Non-haem ligands as functional templates for peptide attachment
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 27-03-2019
2.1 Introduction

Several micro-organisms facilitate the metabolism of various hydrocarbons and aromatic compounds via the activation of molecular oxygen by metalloproteins to provide a vital source of energy for the organism. Many of these enzymes contain a transition-metal ion at the reactive site of the protein. The rate of aerobic degradation of these substrates is in general relatively slow. Metalloenzymes, one class of nature’s catalysts, provide a low energy pathway for oxidation by either enhancing the susceptibility of the substrate towards molecular oxygen, or by increasing the reactivity of dioxygen. A great deal of research has been dedicated to the development of small synthetic catalysts that are also capable of activating molecular oxygen or oxidising hydrocarbons.

Oxidases form a category of enzymes that employ dioxygen as an electron acceptor. The reduction products are either water or hydrogen peroxide, depending on the number of electrons involved. The oxygenases are a distinct class of enzymes that are able to incorporate dioxygen into (in)organic substrates. This conversion is crucial to their aerobic degradation, which is accompanied by, for example, the oxygenation or cleavage of the aromatic ring. These oxidising enzymes can be divided into mono-oxygenases or dioxygenases, depending on the

\[
\begin{align*}
R-H + O_2 & \xrightarrow{\text{mono-oxygenase}} R-OH + H_2O \\
R-H + O_2 & \xrightarrow{\text{dioxygenase}} R(OH)_2 \\
R-H + H_2O_2 & \xrightarrow{\text{peroxidase}} R\cdot + 2H_2O
\end{align*}
\]

Figure 2.1. Classification of oxidising enzymes (R–H = substrate).
number of oxygen atoms that are introduced into the substrate. For mono-oxygenases the remaining oxygen atom is reduced to water. Another class of oxidising enzymes are the peroxidases, like horseradish peroxidase. These natural antioxidants are able to facilitate the oxidation of a variety of (in)organic compounds with the concomitant reduction of hydrogen peroxide or alkyl peroxides to water or alcohols, respectively.

In a number of metalloproteins the transition metal can be positioned in a prosthetic haem group where it is able to activate dioxygen. In other enzymes a mononuclear or dinuclear metal centre is present that functions as a cofactor in a non-haem environment. This leads to a distinction between haem and non-haem enzymes, although this subdivision is not restricted exclusively to oxidising enzymes.

Metalloporphyrins and haem systems in nature can be found in haemoglobin, cytochromes, oxidases and peroxidases. A well-studied family of haem-containing mono-oxygenases includes the cytochromes P-450, which can catalyse the epoxidation of olefins, and the hydroxylation of aromatic or saturated hydrocarbons by molecular oxygen in the presence of the reducing agent NADH or NADPH. These haemoproteins are also capable of oxidising inactive hydrocarbons to functionalised compounds by using oxidants like hydrogen peroxide, iodosylbenzene, and peracids. Therefore, a number of approaches to haem mimics have been presented in recent years.

Rieske oxygenases are able to cis-dihydroxylate inactivated benzene rings. These non-haem mononuclear iron(II) dioxygenases require NADH as a source of electrons to perform the oxidation reaction. The diol products are then converted into catechols, which are subsequently degraded by catechol dioxygenases. These non-haem mononuclear iron(II) enzymes accept the required electrons from the substrate molecules after binding dioxygen.

### 2.2 Bleomycin

Although bleomycin (BLM) is not formally considered to be a protein, this natural glycopeptide is often referred to as a non-haem oxidising enzyme. It has been administered as an antitumour antibiotic for the treatment of different types of cancers, and is most active as the corresponding mononuclear iron(II) complex, Fe–BLM. Its structure, depicted in Figure 2.2,
Models for non-haem iron-containing oxygenases

was deduced from the spectroscopic and NMR investigations of several metallo-BLMs. Recently, X-ray structures of Cu-BLM complexed with a bleomycin-binding protein have also been reported.

An activated species is formed by reacting Fe-BLM with molecular oxygen, and this “activated BLM” is the last detectable intermediate in the catalytic cycle of BLM that leads to an oxidative cleavage of DNA. This reactive intermediate of Fe-BLM is a low-spin iron(III) hydroperoxo (FeIIIOOH, 1) species.

The reaction of Fe-BLM towards DNA proceeds through one of three possible pathways (Scheme 2.1): (a) a heterolytic cleavage of the O–O bond in (BLM)FeIIIOOH furnishing the high-valent FeV=O species; (b) an O–O bond dissociation to produce the FeIV=O species and a hydroxyl radical; or (c) (BLM)FeIIIOOH reacts directly with DNA (R–H) to generate the FeIV=O moiety, a DNA radical, and water without invoking a high-valent intermediate or free hydroxyl radicals. Qualitative investigations on metallo-BLMs tend to favour option (c) as the undiscriminating behaviour of free hydroxyl radicals is inconsistent with the highly specific DNA degradation by Fe-BLM. However, results with non-haem iron oxidation catalysts have been interpreted to indicate that the end-on hydroperoxo ligand in the FeIIIOOH intermediate functions as a precursor for the highly oxidative hydroxyl radical and the FeIV=O species via a homolytic cleavage of the O–O bond.

The chemistry of (BLM)FeIIIOOH is also utilised in reactions other than those involving DNA. Organic substrates can also be oxidised, examples include the epoxidation of styrene, the oxidation of stilbene to the corresponding epoxide with the additional formation of benzaldehyde, and the hydroxylation of aromatic compounds like naphthalene and anisole. In recent years several BLM analogues have been synthesised in order to gain a better understanding of the mechanism, and to study the effect of structural changes on the activity and selectivity of metallo-BLMs.

### 2.3 N4Py synthesis and chemistry

Many research groups have invested a great deal of effort into unravelling the intricate mechanisms of various natural enzymes. Due to their complex composition and immense size, these enzymes and their active species are often studied by investigating the mechanisms of smaller catalytic structures that resemble their behaviour. The data gathered from these
functional mimics can then be compared to that obtained with the native enzyme. For instance, a mononuclear non-haem iron(II) complex has been developed as a functional model for Rieske oxygenases. Non-haem diiron oxygenase mimics have also been designed, as well as metalloporphyrins as versatile oxidation catalysts.

A model has been developed in our group to study the oxidation mechanism of Fe–BLM, as well as to provide an approach to new iron-based oxidation catalysts. The model for the apo-enzyme BLM is a pentadentate ligand, called N4Py. This neutral ligand consists of four pyridine rings that are anchored to a central nitrogen atom (Scheme 2.2). N4Py (6a) and its disubstituted derivatives (6b–d) are obtained by dialkylation of bis(2-pyridinyl)methylamine (2) with the picolyl chlorides (5a–d), respectively, in the presence of a base. The amine 4 can be obtained from the commercially available 2,2'-dipyridyl ketone by conversion to the corresponding ketoxime, and subsequent reduction to afford 4. The synthesis of the picolyl chlorides (5b–d) has previously been described.

\[ \text{Scheme 2.2. Synthesis of N4Py (6a) and disubstituted derivatives (6b–d).} \]

The monosubstituted N4Py derivatives (9) have previously been prepared in a similar fashion. Prior to the alkylation step using a functionalised picolyl chloride, the amine 4 is converted to the secondary amine N3Py (8) via a reductive amination using 2-pyridinecarboxaldehyde (7) (Scheme 2.3).

\[ \text{Scheme 2.3. Synthesis of monosubstituted N4Py ligands.} \]

To encapsulate an iron metal ion, five co-ordination sites are occupied by the ligand 6a, resulting in the iron(II) complex \([\text{[N4Py}Fe\text{(MeCN)](ClO}_4\text{)}_2\text{]}\) (10) (Scheme 2.4). Consequently, the metal ion is positioned in the centre of the generated cavity at 0.2071(5) Å above the mean plane that is formed by the four iron-bound pyridine nitrogen atoms. The sixth co-ordination site is therefore accessible to axial ligands like the solvent-derived acetonitrile molecule in 10.
Although N4Py was developed as a model for bleomycin, the co-ordination environment around the iron metal centre is quite different from that of Fe–BLM (Figure 2.2). The [(N4Py)Fe(MeCN)](ClO₄)₂ complex 10 has been characterised by ¹H NMR and EPR as a low-spin iron(II) complex with a high redox potential (E½ = 1010 mV vs. SCE) for the Fe³⁺/Fe²⁺ couple. The effect of ligand modification on the electron density of the iron metal was determined by measuring the Fe³⁺/Fe²⁺ couples of the low-spin iron(II) complexes of disubstituted N4Py derivatives 6b–d. It was established that substituents at the 5-position of the pyridine ring only have a minor effect on the redox potential of these iron complexes (E½ = 996–1100 mV vs. SCE). The UV-Vis spectrum of 10 displays an absorption band at 458 nm in acetone (ε 4000 M⁻¹cm⁻¹). EPR and NMR studies revealed that the spin state of the iron atom is dependent on the temperature and the charge of the axial ligand. Thus, the replacement of the neutral acetonitrile molecule by a chloride anion leads to a high-spin iron(II) complex. Similarly, a methoxy anion as an axial ligand gives rise to a high-spin iron(III) at room temperature.

When N4PyFe (10) is allowed to react with one equivalent of hydrogen peroxide, 10 is converted to an iron(III) complex. A large excess of hydrogen peroxide is required to produce the iron(III) hydroperoxide intermediate 11. This purple species has been characterised by UV-Vis spectroscopy, EPR, ESI-MS, and resonance Raman spectroscopy as a low-spin iron(III) hydroperoxide in which the hydroperoxo ligand is co-ordinated to the iron centre in an end-on η¹-binding mode (Scheme 2.4). Only in acetone and methanol is the formation of this transient FeIIIOOH species quantitative. The slow and incomplete formation of 11 in acetonitrile is substantiated by the unfavourable equilibrium for the displacement of the axial ligand in 10. The purple intermediate 11 has a characteristic absorption band at 530 nm in acetone (ε 1100 M⁻¹cm⁻¹) and 548 nm in methanol (ε 1100 M⁻¹cm⁻¹). The observed EPR signals at g = 2.17, 2.12, 1.98 resemble those of the low-spin iron(III) species of activated BLM. This FeIIIOOH intermediate is capable of catalytically reducing hydrogen peroxide in the concomitant oxidation of a wide variety of organic compounds. Similar reactivity has been observed with non-haem dinuclear and mononuclear iron complexes of N3Py derivatives, the iron complex of the pentadentate ligand 5Py, as well as many other non-haem iron catalysts.

Furthermore, N4PyFe is able to cleave DNA in the absence of hydrogen peroxide. The efficiency of this oxidation reaction was substantially enhanced by attaching a DNA-intercalating
acridine moiety to the N4Py ligand. The resulting iron complex was found to cleave DNA using molecular oxygen without requiring any additional reducing agents. These findings established that this modified N4PyFe catalyst functioned as an effective mimic for Fe–BLM.

These mechanistic studies have led to a proposed catalytic cycle in which [(N4Py)Fe(MeCN)][ClO4]2 (10) is initially converted into the mononuclear species [(N4Py)FeIIIOH]2+ (14) and the dinuclear species [{(N4Py)FeIII2(μ-O)]4+ (15) (Scheme 2.5).20,30c In methanol as the solvent, these aforementioned intermediates are also in equilibrium with the FeIIIOMe species 16.30c However, upon addition of excess hydrogen peroxide these species are converted into the transient intermediate 11.

Mechanistic studies have provided evidence that the FeIIIOMe intermediate 11 is in fact not the active species, but merely serves as a precursor in the catalysed oxidation of alkanes. Although an oxoferryl (FeIV=O) species with a porphyrin π-radical cation is often observed during the reduction of molecular oxygen by haem peroxidases4,29 and other mononuclear iron centres,8 a heterolytic cleavage of the O–O bond in 11 to a high-valent, formally iron(V)-oxo intermediate is considered energetically too unfavourable to occur (Scheme 2.1).16,34

![Scheme 2.5. Proposed catalytic cycle for the oxidation of alkanes by N4PyFe (10) and hydrogen peroxide.]

A homolytic scission of the O–O bond in 11 would produce two one-electron oxidants, [(N4Py)FeIV(O)]2+ (13) and a hydroxyl radical (•OH). The involvement of hydroxyl radicals in the
catalytic cycle of [(N4Py)Fe(MeCN)](ClO₄)₂ (10) was established by employing radical traps such as acetone, benzene, and dibromomethane during the oxidation reaction. The rationale for these observations is that the neutral pentadentate ligand N4Py is not capable of stabilising the high valent iron in the formally iron(V) oxidation state, and therefore leads to a homolytic rather than a heterolytic scission of the O–O bond. In addition, resonance Raman spectroscopy demonstrated that the O–O bond in the Fe²⁺OOH intermediate 11 is unusually weak.

In the oxidation of alkanes catalysed by N4PyFe the hydroxyl radical produced reacts with a substrate molecule to give rise to a substrate radical after hydrogen abstraction (Scheme 2.5). This intermediate subsequently reacts with dioxygen or 13 to propagate a radical chain auto-oxidation pathway to furnish the oxidation products and the intermediates 14 and 15. The proposed catalytic cycle has been elucidated by employing radical scavengers and mechanistic probes to determine the kinetic isotope effects, the ratio of oxidation at the tertiary over secondary carbon centres in adamantane, and the stereoselectivity in the hydroxylation of trans-1,2-dimethylcyclohexane. However, these mechanistic studies have indicated that another, more selective oxidising intermediate is also involved in the hydrogen abstraction of alkanes catalysed by 10, which is most probably the [(N4Py)FeO²⁺]²⁺ species 13.

When the reactivities of the iron(II) complexes of 6b–d towards the oxidation of alkanes were investigated, the results were comparable to those of N4PyFe. This suggests that a similar mechanism must be involved, irrespective of the electronic properties of the ligand. This is not surprising when taking into account the good correlation between the analytical data of these iron(II) complexes and the corresponding Fe²⁺OOH species. However, the number of free co-ordination sites at the iron centre in the complex has been found to determine the type of chemistry observed during the catalysed oxidation reactions. This is clearly demonstrated by the difference in oxidation behaviour and the proposed mechanism for N3Py derivatives with respect to N4Py ligands (Scheme 2.6). The effect of the co-ordination mode of non-haem ligands, as well as their electronic and steric factors on the oxidation chemistry of the Fe²⁺OOH intermediate has recently been described by Que and co-workers.

![Scheme 2.6](image-url)
The fact that acetone effectively traps hydroxyl radicals can result in different catalytic activities (or turnover number, TON) for the catalyst upon changing the solvent from acetonitrile to acetone. As a result, the selectivity in the oxidation of cyclohexane has been found to be noticeably solvent-dependent. For instance, the ratio of cyclohexanol over cyclohexanone (A/K) changed from 1.4 to 2.6 in acetonitrile and acetone, respectively. When only free radical intermediates are involved in the catalytic cycle, oxidation generally results in A/K ∼ 1.42a By the slow addition of hydrogen peroxide to the reaction mixture the A/K ratio could be increased even further to 5.2, which indicates that oxidation by the non-selective hydroxyl radicals is suppressed in favour of a more selective oxidising species, presumably 13,34

When the axial ligand of the [(N4Py)Fe(MeCN)](ClO4)2 complex is a chloride anion, the purple intermediate 11 cannot be formed. This is presumably due to the strong binding of the anion to the metal, which prevents the displacement of the ligand.30c The catalyst is deactivated to a certain extent during the catalytic cycle, which is probably due to the oxidative degradation of the ligand by the hydroxyl radicals that are formed.

The purple FeIIIOOH intermediate 11 can be converted reversibly into its conjugate base 12 upon the addition of aqueous ammonia in methanol at −45°C. The resulting blue species has been characterised by UV-Vis (λmax 685 nm, ε 520 M⁻¹cm⁻¹), ESI-MS, and resonance Raman spectroscopy as a high-spin [FeIII(O2)]⁺ species.44 In the proposed structure the peroxo ligand is co-ordinated to the iron metal via a side-on binding mode,33b,c,45 whereby the weakly bound tertiary nitrogen is detached from the metal centre (Scheme 2.4).44

Related [FeIII−η²−O₂]⁺ intermediates have also been proposed as the source of nucleophilic peroxides in the oxidation mechanism of haem enzymes (Figure 2.3).46 This [FeIII(O2)]⁺ species is completely unreactive towards the oxidation of alkanes until the FeIIIOOH intermediate 11 is regenerated by the addition of acid.44 This observation seems to support the hypothesis that protonation of the peroxo species is a prerequisite for catalytic activity.47

![Figure 2.3. Possible transition states for oxygen transfer of peroxo ferric haem complexes: closed (left) and open (right) peroxo complexes (X = substrate).46b](image)

**2.4 An initial step towards artificial peroxidases**

The mechanistic studies of [(N4Py)Fe(MeCN)](ClO4)2 (10) and its oxidation chemistry correlate very well with the reactivity and spectroscopic data of Fe−BLM, suggesting that a comparable mechanistic pathway is plausible for both catalysts. Therefore, the iron complex 10 poses as a good functional mimic for Fe−BLM. It will be interesting to ascertain whether or not the incorporation of this model catalyst in a peptide environment will facilitate the construction of a synthetic peroxidase. The outline of this project has already been discussed briefly in Chapter 1.
The four-helix bundle motif has been selected as a potential scaffold to embed the N4PyFe catalyst, where it will hopefully function as a synthetic co-factor. It was illustrated in Chapter 1 that the four-helix bundle appears to be able to accommodate the N4PyFe catalyst. The peptide chains will be attached to a modified N4Py ligand via a cysteine residue in the peptide sequence. Halide displacement on the ligand by the cysteine thiolate is the key to a site-specific attachment of the peptides. This concept has been tested in a preliminary study whereby two unprotected peptides were coupled to the difunctionalised N4Py ligand 18.48

It had already been shown that substituents at the 5-position of the pyridine rings have no major effect on the electron density of the iron centre (vide supra).20 Therefore, it is unlikely that substituents at this position will interfere with the redox potential of the catalyst. Furthermore, these attachment sites will direct the peptides away from the iron centre (Scheme 2.7), which allows the formation of the FeIIIOOH intermediate without affecting its oxidation chemistry. Steric properties in the ligand too close to the iron centre could result in a change in oxidation behaviour, as was described by Que and co-workers for 5- and 6-methyl-substituted tris(2-pyridinylmethyl)amine (TPA) iron catalysts.43

Scheme 2.7. Synthesis of the N4PyFe-dipeptide complex 19.49

The dichloride N4Py derivative 18 was prepared from 6c by initially reducing the ester groups and subsequently converting the alcohol functionalities to the chlorides.48 The reported low yield of only 8% was due to the decomposition of 18 during purification by column chromatography. This can probably be attributed to the benzylic nature of the two chlorides.

The methodology of coupling peptides via a cysteine thioether linkage has several advantages. Firstly, the position of the cysteine residue in the peptide sequence can facilitate a site-specific attachment of the ligand and thereby control the location of the catalyst in the prospective four-helix bundle structure. Secondly, the cysteine thiolate is an efficient nucleophile that can enable the successful coupling of unprotected peptides.50 The presence of other nucleophilic side chains in the sequence (from histidine, lysine, and glutamic acid for instance) can interfere with the chloride displacement in 18. The selected peptide sequence for this experiment consisted of a representative number of residues with nucleophilic side chains (Scheme 2.7). However, the use of caesium carbonate as a base and an excess of peptide afforded a selective and clean conversion to the dipeptide complex 19 after chelation to iron.48 The use of excess peptide was required, as oxidation to the corresponding disulphide occurs readily. UV-Vis, electrospray ionisation mass spectrometry, and protolytic digestion by a
protease verified the incorporation of iron in 19 and the presence of covalently linked peptide chains.48

The retained catalytic activity of 19 was successfully demonstrated by its ability to oxidise the peroxidase activity probes ABTS51 and 1,2,4,5-tetramethoxybenzene (20)52 in water with a performance that was comparable to N4PyFe under identical conditions. In addition, the oxidation of 20 by catalyst 10 gave rise to a benzene radical cation that was also detected by EPR when horseradish peroxidase (HRP) was employed as the catalyst (Scheme 2.8).48

Scheme 2.8. Formation of the radical cation 21 in the oxidation of methoxybenzene 20 catalysed by horseradish peroxidase (HRP).

The pH-dependence of the catalytic system was optimal at pH 4 or lower. At higher pH ranges the activity of the catalyst rapidly diminished. Examination of the reactions over time revealed that after five minutes the oxidation had essentially terminated for both [(N4Py)Fe(MeCN)](ClO4)2 (10) and 19, presumably due to an oxidative degradation. The similar oxidation behaviour of 10 and 19 clearly demonstrated that the attachment of two peptide chains to the catalyst had no significant effect on its performance.

2.5 Conclusions

Earlier work by Gerard Roelfes lead to the formulation of a mechanism for the oxidation catalyst [(N4Py)Fe(MeCN)](ClO4)2 (10). The good correlation between the oxidation chemistry and the spectroscopic data of the reactive intermediates proved that 10 functions as an effective mimic for Fe–BLM.

The oxidation behaviour and performance of the N4PyFe catalyst remains unaffected by the attachment of two peptide chains in close proximity to the iron complex. These findings suggest that the catalyst can be embedded into a peptide environment without substantial loss of activity. This paves the way for the design of water-soluble peroxidase mimetics. At the same time this sets the stage for the challenging task to modify the N4Py ligand in order to host four peptide chains. This will hopefully facilitate the incorporation of the catalyst into a four-helix bundle. The synthesis of such a tetrafunctionalised N4Py derivative will be presented in Chapter 3.

2.6 References and notes


13. For an explanation of the abbreviations used, see Appendix 1 (p. 149).


31. See also Chapter 3 (p. 47) for an overview of synthetic pathways to picolyl chlorides 5b–d.


48. See Appendix 2 (p. 151) for the α-amino acid structures and one-letter abbreviations.