Iron catalyzed oxidation chemistry
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Chapter 7
Discussion, conclusions and future prospects

The major results of the research described in this thesis are summarized and discussed in this chapter. Furthermore, some future prospects are provided.
The glycopeptide bleomycin (BLM) and in particular the compound iron bleomycin (Fe(BLM)) is a highly potent natural compound, which oxidizes DNA readily.\textsuperscript{1-7} Under aerobic conditions, Fe(BLM) is capable of abstracting a hydrogen from the C4'-position of DNA’s sugar backbone, which can result, ultimately, in a scission of the DNA strand.\textsuperscript{8} When only one of the two DNA strands of DNA is cut, the process is referred to as single strand DNA cleavage. Fe(BLM) is also capable of inducing so-called double strand cleavage in the DNA. This occurs when two of these cleavage events have taken place in opposite strands of the DNA in close proximity in rapid succession. \textit{In vivo} double strand DNA cleavage can result in degradation of DNA and consequently in cell death. It is for this reason that bleomycin is administered as a chemotherapeutic treatment for some types of cancer.\textsuperscript{9}

Research directed at elucidating the reaction mechanism points towards the involvement of key intermediates such as (BLM)Fe\textsuperscript{III}OOH and (BLM)Fe\textsuperscript{IV}=O in the hydrogen abstraction step.\textsuperscript{5,10} Many model systems for bleomycin have been published over the years in an attempt to mimic its structure, spectroscopic properties or DNA cleavage activity (or a combination of these) with mixed success. In our group, the pentadentate ligand N4Py has been developed as a functional mimic of bleomycin (Figure 7.1, left).\textsuperscript{11} After coordination to iron(II) (Figure 7.1, complex 1, center), the complex was found to be a good spectroscopic model for Fe(BLM) \textit{(vide infra)}. Moreover, Fe(N4Py) can function as a functional model for Fe(BLM) as this complex was found to be active in the oxidation of DNA. When a DNA intercalating moiety \textit{(i.e. acridine)} was attached covalently to this complex (Figure 7.1, complex 2, right), the complex was, structurally, even more comparable to Fe(BLM). In bleomycin the affinity for DNA was enhanced via intercalation or (minor) groove binding via a bisthiazole moiety, attached covalently to the metal binding domain. The activity of the catalyst 2 in DNA oxidation was increased dramatically, as instant DNA cleavage activity was observed without the need for a sacrificial reductant, which is most often a requirement for DNA cleavage activity with other synthetic complexes as catalysts.\textsuperscript{12}

\textbf{Figure 7.1} The ligand N4Py (left) and two mononuclear Fe\textsuperscript{II}(N4Py) complexes used in this study as catalyst in the oxidative cleavage of DNA.

In DNA cleavage studies, a plasmid DNA is used as a benchmark substrate. The initial superhelical DNA is transformed into relaxed circular DNA (nicked DNA) when only one of the DNA strands is cut. A second cut in the opposite DNA strand within close proximity of the first cut results in the formation of linear DNA. A statistical analysis, based on a Poisson distribution, of the cleavage results obtained \textit{(i.e. the relative amount of supercoiled, nicked and linear DNA)}, allows for discrimination distinct of whether the linear DNA is formed via a single strand cleavage pathway or a double strand cleavage pathway. For proper analysis, it is necessary to quantify the amounts of supercoiled, nicked and linear DNA accurately. This can be achieved by gel electrophoresis and subsequent staining of the gel with ethidium bromide (EtBr). Supercoiled DNA has a reduced uptake capacity of EtBr with respect to the nicked and linear DNA and a factor has to be applied to
correct for this phenomenon. Unfortunately, there are numerous misconceptions in the literature regarding this correction factor. The reality is that a correction factor is a unique property of each type of plasmid DNA and cannot under any circumstances be used interchangeably between different plasmids. In these studies the high copy pUC18 plasmid was used, which allowed for rapid access to suitable amounts of DNA. This in contrast to the more frequently used low copy plasmids in other studies. The correction factor for pUC18 was determined to be 1.31 (Chapter 2).

The two mononuclear Fe(N4Py) complexes 1 and 2 (Figure 7.1) were found to induce DNA strand cuts solely via single strand cleavage pathways. The covalent attachment of a DNA intercalator (complex 2) results in a four-fold increase of the reaction rate with respect to the original Fe(N4Py) complex (complex 1).

The use of enzymatic additives, such as superoxide dismutase (SOD) and catalase, during oxidative DNA cleavage experiments provided insight in the mechanism of the activation of dioxygen. It was found that the reaction was dominated by the formation of reduced oxygen species, in particular superoxide radicals. It is likely that these superoxide radicals react with the iron complex to form iron intermediates in a higher oxidation state, rather than that superoxide itself is involved in the process of hydrogen abstraction from DNA’s sugar backbone. The addition of DMSO to the reaction mixture did not influence the cleavage activity, suggesting a minimal role of hydroxyl radicals as a hydrogen abstracting species.

The next step was to further develop the Fe(N4Py) complex in such a way that also double strand cleavage can be achieved. The approach to achieve this was to attach two of these Fe(N4Py) complexes (two typical single strand cleavage complexes) covalently to one another and in this way obtain a dinuclear complex that is capable of inducing double strand cuts in DNA. The desired ligands were synthesized from known N4Py derivatives and were coordinated to iron(II) in situ prior to use in DNA cleavage experiments (Figure 7.2).

![Figure 7.2](image)

Figure 7.2 Representation of the dinuclear complexes used in this study (left) and the average number of double strand cuts (dsc) per DNA molecule ($m$) as a function of the average number of single strand cuts (ssc) per DNA molecule ($n$) for some representative dinuclear complexes (right). The dashed line is Freifelder-Trumbo equation.13

The DNA oxidation studies, using supercoiled pUC18 plasmid DNA as substrate and employing the new dinuclear complexes as catalysts, showed some interesting features. Initially, only nicked DNA is formed, which is typical of a single strand cleavage agent, such as Fe(N4Py). However, already at an early stage of the reaction significant amounts of
linear DNA are produced, which is not consistent with the activity one would expect from a single strand cleavage agent. By calculating the number of single and double strand cuts in DNA by these dinuclear complexes it was clear that initially only single strand cleavage takes place, but at a later stage of the reaction significant double strand cleavage was observed (Figure 7.2). The number of double strand cuts in DNA deviates significantly from the theoretical value predicted for a single strand cleavage pathway (Freifelder-Trumbo relationship).\(^{13}\)

A significant amount of linear DNA is formed at an early stage of the reaction after a significant amount of nicked DNA was formed. It was, therefore, thought that nicked DNA might be more susceptible towards double strand cleavage. When nicked DNA was used as a substrate instead of the commonly used supercoiled DNA it was found that a significantly higher amount of linear DNA was produced over the same time period with a dinuclear complex compared to a mononuclear complex. Furthermore, the number of double strand cuts obtained with the dinuclear complex is higher than can be accounted for on a statistical basis, whereas with the mononuclear complex the number of double strand cuts is the result of extensive single strand DNA cleavage. The proposed reason for the observation that the nicked DNA is a more accessible substrate than the supercoiled DNA for the dinuclear complexes is that the two active centers are indeed involved in two cleavage events in proximity. However, the superhelicity of the supercoiled DNA might result in these two events occurring close in space, but not close in terms of DNA sequence. Overall, these experiments demonstrate unequivocally that the dinuclear Fe(N4Py) complexes display direct double strand DNA cleavage as opposed to the mononuclear Fe(N4Py) complexes.

There seems to be little dependence on the nature of the spacer between the two active metal centers in the dinuclear complexes. It was found that spacer rigidity, length and orientation do not significantly influence the observed reactivity in terms of overall activity and type of DNA cleavage (single or double strand DNA cleavage).

The introduction of a third Fe(N4Py) bound covalently to the other two moieties showed a small but significant increase in the number of double strand cuts. Surprisingly, the covalent attachment of an acridine to the dinuclear complexes did not lead to an expected rate increase of DNA cleavage, but rather to a retardation of the reaction. Overall, it seems that the DNA cleavage activity of the dinuclear complexes is not affected significantly by DNA affinity parameters (such as increased electrostatics or DNA intercalation).

Generally, it can be concluded that double strand DNA cleavage can be achieved via the covalent connection of two single strand cleavage agents. The dinuclear Fe(N4Py) complexes are the first example of synthetic DNA cleavage agents, which can induce double strand cleavage catalytically. Moreover, these dinuclear iron complexes are the first examples in which double strand cleavage activity is established unequivocally and has been quantified.

The mechanism of the oxidation of DNA by Fe(N4Py) complexes was studied. It was demonstrated that the reaction is dominated primarily by the formation of reduced oxygen species (in particular superoxide radicals), but their exact relationship to the catalyst is still poorly understood. Further spectroscopic study may shed more light on this matter and should be focused on the characterization mechanistically relevant species of Fe(N4Py), which are formed during oxidative DNA cleavage. In turn, the outcome of such a study could prove useful in understanding the activity of other systems in more detail, e.g. Fe(BLM).

A change of the acridine for another more selective DNA binding moiety might be worth investigating as well. In this way, selective DNA cleavage can be accessed. Another aspect
that might be interesting for future research is examining the actual activity of the Fe(N4Py) complexes against (cancer) cell lines. Initially, this requires study and optimization of the system in terms of cell permeability. The ultimate goal in these studies will be the development of a DNA cleavage system, which can discriminate between different cell lines.

Another topic, which has been investigated in this thesis, is the catalytic oxidation of alkanes catalyzed by 1. Prior studies in our group have demonstrated that the treatment of 1 with H2O2 in acetone led to the formation of (N4Py)FeIIIOOH, which has spectroscopic features comparable to those of (BLM)FeIIIOOH. It was established that this so-called ‘purple intermediate’ plays a crucial role in the catalytic cycle of the C-H bond activation of alkanes. Furthermore, it was proposed that this intermediate undergoes a homolysis of the O-O bond to form a high-valent (N4Py)FeIV=O (3) species together with a hydroxyl radical. Both of these species can engage in hydrogen abstraction from a target substrate during catalysis. More recent investigations have demonstrated that this (N4Py)FeIV=O species could be accessed independently and that this species was indeed capable of inducing hydrogen abstraction from a target substrate under single turnover conditions.

The catalytic oxidation of cyclohexane to cyclohexanol and cyclohexanone with peracids and 1 as catalyst was used as a benchmark reaction. It was found that the use of mCPBA instead of H2O2 as oxidant resulted in a more selective reaction (higher alcohol to ketone ratio; A/K ratio), whilst retaining a comparable overall oxidation activity. This higher A/K ratio points towards a more selective oxidation than that involving a Russell type process, which is thought to be largely responsible for oxidation when H2O2 is employed as terminal oxidant. Other catalytic probes (such as adamantane oxidation, high kinetic isotope effects (KIE) and the absence of benzene oxidation) confirm the hypothesis that a more selective process is responsible for the oxidation of cyclohexane. Spectroscopic techniques have demonstrated the presence of the high-valent (N4Py)FeIV=O (3) intermediate throughout the course of the oxidation activity. Therefore, it is proposed that this species is largely responsible for the hydrogen abstraction from the target substrate resulting in an alkyl radical, which can be oxidized.

A catalytic cycle can be proposed based on the available data (Scheme 7.1). In this catalytic cycle, the key intermediate (N4Py)FeIV=O can act as either a hydrogen abstracting species (for which there is evidence) and as the actual oxidant of the resulting alkyl radical (which is speculative). A discrepancy in this proposal is that the overall catalysis rate is significantly higher than would be expected from the reported rate constant for the hydrogen abstraction from cyclohexane by 3 (kobs = 0.00039 s⁻¹). These kinetic data demonstrate that not all aspects are understood exactly and that further research is required to obtain more information about the exact role of the high-valent intermediate 3 in the catalytic cycle of alkane oxidation.

Another important finding in this study was the involvement of dioxygen in the reaction. When dioxygen was excluded from the reaction, the catalyst slowly reduces back to the initial FeIII(N4Py) species. However, if the reaction was performed under air, this spontaneous reduction does not take place and a catalytically inactive species was formed.
Scheme 7.1 Proposed catalytic cycle for the oxidation of alkanes with 1 as catalyst and mCPBA as oxidant

Several questions have remained unanswered and could form the basis of further study. First of all, what is or are the exact role(s) of (N4Py)Fe IV=O? Is only this species responsible for the hydrogen abstraction of the target substrate or can it act as a terminal oxidant for the formed alkyl radicals also? Perhaps isotope labeling studies could provide more insight. Another poorly understood aspect is the interaction of dioxygen with the catalyst during catalysis. A catalytically inactive species is formed, but the exact structure is remains elusive to date. The spontaneous reduction to the initial catalyst 1 has been the subject of a preliminary investigation, but it requires more attention in order to fully understand this process.

It would be of considerable interest if the results of the Fe(N4Py) catalyst could be placed in a broader perspective. Fe(N4Py) has been developed as a model for Fe(BLM). Therefore, it would be useful to establish whether the chemistry displayed by Fe(N4Py) is similar to that of Fe(BLM). If it can be demonstrated that the results obtained with Fe(N4Py) have a strong parallel with Fe(BLM), it would provide further evidence for the existence of high-valent Fe(BLM) intermediates in nature.

Overall, Fe(N4Py) has proven to be one of the most versatile and active non-heme iron catalysts in the oxidation of substrates ranging from cyclohexane to DNA. However, lots of challenging research remains in this area and for this reason further investigation of Fe(N4Py) and it’s applications is warranted.

References
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