Biotransformations

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Regio- and Chemoselective Enzymatic N-Oxygenation In Vivo, In Vitro, and in Flow**

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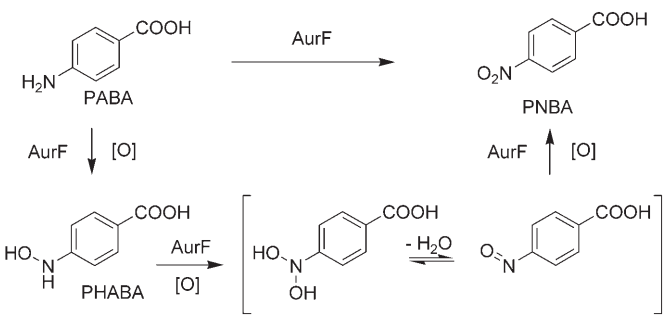
The use of oxygenases in biotransformations can expand vastly the repertoire of synthetic methods.[1] A hallmark of these enzymes is their exquisite specificity and their application under environmentally friendly and technically safe conditions.[2–4] However, the use of these biocatalysts may be restricted by the need for cofactor recycling,[5] which can be circumvented by whole-cell biotransformations.[1] The most notable success of oxygenases has come with the oxygenation of nonactivated carbon or the regioselective oxidation of polyhydroxy compounds.[1]

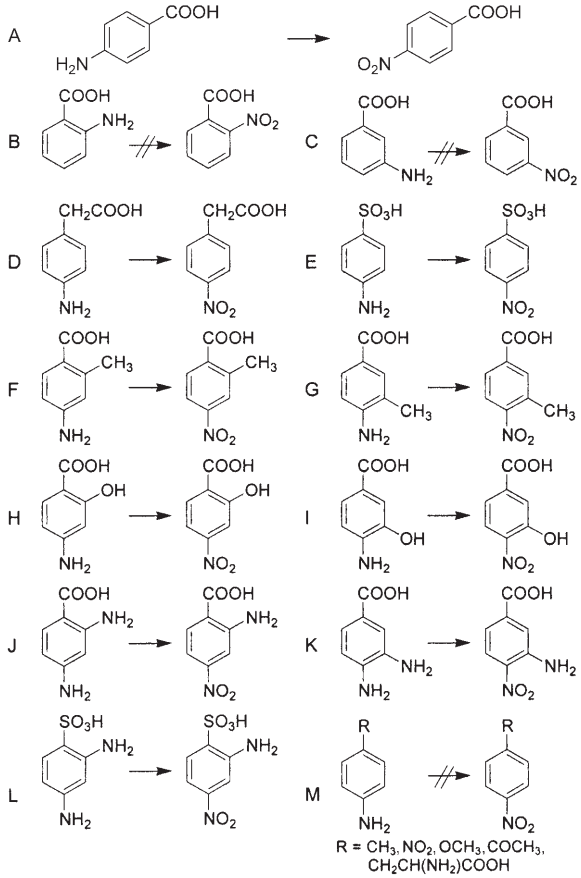
Considering the breadth of biotransformations available, it is remarkable that enzymes catalyzing the selective oxidation of amino groups are only little explored. To date, only two genuine nitro-group-forming N-oxygenases have been identified. Aminopyrrolnitrin N-oxygenase (PrnD) is involved in pyrrolnitrin biosynthesis in *Pseudomonas fluorescens*, and belongs to the Rieske-type oxygenases.[6] In contrast, *para*-aminobenzoate N-oxygenase (AurF) from *Streptomyces thioluteus* represents an unparalleled enzyme that does not show any sequence similarity to known oxygenases.[7] This unusual biocatalyst transforms *para*-aminobenzoic acid (PABA) into *para*-nitrobenzoic acid (PNBA), the rare aureothin polyketide synthase starter unit (Scheme 1).[7,8] Recent in vivo studies coupled to an "at-line" liquid chromatographic (LC) analysis revealed that the oxygenation reaction occurs stepwise via hydroxylamine and, most probably, nitroso intermediates.[9,10]

With the intention of exploring the synthetic potential of AurF, we have addressed the scope of this unique oxygenase, and its chemo- and regioselectivity, and have established an in vitro N-oxygenation method that circumvents the need for cumbersome cofactor regeneration. We first carried out a comparative study in a validated whole-cell approach using the geometrical isomers of aminobenzoate. While AurF readily transforms PABA into PNBA, neither of the two



Scheme 1. Proposed mechanism of N-oxygenation by AurF. PHABA = *para*-(hydroxyamino)benzoic acid.

isomers, *ortho*- and *meta*-aminobenzoic acid, was oxygenated (Scheme 2, entries B and C; Figure 1). This result clearly



Scheme 2. Substrate specificity of AurF.

indicates a high *para* regioselectivity of AurF. Most PABA analogues substituted at the carboxy moiety with various substituents, such as methoxy (*para*-anisidine), acetyl (*para*-aminoacetophenone), alaninyl (*para*-amino-L-phenylalanine), and methyl (*para*-toluidine), are not transformed (Scheme 2, entry M). However, AurF also accepts *para*-aminophenyl acetic acid and *para*-aminophenyl sulfonic acid as substrates and converts them into *para*-nitrophenyl acetic acid and *para*-nitrophenyl sulfonic acid, respectively (Scheme 2, entries D and E). Hence, it can be concluded

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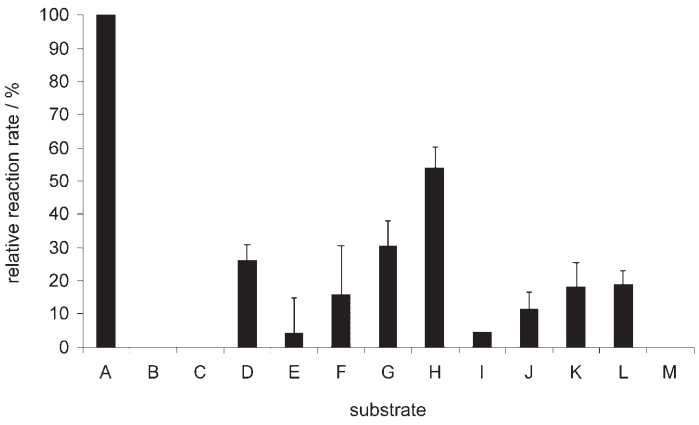

Figure 1. Relative reaction rates (with reference to PABA (substrate A)).

that the enzyme allows only some degree of variation at the carboxy group. Comparing these probes, it is notable that all converted substrates bear an acidic group adjacent to the aromatic ring. This fact strongly suggests that the presence of an acidic residue is important for substrate binding.[11]

To investigate the impact of additional ring substitutions, substrates with extra methyl and hydroxy groups in either the *ortho* or *meta* position to the carboxy group were administered. All the tested monosubstituted PABA derivatives resulted in the corresponding nitro product (Scheme 2, entries F–I)). Acceptance of both methyl and hydroxy substituents on either the *ortho* or *meta* position is amazing, as the first accounts for a $+I$ effect and the latter for a $-I$ effect. Apparently some variation of the electrochemical state of the substrate seems to be tolerated. The substrate-binding site allows for a certain range of flexibility, which was already indicated by the successful transformation of *para*-aminophenyl acetic acid.

The absent oxygenation of 3-amino-4-methylbenzoic acid and 3-amino-5-methylbenzoic acid (not shown) further supports the strict regioselectivity of AurF in regard to the amino group, whilst the failure in oxidizing 4-amino-2,3,5,6-tetrafluorobenzoic acid (not shown) could be a result of exaggerated electron withdrawal from the ring system, or may be caused by space constraints at the active site.

To exploit the limited substrate tolerance in conjunction with the strict *para* specificity, the diamino surrogates 2,4-diaminobenzoic acid, 3,4-diaminobenzoic acid, and 2,4-diaminobenzenesulfonic acid were probed. Strikingly, all transformations resulted exclusively in products with a nitro group at the *para* position and unaltered amino groups at the *meta* and *ortho* positions (Scheme 2, entries J–L). Such strict site-directed N-oxygenations are unprecedented and have not been achieved by standard synthetic protocols.

To establish a convenient in vitro N-oxygenation method for this unique biocatalyst, various attempts to yield pure enzyme were undertaken. Initial trials to overexpress native or 6×His-tagged protein in *S. lividans* or *Escherichia coli* failed. However, soluble MalE–AurF fusion protein was produced at high levels in *E. coli*, and in vivo tests proved the catalytic activity of the chimeric enzyme. Large amounts of the enzyme were produced by high-cell-density cultivation (HCDC) in minimal salt medium. Enrichment of acetate was prevented by controlled feeding of glucose,[12] which yielded a final wet cell mass of 133 g L^{-1}. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) and performed at 26 °C to support correct protein folding by chaperones.[13] One-step affinity purification of clarified disintegrates from a 20-g cell pellet on a 17-mL amylose column yielded over 100 mg of fusion protein with more than 90 % purity (SDS-PAGE) per batch.

Interestingly, UV/Vis and fluorescence spectroscopy gave no hint of flavin, heme, or other typical oxygenase cofactors. However, inductively coupled plasma optical emission spectrometry (ICP-OES) investigations showed significant contents of iron and manganese in the purified protein. This finding was confirmed by colorimetric assays for iron and manganese and comparison with the culture media, which also revealed that manganese is enriched by a factor of about 20 in relation to iron. Such a high selectivity of the protein for manganese provides strong evidence that AurF employs manganese as an oxygen-activating cofactor.

It is astonishing that the addition of H_2O_2 to MalE–AurF preparations restored the enzymatic activity in vitro without the need for other supplements. This is in strong contrast to the N-oxygenase PrnD, which requires proteolytic cleavage of the fusion enzyme and additional coenzymes for in vitro activity.[14] Presumably, the reaction mechanism employed by AurF is similar to that of heme or iron-dependent oxygenases, which allow bypassing of the native enzyme regeneration by H_2O_2 via a so-called "peroxide shunt".[15]

The strict *para* selectivity of AurF can be rationalized by a simplified model (Figure 2). The substrate is most likely

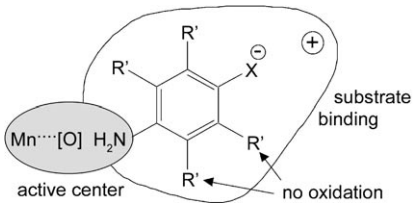

Figure 2. Model for regioselective N-oxygenation by AurF; R′ = H, NH_2; X = COO, CH_2COO, SO_3.

tethered by ion interactions to a positively charged protein residue, which allows the *para*-substituted amino group to enter the reactive hot spot, probably a Mn–oxo species. In stark contrast to AurF, haloperoxidases, which are not genuine N-oxygenases, catalyze nitro group formation in a nonspecific way through the formation of reactive peroxo species in the absence of halide ions.[16–18]

Apart from its remarkable selectivity, AurF has another advantage over other systems. Most conveniently, the loaded amylose column with immobilized enzyme can be used directly as an N-oxygenator "in flow". This is simply achieved by addition of substrate and H_2O_2 to the loading buffer. In a pilot experiment, a single pass of PABA and H_2O_2 immediately resulted in 27 % conversion to PNBA. To challenge the

method, a mixture of 2-, 3-, and 4-aminobenzoic acids was circulated about 140 times. LC analyses demonstrated that a steady state is reached at 47% conversion to PNBA, and that only 4-aminobenzoic acid is oxidized, while the regioisomers remain unchanged (Figure 3).

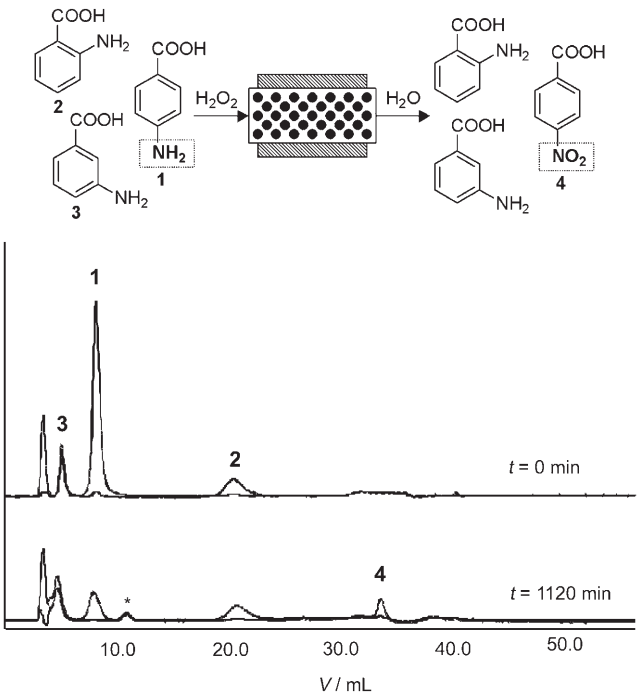

Figure 3. Top: Continuous regio- and chemoselective N-oxygenation on a fixed bed (column with thermostat) with immobilized MalE–AurF enzyme (spheres). Bottom: Chromatographic profiles of selective biotransformation; the asterisk indicates an unknown side product from PABA. The peak of **4** is significantly reduced in relation to that of **1** as a result of lower absorption of the nitro compound at 280 nm.

The possibility of using an immobilized enzyme, supplied with only H_2O_2, for the selective biotransformation of aromatic amines is notable, because it allows for a number of applications, such as the design of continuous flow processes, which are increasingly relevant to the future of chemoenzymatic syntheses. The urgent need for such novel techniques is a contemporary challenge in organic syntheses.[19] Possible uses of enzymatic N-oxygenation are "green" regio- and chemoselective modification of valuable fine chemicals,[20] or the separation and extraction of aromatic amines by previous oxidation, as well as in analytical applications.

In summary, various substrates have been probed for N-oxygenation by AurF, which revealed a strict regio- and chemoselectivity for an aromatic amino group *para* to an acidic group. A number of modifications on the carboxy group (carboxymethyl, sulfonate) and additional ring substituents (CH_3, OH, NH_2) are tolerated by AurF, although reaction rates are reduced compared to those of PABA (Figure 1). The most remarkable result is the selective oxygenation of diamino compounds. The excellent *para* specificity can be rationalized on the basis of an active-site model.

Preparative amounts of an active MalE–AurF fusion protein were manufactured and the enzyme was proven functional in vitro by addition of H_2O_2 (peroxide shunt). The chimeric enzyme could be immobilized and successfully applied in a fixed-bed tube reactor for regioselective amine transformation. To our knowledge, this is the first report of an enzymatic process for the chemo- and regioselective oxygenation of aromatic amines to nitro compounds, which could not be emulated in a single step using current synthetic protocols. Future studies of AurF will include elucidation of the structure of the enzyme and protein engineering techniques, with the goal of increasing reaction rates and altering the scope of accepted substrates.

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