Conclusions

And

Future Outlook
Conclusions?

After nine chapters with conclusion sections, what is left to conclude? We knew that we know nothing 2500 years ago, and—arguably—it hasn’t changed a lot in absolute terms. The very foundation of research is the curiosity-driven quest for discoveries. However, what we discover, often doesn’t answer anything, and just leads to more questions. Thus, with the knowledge accumulated in a PhD arguably positioning one at the minimum of the Dunninger-Kruger plot, it seems adequate to also end this thesis with questions, instead of answers.

![Figure 1. The Dunninger-Kruger effect.](image)

Biocatalysis to the rescue?

The extensive literature on monooxygenases (Chapters 1-2) reflect the continuous interest in these catalysts. Though some representatives obviously emerged from investigations of biosynthetic pathways, many monooxygenases have been explored exclusively for their potential in application. This development gives hope that monooxygenases may be at the forefront of a new industrial revolution. In the future, we need to drastically change our production systems. This is especially true for the chemical industry that produces so many of the goods that sustain the living standards of this planet’s population, which approaches eight billion people in 2019. Considering the frustratingly slow progress of political action to our global problems, technological advances might be our best bet to stop the downward spiral caused by the combination of overpopulation and increased consumption with resource depletion and environmental damage. As the chemical industry is a major player in this development, catalysis with green reagents can make an enormous impact of global scale. Biocatalysis has the potential to emerge as a “game changer”, as it combines sustainability with efficiencies. The major technological limitations that remain to be overcome are largely the same for monooxygenases as for other enzyme classes: stability and specificity. The here
presented work shows that a combination of classical enzyme discovery and characterization with computational approaches can successfully tackle these issues. Research and development in both techniques causes a mutually beneficial advance, an effect exemplified also in various parts of this thesis.

**Cytochrome P450s—what lurks in the shadow of the king of catalysis?**

P450s are extremely popular enzymes in the biocatalysis community, because they are such an obvious example of superiority of enzyme catalysis. As one of the holy grails of organic chemistry, their ability of selective carbon activation means a tremendous synthetic (and thus commercial) potential. The other side of the medal is only too well known to enzymologists with hands-on experience: poor stability and unpredictable catalytic behavior. Thermostability can hopefully be achieved—with new variants, such as the one described in Chapter 3, and using engineering techniques as outlined in Chapters 5 and 6. However, the greater challenge will be to alter the stability of the reactive heme and the associated fate of a substrate to be either accepted or induce uncoupling. Natively, P450s will never display capabilities that are ideally suited for application: as nature knows that with great power comes great responsibility, it has fenced its most reactive catalysts with deliberate restrictions. These probably are also reflected in catalytic control through enzyme flexibility (Chapter 1), which add a layer of complexity to the intricate catalytic mechanism. These reasons make also computational predictions vastly more complicated, where the fear is that only extensive simulation times can cover all mechanistic steps leading to substrate acceptance, while at the same time only theoretical descriptors at the highest level of theory can adequately calculate the electronic nature of the catalytic heme species and thus predict the reaction outcome. With technological advances progressing in a reliably astonishing pace and new and creative methodologies improving simulation accuracies at ever longer timescales, we may yet see these hurdles overcome. On the other hand, if the development stalls, a serious competitor in the enzyme world—peroxygenases—has emerged as a promising alternative.

**Baeyer-Villiger monooxygenases—is the field exhausted?**

Although to perhaps a lesser extent, many of the above considerations apply also to BVMOs. Even though a scan of the recent literature can sometimes give the impression of a von Baeyer-like confidence in the field, it seems appropriate to doubt which of the two maxima in Figure 1 our current knowledge is approaching. The most important open questions lie in the mechanism and they may or may not all be connected to each other. The uncertainty about the kinetic step of BVMOs found to be rate-limiting (Chapter
needs to be resolved. If it turns indeed out to be a conformational change, its nature (side-chains, loops, cofactors, domains?) needs to be elucidated. In relation to that, the exact position of the substrate during catalysis must be clarified (Chapter 1). Only if these uncertainties can be dispelled, computational analyses can be taken seriously and be used in a predictive way. This will hopefully largely reduce the workload currently required to engineer desired activities (Chapter 9) and allow the design of tailor-made mutants for specific substrates. Similarly, the puzzle of substrate promiscuity will then need to be connected to these insights. Possibly related is another mechanistic open question—the reduction of the flavin by NADPH. The incoherency of the stereochemistry of hydride transfer with the sliding mechanism is not just a curiosity, it will also be essential in enabling the engineering of true dependency on the dephosphorylated cofactor.

Next, the issue of substrate and product inhibition has been largely unaddressed. The reason may be that so far, the low stability often has masked this limitation. However, with new homologs and engineered variants, this issue has become and will be the more important new bottleneck. Also here, an approach aiming to tackle the underlying cause, and not just the symptoms would be desirable. However, until cleverly designed experiments are able to establish the mechanism of inhibition and protein engineering can be applied to overcome it, another focus will lie in process design and engineering. Chemoenzymatic systems employing (co)solvents and cascade reactions have already become popular and many more examples are expected to be developed in the future. An extended knowledge will also be valuable for stability engineering, where seemingly distant mutations can sometimes abolish activity (Chapter 7). And although the stability of BVMOs has been tackled (Chapters 4-7), it can be doubted that this is enough to reach a broad application. However, with so many thermo- and hyperthermostable enzymes known from other enzyme families, it seems fair to speculate that it is only a matter of time until a BVO representative will be discovered as well. Other approaches such as ancestral sequence reconstruction could also create thermostable BVMOs that likely show a broad substrate scope.

Lastly, the stability of the peroxyflavin should be better investigated. Uncertainties about variations in the mode of uncoupling exist, and the influencing factors are largely unknown. Although not as pressing as in P450s, improvements in oxygenation coupling will make BVMO reactions more reliable and efficient.

References