsten is coordinated by a cysteine and a small ligand in addition to the two pyranopterins. The small inorganic ligand, however, remained unresolved when the enzyme structure was determined by X-ray crystalography.[53] When investigating the mechanistic details of benzoyl-CoA reduction by BamB, I considered four potential candidates for the unresolved ligand, including diatomic CO and CN proposed by EXAFS spectroscopy[53] together with common sulfur and oxygen (Manuscript D). According to my calculations, an aqua ligand seems to support the most plausible reaction mechanism and the BamB active site might thus resemble the one of the acetylene hydratase. An electron from the tungsten center along with a proton from the aqua ligand are transferred to the aromatic ring forming a radical intermediate. The second reduction step

is promoted by a proton transfer from nearby histidine residue. The proposed mechanism of BamB, a formal hydride transfer in two steps, is in line with the two-electron chemistry commonly catalyzed by tungsten enzymes.

4. Synopsis of the Manuscripts

The main theme of this thesis is development and application of computational methods for studying mechanisms of enzyme catalysis, in particular mechanisms of key steps in aromatic compound metabolism. Over the forty years of computational enzymology history, a vast number of approaches to study the details of enzymatic mechanisms has been developed. The broad variety of computational enzymology approaches has been summarized in a review article (Manuscript A). Unfortunately, diverse scientific groups implement their newly developed computational methods in different software packages. This limits the applicability of otherwise interesting methods, because some method combinations are impossible to achieve. Conjugate peak refinement (CPR) method is a robust approach to locate transition state structures along the reaction pathway in complex systems. CPR has been, however, so far only implemented in the program CHARMM, which was not freely available at the time when I begun the work on my thesis. Furthermore, a proper usage of CHARMM, especially with hybrid QM/MM methods, is not always straightforward. Therefor, I participated on modification and implementation of the CPR method into a modern python-based framework pDynamo. The modified CPR implementation (called PyCPR) together with two application examples and a comparison to a broadly used nudged elastic band (NEB) method is described in Manuscript B. PyCPR completed the toolbox of QM/MM and continuum-electrostatic methods, which was used to investigate reactions involved in aromatic compound metabolism. Continuum electrostatics was used to determine protonation behavior of the proteins and their cofactors and also energetics of protonation from solvent within the reaction energy profiles. Reaction path alternatives were studied in the hybrid QM/MM environment and the PyCPR was used to locate the transition states.

In Manuscript B, a better highest transition state of the radical decarboxylation of 4-hydroxyphenylacetate than previously reported is found using PyCPR.

PhzF described in Manuscript C is part of the phenazine biosynthesis pathway. The aromatic phenazines serve as electron transporters and have antibiotic properties that promote survival of the phenazine-producing bacteria in competitive environment. Together with our experimental collaborators, we were able to clearly identify the mechanism of two subsequent isomerization steps catalyzed by PhzF. The BamB enzyme described in Manuscript D is a benzoyl-CoA reductase. Since benzoyl-CoA is a central intermediate of aromatic acid degradation metabolism in anaerobic bacteria, the knowledge of BamB mechanism is of high interest. Moreover, the BamB is able to reduce the aromatic ring without the usage of ATP. Instead, it is part of a large electron transfer complex (BamB-I) and contains an unusual tungstopterin cofactor in the active site. I was able to find the most feasible mechanism alternative and, in addition, bring more light into a question of uncertain small inorganic ligand of the tungsten in the active site.

Manuscript A: Computational Biochemistry – Enzyme Mechanisms Explored. Approaches to unravel the detailed mechanisms of enzyme catalysis using computer models are discussed in this review article. The field of computational biochemistry is put into a broader context of biochemical research and the indispensable role of computations as a complement to the experimental techniques is emphasized. We first list various structural model environments used to study the enzyme mechanisms together with their strengths and limits. In the second part, we describe the methods for calculation of kinetic and thermodynamic parameters of enzyme mechanisms. In particular, we discuss the approaches for obtaining the reaction pathway on the potential energy landscapes and the methods to get proper free energy estimates.

Manuscript B: PyCPR – a python-based implementation of the Conjugate Peak Refinement (CPR) algorithm for finding transition state structures. When investigating the enzyme mechanism details, a crucial task is to identify stable intermediate structures and transition state structures that lie between them. The free energy difference between a stable structure and the transition state structure on the way to the next stable state determines the rate of this transformation. A structural computer model (such as hybrid QM/MM model of an enzyme) assigns potential energy to a given structure and calculates

its derivatives. The energies of all possible conformations and chemical transformations of a molecular system plotted against atom coordinates form so-called potential energy surface (PES). The PES can be seen as a landscape with valleys and mountain passes (saddle points) that represent stable states and transition states. CPR is a method that identifies the minimum energy reaction pathway by locating the lowest saddle points on the path between two stable states. Although this powerful and robust method has been known for more than twenty years, the only implementation in the CHARMM program is far from easy to use.

In Manuscript B, we revise the theory behind the CPR method and thoroughly describe the new PyCPR implementation into a versatile python-based framework pDynamo. The CPR uses similar formalism as the conjugate gradient minimizer with an additional term to stay conjugate to the reaction path vector. Every cycle, the PyCPR algorithm first increases the discretization of the current reaction path by linear interpolation and picks to global energy maximum for saddle point optimization. The chosen structure is maximized along the path vector and subsequently minimized along the conjugate of steepest gradient direction, the path vector and the previous line minimization vector. In ideal case, the saddle point should be located in one cycle. In practice, especially for complex systems like enzymes, the saddle point optimization has to be broken due to the lost of conjugacy to the path vector. The semi-optimized structure is nevertheless added to the path and new CPR cycle starts. By an iterative process the path vector around the saddle point is improved and the saddle point is occasionally located. The high energy path points that cannot by optimized to saddle points are deleted in course of the iterative process. The PyCPR terminates once all maxima on the reaction path have been optimized to saddle points.

Our implementation provides a broad flexibility in algorithm parameter adjustments and the pDynamo framework ensures that the PyCPR can easily be used with different models (QM, MM, QM/MM) and combined with various other methods. To demonstrate the strength of PyCPR, two examples of application together with comparison to a broadly used NEB method are presented in Manuscript B. The gauche to anti-periplanar transition in butane is presented as a proof-of-concept example. In the second example, the difficult radical enzymatic decarboxylation of 4-hydroxyphenylacetate is revised. It is shown that PyCPR performs equivalently to NEB in the majority of the steps, yet it is able to locate the proper transition state for the rate-limiting final step where the NEB previ-

ously failed. In the end of Manuscript B, a practical guide how to use the PyCPR to efficiently find the reaction pathway is provided. Further two applications of the PyCPR method on yet unresolved mechanisms of enzymes participating in aromatic compound anabolism and catabolism are discussed in Manuscripts C and D, respectively.

Manuscript C: Mechanisms and Specificity of Phenazine Biosynthesis Protein PhzF. In this work, a combination of computational and experimental approaches is used to probe mechanistic alternatives of PhzF – an enzyme that is part of the biosynthetic pathway of tricyclic aromatic phenazines. PhzF catalyzes a two step isomerization of 2,3-dihydro-3-hydroxy anthranilate (DHHA) to 6-amino-5-oxo-2-cyclohexene-1-carboxylic acid (AOCHC). The first step is a suprafacial [1,5]-hydrogen shift between C3 and C1 of DHHA producing a metastable enol intermediate, which is subsequently tautomerized to the ketone product. Previous NMR experiments suggested a sigmatropic shift mechanism for the first step, while the mutagenesis experiments underlined crucial role of an active site glutamate (E45) in catalysis.

I performed continuum electrostatic calculations of the PhzF structure and found out that E45 has titration midpoint shifted to 9 in closed substrate-bound structure, while it remains around 5 in an open substrate-free structure. This shift indicates on high proton affinity of E45 once the substrate is bound. Subsequent QM/MM calculations confirm the crucial role of E45 as a proton shuttle in the first step. The sigmatropic shift with a direct proton transfer between the C3 and C1 shows prohibitive energy barriers, presumably due to the strain in four-membered ring of the transition state. The first step intermediate state with carbanion on the substrate and protonated E45 turns out to be quite labile, which could explain the unlikeliness of the proton exchange with the solvent and the previous interpretation of the macroscopic process as a sigmatropic shift. The kinetic measurements and my calculations show that E45D mutant is inactive. The proximity of protonated carboxyl to the carbanion in the intermediate state is obviously crucial for the proton transfer process, since the intermediate with protonated aspartate has much higher energy compared to the wild-type intermediate with glutamate. The QM/MM calculations further reveal a role of PhzF in the tautomerization step, where E45 together with a tightly bound water molecule is involved in the enol-ketone proton transfer in addition. These findings

are corroborated by Nuclear Overhauser Effect (NOE) NMR measurements done by our collaborators that suggest on stereospecific tautomerization. The overall barrier of the calculated energy profile corresponds perfectly to the experimental kinetic data presented together with my computational data in Manuscript C. In addition, a large kinetic isotope effect on C3-deuterated DHHA indicates that the initial proton abstraction is rate-limiting, as the QM/MM calculations predicted. In addition, my calculations are able to explain that the modified substrate (2,3-dihydro-3-dihydroxy salicylic acid) is not metabolized due to a decrease in acidity of the C3-bound hydrogen.

Manuscript D: Breaking the Aromaticity – Computational Insights Into Benzoyl-CoA Reduction by the Tungsten Enzyme BamB. This work deals with a reversible mechanism of tungsten-containing benzoyl-CoA reductase from strict anaerobic bacterium *Geobacter metallireducens*. X-ray crystalography was not able to unequivocally identify a small inorganic ligand (denoted X), which points towards the substrate and thus might play a crucial role in the reduction of the aromatic ring. The ligand X can be either sulfur or oxygen, common small ligands of tungsten enzymes, or a diatomic ligand such as cyanide or carbon monoxide, as suggested by an EXAFS study.
I performed continuum-electrostatic calculations and found a connection between substrate binding, tungsten oxidation state and active site protonation. Then I investigated a large number of mechanism scenarios in QM/MM environment with respect to tungsten oxidation state, ligand X nature and proton donor identity. Based on the calculation results, we are able to propose an aqua ligand as the most probable ligand X with reversible and energetically feasible reaction energy profile. An aqua ligand stabilizes low oxidation numbers of the tungsten and thus enables its reduction before the actual reaction. In the first reduction step, the coordinated water donates one proton to the substrate and a radical intermediate is formed. The second reduction step is accompanied by proton transfer from an active site histidine side chain. Although we found some promising reaction energy profiles for sulfur and cyanide as ligand X, they had to be ruled-out when analyzing the catalytic cycle in broader context. The sulfur ligand shows reversible energy profile only with double protonation. However, it is thermodynamically unfeasible to get the dihydrosulfate ligand in the regenerative phase of the catalytic cycle. The problem of the cyanide ligand is its protonated

form, which turns out to be an unavoidable thermodynamic trap. The proposed reversible mechanism involving aqua ligand as proton donor is in line with available experimental findings. My calculations also suggest on unprecedented direct involvement of the pyranopterin cofactor in the catalytic process.

Bibliography

[1] M. Chatzou, C. Magis, J.-M. Chang, C. Kemena, G. Bussotti, I. Erb, and C. Notredame. Multiple sequence alignment modeling: methods and applications. *Brief. Bioinform.*, 17:1009–1023, 2016.

[2] C. L. Mills, P. J. Beuning, and M. J. Ondrechen. Biochemical functional predictions for protein structures of unknown or uncertain function. *Comput. Struct. Biotechnol. J.*, 13:182–191, 2015.

[3] J. Colinge and K. L. Bennett. Introduction to Computational Proteomics. *PLoS Comput. Biol.*, 3:e114, 2007.

[4] M. Vaudel, K. Verheggen, A. Csordas, H. Ræder, F. S. Berven, L. Martens, J. A. Vizcaíno, and H. Barsnes. Exploring the potential of public proteomics data. *Proteomics*, 16:214–225, 2016.

[5] S. Uchida. Image processing and recognition for biological images. *Dev. Growth Differ.*, 55:523–549, 2013.

[6] Y. Cheng, N. Grigorieff, P. A. Penczek, and T. Walz. A primer to single-particle cryo-electron microscopy. *Cell*, 161:439–449, 2015.

[7] A. B. Tucker. *Computer science handbook*. Chapman & Hall/CRC, Boca Raton, 2004.

[8] R. O. Dror, R. M. Dirks, J. P. Grossman, H. Xu, and D. E. Shaw. Biomolecular simulation: a computational microscope for molecular biology. *Annu. Rev. Biophys.*, 41:429–452, 2012.

[9] D. J. Cole and N. D. M. Hine. Applications of large-scale density functional theory in biology. *J. Phys. Condens. Matter*, 28:393001, 2016.

[10] M. W. van der Kamp and A. J. Mulholland. Combined Quantum Mechanics/Molecular Mechanics (QM/MM) Methods in Computational Enzymology. *Biochemistry*, 52:2708–2728, 2013.

[11] R. T. Ullmann and G. M. Ullmann. GMCT : a Monte Carlo simulation package for macromolecular receptors. *J. Comput. Chem.*, 33:887–900, 2012.

[12] E. Bombarda and G. M. Ullmann. Continuum electrostatic investigations of charge transfer processes in biological molecules using a microstate description. *Faraday Discuss.*, 148:173–193, 2011.

[13] D. L. Nelson and M. M. Cox. *Lehninger Principles of Biochemistry 5th ed.* W. H. Freeman & Co, New York, 2008.

[14] K. M. Herrmann. The shikimate pathway as an entry to aromatic secondary metabolism. *Plant Physiol.*, 107:7–12, 1995.

[15] V. Tzin and G. Galili. The Biosynthetic Pathways for Shikimate and Aromatic Amino Acids in Arabidopsis thaliana. *Arab. B.*, 8:e0132, 2010.

[16] L. S. Pierson and E. A. Pierson. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl. Microbiol. Biotechnol.*, 86:1659–1670, 2010.

[17] G. Ladino-Orjuela, E. Gomes, R. da Silva, C. Salt, and J. R. Parsons. Metabolic Pathways for Degradation of Aromatic Hydrocarbons by Bacteria. In *Rev. Environ. Contam. Toxicol. Vol. 237*, pages 105–121. Springer International Publishing, 2016.

[18] D. Ghosal, S. Ghosh, T. K. Dutta, and Y. Ahn. Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Front. Microbiol.*, 7:1369, 2016.

[19] M. Gaur, R. Singh, and A. Shukla. Variability in the Levels of BTEX at a Pollution Hotspot in New Delhi, India. *J. Environ. Prot.*, 7:1245–1258, 2016.

[20] T. Lueders. The ecology of anaerobic degraders of BTEX hydrocarbons in aquifers. *FEMS Microbiol. Ecol.*, 93:fiw220, 2017.

[21] J.-S. Seo, Y.-S. Keum, and Q. X. Li. Bacterial degradation of aromatic compounds. *Int. J. Environ. Res. Public Health*, 6:278–309, 2009.

[22] J. M. Berg, J. L. Tymoczko, L. Stryer, and L. Stryer. *Biochemistry 5th ed.* W.H. Freeman, New York, 2002.

[23] H. Maeda and N. Dudareva. The Shikimate Pathway and Aromatic Amino Acid Biosynthesis in Plants. *Annu. Rev. Plant Biol.*, 63:73–105, 2012.

[24] G. Gosset. Production of aromatic compounds in bacteria. *Curr. Opin. Biotechnol.*, 20:651–658, 2009.

[25] K. Caetano-Anollés and Caetano-Anollés. Structural Phylogenomics Reveals Gradual Evolutionary Replacement of Abiotic Chemistries by Protein Enzymes in Purine Metabolism. *PLoS One*, 8:e59300, 2013.

[26] J. Zhan. Biosynthesis of bacterial aromatic polyketides. *Curr. Top. Med. Chem.*, 9:1958–1610, 2009.

[27] T. P. Begley, C. Kinsland, S. Taylor, M. Tandon, R. Nicewonger, M. Wu, H.-J. Chiu, N. Kelleher, N. Campobasso, and Y. Zhang. Cofactor Biosynthesis: A Mechanistic Perspective. In F. J. Leeper and J. C. Vederas, editors, *Biosynth. Polyketides Vitam.*, pages 93–142. Springer Berlin Heidelberg, 1998.

[28] M. Mentel, E. G. Ahuja, D. V. Mavrodi, R. Breinbauer, L. S. Thomashow, and W. Blankenfeldt. Of two make one: The biosynthesis of phenazines. *ChemBioChem*, 10:2295–2304, 2009.

[29] A. Price-Whelan, L. E. P. Dietrich, and D. K. Newman. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nat. Chem. Biol.*, 2:71–78, 2006.

[30] D. V. Mavrodi, J. A. Parejko, O. V. Mavrodi, Y.-S. Kwak, D. M. Weller, W. Blankenfeldt, and L. S. Thomashow. Recent insights into the diversity, frequency and ecological roles of phenazines in fluorescent Pseudomonas spp. *Environ. Microbiol.*, 15:675–686, 2013.

[31] G. W. Lau, D. J. Hassett, H. Ran, and F. Kong. The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends Mol. Med.*, 10:599–606, 2004.

[32] G. W. Lau, H. Ran, F. Kong, D. J. Hassett, and D. Mavrodi. Pseudomonas aeruginosa pyocyanin is critical for lung infection in mice. *Infect. Immun.*, 72:4275–4278, 2004.

[33] O. V. Mavrodi, D. V. Mavrodi, J. A. Parejko, L. S. Thomashow, and D. M. Weller. Irrigation differentially impacts populations of indigenous antibiotic-producing Pseudomonas spp. In the rhizosphere of wheat. *Appl. Environ. Microbiol.*, 78:3214–3220, 2012.

[34] D. V. Mavrodi, O. V. Mavrodi, J. A. Parejko, R. F. Bonsall, Y. S. Kwak, T. C. Paulitz, L. S. Thomashow, and D. M. Weller. Accumulation of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of dryland cereals. *Appl. Environ. Microbiol.*, 78:804–812, 2012.

[35] N. R. Glasser, S. E. Kern, and D. K. Newman. Phenazine redox cycling enhances anaerobic survival in Pseudomonas aeruginosa by facilitating generation of ATP and a proton-motive force. *Mol. Microbiol.*, 92:399–412, 2014.

[36] W. Blankenfeldt, A. P. Kuzin, T. Skarina, Y. Korniyenko, L. Tong, P. Bayer, P. Janning, L. S. Thomashow, and D. V. Mavrodi. Structure and function of the phenazine biosynthetic protein PhzF from Pseudomonas fluorescens. *Proc. Natl. Acad. Sci. U. S. A.*, 101:16431–16436, 2004.

[37] E. G. Ahuja, P. Janning, M. Mentel, A. Graebsch, R. Breinbauer, W. Hiller, B. Costisella, L. S. Thomashow, D. V. Mavrodi, and W. Blankenfeldt. PhzA/B catalyzes the formation of the tricycle in phenazine biosynthesis. *J. Am. Chem. Soc.*, 130:17053–17061, 2008.

[38] C. Eberlein, J. Johannes, H. Mouttaki, M. Sadeghi, B. T. Golding, M. Boll, and R. U. Meckenstock. ATP-dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of naphthalene. *Environ. Microbiol.*, 15:1832–1841, 2013.

[39] M. M. E. Huijbers, S. Montersino, A. H. Westphal, D. Tischler, and W. J. H. Van Berkel. Flavin dependent monooxygenases. *Arch. Biochem. Biophys.*, 544:2–17, 2014.

[40] R. E. Parales and S. M. Resnick. Aromatic hydrocarbon dioxygenases. In *Biodegrad. Bioremediation*, pages 175–195. Springer Berlin Heidelberg, 2004.

[41] S. Fetzner. Ring-cleaving dioxygenases with a cupin fold. *Appl. Environ. Microbiol.*, 78:2505–2514, 2012.

[42] C. Vogt, S. Kleinsteuber, and H. H. Richnow. Anaerobic benzene degradation by bacteria. *Microb. Biotechnol.*, 4:710–724, 2011.

[43] B. Philipp and B. Schink. Evidence of two oxidative reaction steps initiating anaerobic degradation of resorcinol (1,3-dihydroxybenzene) by the denitrifying bacterium Azoarcus anaerobius. *J. Bacteriol.*, 180:3644–3649, 1998.

[44] K. Breese and G. Fuchs. 4-hydroxybenzoyl-CoA reductase (dehydroxylating) from the denitrifying bacterium Thauera aromatica - Prosthetic groups, electron donor, and genes of a member of the molybdenum-flavin-iron-sulfur proteins. *Eur. J. Biochem.*, 251:916–923, 1998.

[45] S. Wischgoll, D. Heintz, F. Peters, A. Erxleben, E. Sarnighausen, R. Reski, A. Van Dorsselaer, and M. Boll. Gene clusters involved in anaerobic benzoate degradation of Geobacter metallireducens. *Mol. Microbiol.*, 58:1238–1252, 2005.

[46] R. Teufel, V. Mascaraque, W. Ismail, M. Voss, J. Perera, W. Eisenreich, W. Haehnel, and G. Fuchs. Bacterial phenylalanine and phenylacetate catabolic pathway revealed. *Proc. Natl. Acad. Sci. U. S. A.*, 107:14390–5, 2010.

[47] L. J. Rather, T. Weinert, U. Demmer, E. Bill, W. Ismail, G. Fuchs, and U. Ermler. Structure and mechanism of the diiron benzoyl-coenzyme A epoxidase BoxB. *J. Biol. Chem.*, 286:29241–29248, 2011.

[48] G. Fuchs, M. Boll, and J. Heider. Microbial degradation of aromatic compounds – from one strategy to four. *Nat. Rev. Microbiol.*, 9:803–816, 2011.

[49] M. Boll and G. Fuchs. Benzoyl-coenzyme A reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism. ATP dependence of the reaction, purification and some properties of the enzyme from Thauera aromatica strain K172. *Eur. J. Biochem.*, 234:921–933, 1995.

[50] M. Boll, G. Fuchs, and D. J. Lowe. Single turnover EPR studies of benzoyl-CoA reductase. *Biochemistry*, 40:7612–7620, 2001.

[51] J. W. Kung, C. Löffler, K. Dörner, D. Heintz, S. Gallien, A. Van Dorsselaer, T. Friedrich, and M. Boll. Identification and characterization of the tungsten-containing class of benzoyl-coenzyme A reductases. *Proc. Natl. Acad. Sci. U. S. A.*, 106:17687–17692, 2009.

[52] W. Buckel and R. K. Thauer. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na^+ translocating ferredoxin oxidation. *Biochim. Biophys. Acta - Bioenerg.*, 1827:94–113, 2013.

[53] T. Weinert, S. G. Huwiler, J. W. Kung, S. Weidenweber, P. Hellwig, H.-J. Stärk, T. Biskup, S. Weber, J. J. H. Cotelesage, G. N. George, U. Ermler, and M. Boll. Structural basis of enzymatic benzene ring reduction. *Nat. Chem. Biol.*, 11:586–591, 2015.

[54] R. Hille. Molybdenum and tungsten in biology. *Trends Biochem. Sci.*, 27:360–367, 2002.

[55] L. B. Maia, I. Moura, and J. J. G. Moura. Molybdenum and Tungsten-Containing Enzymes: An Overview. In *Molybdenum Tungsten Enzym.*, pages 1–80. Royal Society of Chemistry, Cambridge, 2016.

[56] R. U. Meckenstock, R. Krieger, S. Ensign, P. M. Kroneck, and B. Schink. Acetylene hydratase of Pelobacter acetylenicus. Molecular and spectroscopic properties of the tungsten iron-sulfur enzyme. *Eur. J. Biochem.*, 264:176–182, 1999.

[57] B. M. Rosner and B. Schink. Purification and characterization of acetylene hydratase of Pelobacter acetylenicus, a tungsten iron-sulfur protein. *J. Bacteriol.*, 177:5767–5772, 1995.

[58] S. Leimkühler and R. R. Mendel. Molybdenum Cofactor Biosynthesis. In *Molybdenum Tungsten Enzym.*, pages 100–116. Royal Society of Chemistry, Cambridge, 2016.

[59] R. A. Rothery, B. Stein, M. Solomonson, M. L. Kirk, and J. H. Weiner. Pyranopterin conformation defines the function of molybdenum and tungsten enzymes. *Proc. Natl. Acad. Sci.*, 109:14773–14778, 2012.

[60] M. G. Bertero, R. A. Rothery, M. Palak, C. Hou, D. Lim, F. Blasco, J. H. Weiner, and N. C. J. Strynadka. Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. *Nat. Struct. Biol.*, 10:681–687, 2003.

[61] D. P. Kloer, C. Hagel, J. Heider, and G. E. Schulz. Crystal Structure of Ethylbenzene Dehydrogenase from Aromatoleum aromaticum. *Structure*, 14:1377–1388, 2006.

[62] D. M. A. M. Luykx, J. A. Duine, and S. De Vries. Molybdopterin radical in bacterial aldehyde dehydrogenases. *Biochemistry*, 37:11366–11375, 1998.

[63] S.-Y. Wu, R. A. Rothery, and J. H. Weiner. Pyranopterin Coordination Controls Molybdenum Electrochemistry in Escherichia coli Nitrate Reductase. *J. Biol. Chem.*, 290:25164–25173, 2015.

[64] C. Dong, J. Yang, S. Leimkuehler, and M. L. Kirk. Pyranopterin Dithiolene Distortions Relevant to Electron Transfer in Xanthine Oxidase/Dehydrogenase. *Inorg. Chem.*, 53:7077–7079, 2014.

[65] M. K. Chan, S. Mukund, A. Kletzin, M. W. Adams, and D. C. Rees. Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science*, 267:1463–1469, 1995.

[66] L. E. Bevers, P.-L. Hagedoorn, and W. R. Hagen. The bioinorganic chemistry of tungsten. *Coord. Chem. Rev.*, 253:269–290, 2009.

[67] R.-Z. Liao, J.-G. Yu, and F. Himo. Tungsten-dependent formaldehyde ferredoxin oxidoreductase: Reaction mechanism from quantum chemical calculations. *J. Inorg. Biochem.*, 105:927–936, 2011.

[68] S. Mukund and M. W. W. Adams. Glyceraldehyde-3-phosphate Ferredoxin Oxidoreductase, a Novel Tungsten-containing Enzyme with a Potential Glycolytic Role in the Hyperthermophilic Archaeon Pyrococcus furiosus. *J. Biol. Chem.*, 270:8389–8392, 1995.

[69] R. Roy, S. Mukund, G. J. Schut, D. M. Dunn, R. Weiss, and M. W. Adams. Purification and molecular characterization of the tungsten-containing formaldehyde ferredoxin oxidoreductase from the hyperther-

mophilic archaeon Pyrococcus furiosus: the third of a putative five-member tungstoenzyme family. *J. Bacteriol.*, 181:1171–1180, 1999.

[70] L. E. Bevers, E. Bol, P.-L. Hagedoorn, and W. R. Hagen. WOR5, a Novel Tungsten-Containing Aldehyde Oxidoreductase from Pyrococcus furiosus with a Broad Substrate Specificity. *J. Bacteriol.*, 187:7056–7061, 2005.

[71] H. Raaijmakers, S. Macieira, J. M. Dias, S. Teixeira, S. Bursakov, R. Huber, J. J. G. Moura, I. Moura, and M. J. Romão. Gene sequence and the 1.8 A crystal structure of the tungsten-containing formate dehydrogenase from Desulfovibrio gigas. *Structure*, 10:1261–1272, 2002.

[72] N. M. F. S. A. Cerqueira, P. A. Fernandes, P. J. Gonzalez, J. J. G. Moura, and M. J. Ramos. The Sulfur Shift: An Activation Mechanism for Periplasmic Nitrate Reductase and Formate Dehydrogenase. *Inorg. Chem.*, 52:10766–10772, 2013.

[73] A. Hochheimer, R. A. Schmitz, R. K. Thauer, and R. Hedderich. The tungsten formylmethanofuran dehydrogenase from Methanobacterium thermoautotrophicum contains sequence motifs characteristic for enzymes containing molybdopterin dinucleotide. *Eur. J. Biochem.*, 234:910–20, 1995.

[74] T. Wagner, U. Ermler, and S. Shima. The methanogenic CO_2 reducing-and-fixing enzyme is bifunctional and contains 46 [4Fe-4S] clusters. *Science*, 354:114–117, 2016.

[75] G. B. Seiffert, G. M. Ullmann, A. Messerschmidt, B. Schink, P. M. H. Kroneck, and O. Einsle. Structure of the non-redox-active tungsten/[4Fe:4S] enzyme acetylene hydratase. *Proc. Natl. Acad. Sci. U. S. A.*, 104:3073–3077, 2007.

[76] R.-Z. Liao, J.-G. Yu, and F. Himo. Mechanism of tungsten-dependent acetylene hydratase from quantum chemical calculations. *Proc. Natl. Acad. Sci. U. S. A.*, 107:22523–22527, 2010.

Manuscripts

Individual Contributions to the Manuscripts

A **Martin Culka**[*], Florian J. Gisdon[*] and G. Matthias Ullmann (2017): Computational Biochemistry – Enzyme Mechanisms Explored. *Adv. Protein Chem. Struct. Biol.*, 109, 77–112

The two core chapters of the review – about types of structural computer models of enzymes and about techniques to model enzyme catalysis on computer – were written jointly by myself and Florian J. Gisdon. G. Matthias Ullmann wrote the introductory and concluding paragraphs and helped to prepare the final version of the manuscript.

B Florian J. Gisdon[*], **Martin Culka**[*] and G. Matthias Ullmann (2016): PyCPR – a python-based implementation of the Conjugate Peak Refinement (CPR) algorithm for finding transition state structures. *J. Mol. Model.*, 22, 242

The PyCPR algorithm was modified and implemented by myself and Florian J. Gisdon. The calculations on butane application example were performed by Florian J. Gisdon, while the calculations on 4-hydroxyphenylacetate decarboxylase were performed by myself. The manuscript was written by myself and Florian Gisdon together with G. Matthias Ullmann.

C Christina Diederich[*], Mario Leypold[*], **Martin Culka**[*] , Hansjörg Weber, Rolf Breinbauer, G. Matthias Ullmann and Wulf Blankenfeldt (2017): Mechanism and specificity of phenazine biosynthesis protein PhzF. *Sci. Rep.*, 7, 6272

The experimental data were acquired and analyzed by Christina Diederich, Mario Leypold, Hansjörg Weber, Rolf Breinbauer and Wulf Blankenfeldt. I performed all the continuum-electrostatic, QM and QM/MM calculations presented in the manuscript. The computational results were analyzed and put into context with the experimental data by myself, G. Matthias Ullmann and Wulf Blankenfeldt. The manuscript was written by Wulf Blankenfeldt. All authors have discussed and contributed to the final version of the manuscript.

D **Martin Culka**, Simona G. Huwiler, Matthias Boll and G. Matthias Ullmann (2017): Breaking Benzene Aromaticity - Computational Insights into the Mechanism of the Tungsten-Containing Benzoyl-CoA Reductase. *J. Am. Chem. Soc.*, 139, 14488–14500

All calculations presented in the manuscript were performed by myself. The calculation results were analyzed and interpreted by myself together with G. Matthias Ullmann. Simona G. Huwiler contributed the multiple sequence alignment. Simona G. Huwiler and Matthias Boll contributed to the discussions about the mechanistic variants in context with the experimental data. The manuscript was prepared by myself and G. Matthias Ullmann. All authors have discussed and contributed to the final version of the manuscript.

* These authors contributed equally to the manuscript

Manuscript A

"Computational Biochemistry – Enzyme Mechanisms Explored"

Martin Culka*, Florian J. Gisdon*, G. Matthias Ullmann

Adv. Protein Chem. Struct. Biol., 109, 77–112 (2017)

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* These authors contributed equally to the manuscript

Manuscript B

"PyCPR – a python-based implementation of the Conjugate Peak Refinement (CPR) algorithm for finding transition state structures"

Florian J. Gisdon*, Martin Culka*, G. Matthias Ullmann

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* These authors contributed equally to the manuscript

Manuscript C

"Mechanisms and Specificity of Phenazine Biosynthesis Protein PhzF"

Christina Diederich*, Mario Leypold*, Martin Culka* , Hansjörg Weber, Rolf Breinbauer, G. Matthias Ullmann and Wulf Blankenfeldt

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* These authors contributed equally to the manuscript

Manuscript D

"Breaking Benzene Aromaticity - Computational Insights into the Mechanism of the Tungsten-Containing Benzoyl-CoA Reductase"

Martin Culka, Simona G. Huwiler, Matthias Boll, G. Matthias Ullmann

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