Biocatalysis

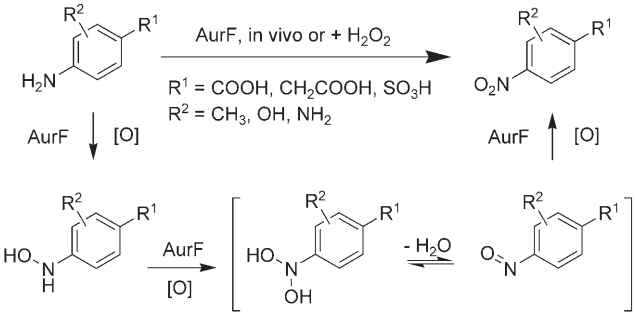
DOI: 10.1002/anie.200703089

A Binuclear Manganese Cluster That Catalyzes Radical-Mediated N-Oxygenation**

*Robert Winkler, Georg Zocher, Ingrid Richter, Thorsten Friedrich, Georg E. Schulz, and Christian Hertweck**

Manganese-catalyzed reactions play a vital role in organic synthesis, in particular when applied to selective oxygenation reactions. The best known examples are Mn–porphyrins[1–3] and the Jacobsen–Katsuki Mn–salen catalysts,[4,5] which have emerged as indispensable tools for the epoxidation of non-functionalized alkenes. More recently, a dimanganese catalyst has been developed for the selective C–H activation.[6] Surprisingly, although a number of Mn-dependent catalysts from biological systems are known, such as catalase and superoxide dismutase, no Mn-dependent monooxygenases have been described.[7] This is striking considering that manganese is the element with the highest number of possible valence states, namely 11.[8] Herein we present the coordination sphere and catalyst geometry of the first reported Mn-dependent monooxygenase, the N-oxygenase AurF from *Streptomyces thioluteus*.[9–12] Recently we showed that AurF is able to selectively oxidize *p*-aryl amino groups to the corresponding nitro groups (Scheme 1). The enzyme can be applied in various ways—in vivo, in vitro, and immobilized in flow driven by H_2O_2—highlighting its potential application as a biocatalyst.[13]

Three lines of evidence indicated that the unusual N-oxygenase AurF contains manganese. Total element scans by inductively coupled plasma (ICP) mass spectrometry (MS) determined only Fe and Mn in significant amounts. Through sequential dialysis against ethylenediaminetetraacetic acid (EDTA) and water, the Fe content was minimized while the Mn content remained constant as monitored by ICP optical emission spectroscopy (OES). This result implies nonspecific binding of so-called junk iron from media and a role of Mn in



Scheme 1. Proposed mechanism of stepwise and regioselective N-oxygenation by AurF.

catalysis. These findings were corroborated by colorimetric assays of solutions acquired from enzyme preparations denatured by acid and heat, which showed 20-fold preference of Mn over Fe, on the basis of the culture media.[13] Other authors also detected Mn by ICP-MS and electron spin resonance (ESR) spectroscopy. However, on the basis of a putative conserved amino acid sequence they concluded that AurF is a diiron enzyme.[14] Their interpretation is in disagreement with our crystal structure data for AurF, which provides the ultimate proof for the presence of manganese in the active site. We were able to solve the crystal structure at 2.1-Å resolution to reveal that AurF consists of two subunits, each containing a binuclear metal cluster.[15] On the basis of potential metal candidates and the availability of the enzyme structure we determined the exact position and identity of metals by investigation of AurF crystals by anomalous X-ray diffraction. In both active centers a homobinuclear manganese cluster was confirmed. No typical organic cofactors such as flavins or porphyrins[16,17] could be detected. On the basis of the initial structural analysis we noted that Mn atoms are embedded in a rigid matrix of four peptide helices with several potential amino acid side chains protruding (Figure 1).

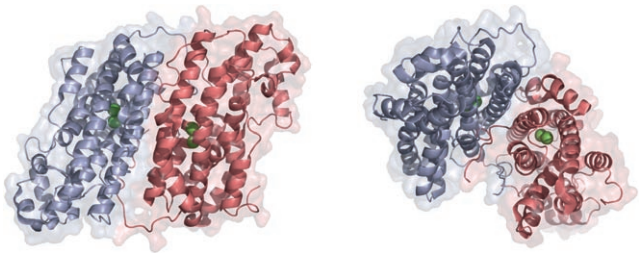


Figure 1. Crystal structure of AurF showing the dimanganese centers (green) embedded in a rigid tetrahelical environment.

[*] R. Winkler, Prof. Dr. C. Hertweck
Department of Biomolecular Chemistry
Leibniz Institute for Natural Product Research and Infection Biology (HKI)
Beutenbergstrasse 11a, 07745 Jena (Germany)
Fax: (+49) 3641-656-705
E-mail: christian.hertweck@hki-jena.de
Dr. G. Zocher, Prof. Dr. T. Friedrich, Prof. Dr. G. E. Schulz
Institute for Organic Chemistry and Biochemistry
Albert-Ludwigs University
Freiburg (Germany)
I. Richter, Prof. Dr. C. Hertweck
Friedrich-Schiller University
Jena (Germany)

[**] Financial support by the Leibniz Gemeinschaft, the Otto Röhm Foundation, and the DFG is gratefully acknowledged.

Supporting information (experimental details) for this article is available on the WWW under http://www.angewandte.org or from the author.

To characterize the complex, the proximity of the manganese atoms was examined for potential ligands. Seven amino acid residues were identified within 4 Å of the manganese center that could potentially be involved in metal coordination:[18] three histidines (H) and four glutamic acids (E). These target amino acids were mutated to verify their role in catalytic function or coordination. Histidine residues provide only a single coordination site and were mutated into alanine (A). In contrast, glutamate could contribute to two coordination axes (bidentate) and thus each amino acid was mutated to alanine (no coordination possible) and glutamine (Q) (one coordination site). To address the influence of ligand distance, glutamate residues were also changed to aspartate (D) and asparagine (N). Successful heterologous expression of the mutated fusion proteins in *E. coli* was verified by SDS-PAGE. For all mutants product formation by oxidation of PABA was measured by LC and compared with the unaltered ("native") protein (Figure 2).

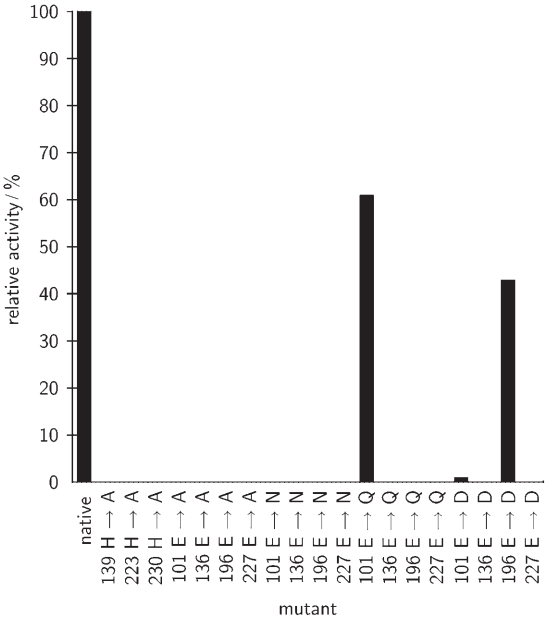


Figure 2. Effect of mutations on enzyme activity.

In all cases the mutation of the seven selected amino acids into alanine resulted in a nonfunctional enzyme. This initial experiment highlights the importance of these amino acid side chains for coordination and catalysis (see Table 1, entries 2–8 in the Supporting Information). The glutamate residues were next mutated into aspartate to evaluate the influence of the distance of the ligand to the metal. The E136D, E227D, and the E101D mutants lost their ability to oxidize the PABA amino group, revealing that the coordination geometry is crucial in these cases. In contrast, the E196D mutant retained about 43 % activity, indicating a degree of tolerance in this position. To test whether the carboxy group is critical for catalyst function we mutated glutamate to glutamine and glutamate to asparagine. In all cases in which glutamate was changed into asparagine, the mutants lost catalytic activity. Also, mutation of the E136, E196, and E227 into glutamine yielded nonfunctional variants of AurF. However, the E101Q mutant retained 61 % relative activity.

The results of the mutagenesis experiments shed light onto the coordination of the manganese sites. The three His and four Glu residues in the active site of AurF provide five and four peptide ligands of the manganese centers, respectively. Although the presence of these seven residues is essential, a limited degree of flexibility is allowed. All three predicted histidine ligands are important for activity and may not be altered.

The glutamate and aspartate carboxylate groups are known for their ability to function as bidentate ligands. For E227 and E136 no tolerance was observed, indicating that both chain lengths and the nature of the functional group are important. Loss of function if those glutamates are exchanged with glutamine provided the strongest evidence for bidentate or bridging coordination. In contrast, the E101 carboxylate group can be mutated into an amide with about 40 % loss of activity, indicating that only one coordination site is provided by this residue. For E196 it appears that the presence of the carboxy group is far more important than the length of the side chain. However, the coordination angle and the Mn–O1 bond length suggest that Mn is coordinated to one rather than two oxygen atoms of the carboxy ligand. The second oxygen atom (Oε2) of E196 appears to be essential for H transfer and/or substrate binding.

The crystal structure indicated the presence of a water molecule at a distance of 2.6 Å from each metal ion (Figure 3). The nine coordination sites of the amino acids are supported by two additional sites of this water molecule, which form a μ-aqua bridge. Thus, each manganese center has three different types of ligand. All distances between Mn and amino acid and water ligands range between 2.0 and 2.6 Å, whereas the distance between the two metal ions is 3.6 Å. The angle distribution of the amino acid ligands at each Mn center is equivalent to 93° ± 5°, which approaches the value of 90° expected for perfect octahedral geometry (Figure 3 and Figure S1 in the Supporting Information). The octahedra are condensed and share one edge between E227 and the water ligand. The enforced deviation from the geometrical ideal by the protein structure is typical for metal clusters in enzymes. Such an entatic (or strained) complex is more likely to support electron transfer than would one with perfect coordination geometry.[19]

Our results are in stark contrast to a previously hypothesized diiron cluster. Zhao and co-workers suggested two putative conserved iron binding motifs ($Ex_{28-37}DExxH$).[14] However, mapping these proposed aspartate ligands in the crystal structure clearly shows that residues of D135 and D226 in fact point away from the active site and the substrate channel and hence cannot be involved in metal coordination (Figure S2 in the Supporting Information).[20] Furthermore, the biochemical verification of iron by Zhao and co-workers is in question because of the metal chelating His tag, which was not removed prior to the biochemical analyses.

Our structure-based mutagenesis clearly established the architecture of the novel binuclear Mn cluster. Next, we aimed at proving its involvement in N-oxygenation. For this

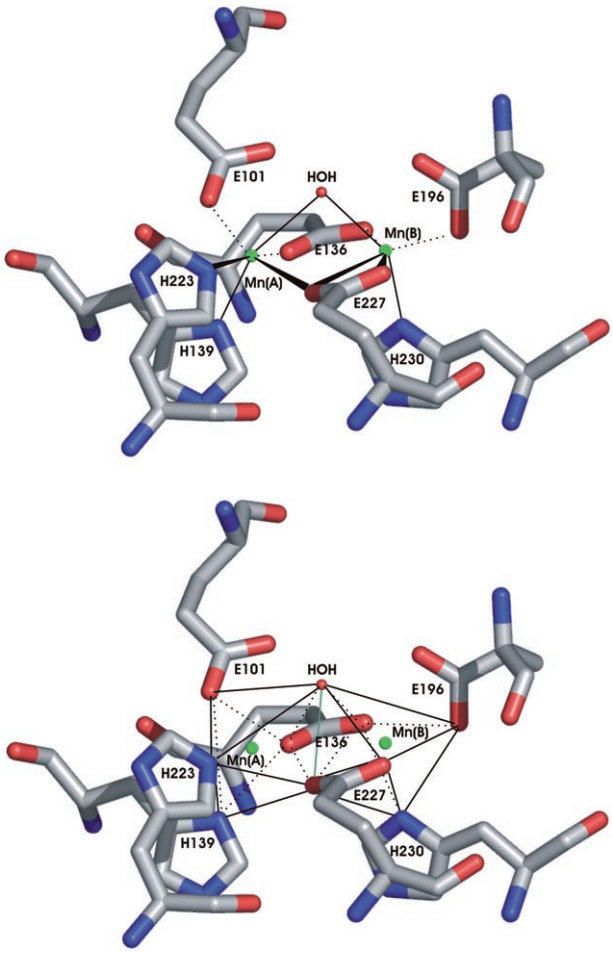


Figure 3. Manganese coordination sphere in AurF; green Mn, red O, blue N.

purpose, we performed individual ESR measurements on purified AurF alone, with oxidant, and supplemented with the natural substrate (Figure 4 a–c). Native AurF shows a clear high-spin Mn^{2+} feature (spin state 5/2) with a sextet between 3076.54 and 3540.86 G and a center at about $g = 2.05$. The average distance between the Mn maxima is 88.5 G. These values are very similar to those of the mononuclear Mn center of oxalate decarboxylase (Mn^{2+}).[21] It should be noted that binuclear Mn centers might also appear as mononuclear centers in ESR. This was demonstrated for phosphodiesterase, which shows reversible changes in its spectroscopic properties with pH.[22] The weak signal at $g = 4.3$ is most likely from junk Fe^{3+} with low ligand symmetry.[21–24] Protein-bound iron does not give any signal at $g = 4.3$.[24] We then activated AurF with H_2O_2 and observed a sharp radical signal with a maximum at about $g = 2.003$ and a peak-to-peak width of about 24 G. Concomitantly, the Mn sextet disappears, which indicates a change in oxidation state. Both facts support an Mn-based radical mechanism. Finally, when both H_2O_2 and PABA were administered, no radical is visible, but the Mn signal is restored. These results provide strong evidence that manganese is involved in the oxygenation reaction, and not Fe, as postulated by others.

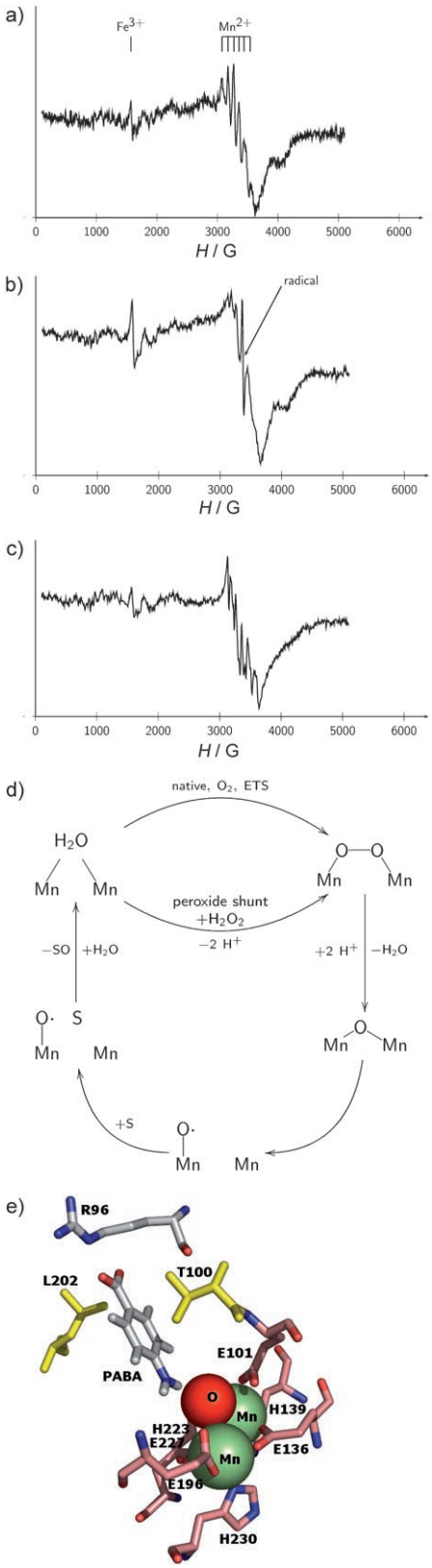


Figure 4. ESR spectra of: a) native AurF; b) AurF and H_2O_2; c) AurF, H_2O_2, and PABA. d) Catalytic cycle for radical-based mechanism of N-oxygenation by AurF; e) model for substrate binding with *p*-amino group protruding into the active site of AurF.

On the basis of the ESR data, a radical-based mechanism analogous to binuclear iron-dependent methane monooxygenases appears most plausible (Figure 4d). This is further supported by the working peroxide shunt.[25] Notably, the radical seems to be located close to the manganese center of the enzyme and not at the substrate, which in turn quenches the radical. A tyrosyl-stabilized radical similar to ribonucleotide reductases could be excluded because the typical sharp UV absorbance signal at 408 nm upon oxidation is missing.[26,27] Since the apparent K_m value for H_2O_2 of 0.2 M is well above the range expected for physiological substrates, H_2O_2 is not the native cosubstrate. However, H_2O_2 alone in the presence of boiled enzyme is not able to catalyze any oxidation of PABA. Under native conditions, AurF likely employs molecular oxygen[28–30] and might be regenerated by an electron-transfer system similar to P450 oxygenases and binuclear monooxygenases. Since AurF is functional in both *E. coli* and *S. lividans* it is likely that the electron-transfer system is universal. Finally, the results from our mutational studies strongly suggest that protons are delivered through the E196 carboxy moiety. A model for substrate binding in the active site of AurF is shown in Figure 4e.

In conclusion, we have characterized a novel binuclear manganese biocatalyst involved in the specific oxidation of amino to nitro groups. To explore the ligand sphere of the unique Mn centers we performed 19 structure-based rational site-directed mutagenesis experiments and tested the activity of the mutants. From these results we could clearly assign the essential residues in the complex for manganese coordination and enzyme activity. In conjunction with the X-ray structure we could deduce the exact geometry of a novel dimanganese cluster that is capable of regio- and chemoselective N-oxygenation. The involvement of Mn in catalysis and radical formation was monitored by ESR spectroscopy. On the basis of these results a mechanism analogous to cytochromes and binuclear Fe monooxygenases was proposed. This is the first report on the functional analysis of an Mn-dependent monooxygenase. The elucidation of this novel biocatalyst may now set the basis for developing synthetic biomimetic oxygenation catalysts.

Received: July 11, 2007
Published online: October 9, 2007

Keywords: biocatalysis · enzymes · manganese · nitro groups · oxygenation

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