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Pyridine as novel substrate for regioselective oxygenation with aromatic peroxygenase from *Agrocybe aegerita*

René Ullrich*, Christoph Dolge, Martin Kluge, Martin Hofrichter*

Unit of Environmental Biotechnology, International Graduate School of Zittau, Markt 23, 02763 Zittau, Germany

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Abstract ***Agrocybe aegerita*** **peroxidase (AaP) is a versatile extracellular biocatalyst that can oxygenate aromatic compounds. Here, we report on the selective oxidation of pyridine (PY) yielding pyridine *N*-oxide as sole product. Using $H_2{}^{18}O_2$ as co-substrate, the origin of oxygen was confirmed to be the peroxide. Therefore, AaP can be regarded as a true peroxygenase transferring one oxygen atom from peroxide to the substrate. To our best knowledge, there are only two types of enzymes oxidizing PY at the nitrogen: bacterial methane monooxygenase and a few P450 monooxygenases. AaP is the first extracellular enzyme and the first peroxidase that catalyzes this reaction, and it converted also substituted PYs into the corresponding *N*-oxides.**

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Keywords: Haloperoxidase; Oxygenation; P450; Pyridine; Pyridine *N*-oxide; *Agrocybe*

1. Introduction

Various *N*-heterocyclic aromatic compounds such as substituted pyridines (PYs), pyrimidines, quinolines or indoles are found in all living organisms where they have important biological functions (electron carriers, nucleotides, vitamins, porphyrines, alkaloids, etc.) [1]. In addition, numerous man-made *N*-heteroaromatics, mainly produced at industrial scale from coal tar [2], are used as solvents, pharmaceuticals, pesticides, dyes or explosives, and thus are released in large amounts to the environment. Their microbial degradation and transformation was subject of comprehensive studies and shown to occur both under aerobic and anaerobic conditions [1,3–6]. Some of the responsible bacteria have become subjects of biotechnological interest (e.g. *Agrobacterium* sp., *Comamonas testosteroni*) and are used for the preparation or functionalization of *N*-heterocycles [7].

PY, widely used as industrial solvent and starting compound in diverse chemical syntheses [8], is the simplest six-membered *N*-heterocycle and a rather unactivated, aromatic molecule that is chemically difficult to oxygenate, especially at the *N*-position [9]. There are a few PY-utilizing bacteria (e.g. *Rhodococcus opacus, Arthrobacter crystallopoietes*) which seemingly hydroxylate the heterocycle, however, only at 2- and 3-positions, and it is unknown which enzymes are responsible for these reactions [5,10–12].

The chemistry and applications of PY *N*-oxides have received more and more attention over the last two decades due to their usefulness as synthetic intermediates and their biological importance [8,9,13]. There are several chemical methods to achieve PY *N*-oxidation/oxygenation including ring transformations, oxidation by peracids or metallo-organic oxidants and cycloaddition [9]. All these reactions, however, are relatively unspecific and elaborate. Therefore, it appears to be promising to look for biocatalysts which selectively oxygenate the *N*-position of PY and its derivatives. So far merely methane monooxygenases (MMO; EC 1.4.13.25) and membrane bound cytochrome P450 monooxygenases (P450s; EC 1.14.13.x) have been reported to act in that way [14,15].

Due to their high stability, microbial peroxidases would be more suitable biocatalysts to study both PY oxidation as such and to develop biocatalysts for respective applications. So far, however, no peroxidase has been described to oxidize PY at neither the *N*- nor any other position. Here, we describe the *N*-oxygenation of PY and related compounds by *Agrocybe aegerita* peroxidase/peroxygenase (AaP), a versatile fungal enzyme that has recently been found to efficiently and selectively transfer oxygen to naphthalene and other aromatic substrates [16–19].

2. Materials and methods

2.1. Organism and peroxygenase preparation

A. aegerita (V. Brig.) Singer, 1951 (synonym *A. cylindrica*) strain A 1 is an agaric mushroom that grows on wood of deciduous trees (preferably on poplar stumps), bark and mulch, and rapidly develops edible fruiting bodies [20]. Stock cultures of the fungal mycelium were grown and maintained on 2% malt extract agar and stored at 4 °C in the dark. For enzyme production, the fungus was cultured in a 10-L stirred-tank bioreactor using a soybean-based complex medium [21].

AaP (AaP; also referred to as haloperoxidase or haloperoxidase-peroxygenase and nowadays named aromatic peroxygenase) [16–19,22] was purified by several steps of ion exchange chromatography as described previously [21]. The final AaP preparation (isoform AaP II) used in the present study had a specific activity of 75 Units per mg protein (U mg^{-1}, substrate veratryl alcohol) and an $RZ_{420/280}$ value of 1.7; its physico-chemical properties are described elsewhere [16,21–23].

2.2. Enzymatic oxidation of PY

Reactions were performed in 1.5-ml glass vials containing in a total of 1 ml 10 mM phosphate buffer (pH 7.0), 10% acetonitrile, 2 mM PY (added dissolved in acetonitrile) and 0.58 μM AaP (= 26.6 μg ml^{-1});

*Corresponding authors. Fax: +49 3583 612734 (M. Hofrichter).
E-mail address: ullrich@ihi-zittau.de (R. Ullrich).

Abbreviations: AaP, *Agrocybe aegerita* peroxygenase; CPO, chloroperoxidase; P450, cytochrome P450 dependent enzyme; MMO, methane monooxygenase; PY, pyridine

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the reactions were started with 1 mM H_2O_2 and stopped by the addition of 50 μl NaOH (5 M). Reactions mixtures were constantly stirred with a magnetic stirrer (1 × 3 mm) at 600 rpm.

Reaction products were analyzed by high performance liquid chromatography (HPLC) using a model HP 1200 liquid chromatograph (Agilent, Waldbronn, Germany) equipped with diode array (DAD) and mass detectors (LC/MSD VL). For the quantification of PY, just the DAD detector was used along with a Synergi Fusion RP-80A reversed phase column (4.6 × 150 mm, 4 μm particle size, Phenomenex Ltd., Aschaffenburg, Germany) and a C18 Security Cartridge (4 × 2 mm, Phenomenex). A mixture of acetonitrile and 20 mM ammonium formate buffer (pH 9.4) (7:93, vol/vol) was used as solvent at a flow rate of 1.0 ml min^{-1} under isocratic conditions. Products formed in the course of enzymatic PY oxidation were identified using concomitant DAD and MS detection and a Phenomenex Gemini-NX C18 110A column (150 × 2 mm, 3 μm particle size). Methanol and 20 mM ammonium formate buffer (pH 9.4) (7:93, vol/vol) served as mobile phase at a flow rate of 0.2 ml min^{-1} under isocratic conditions. Eluted substances were detected in the wavelength range from 200 to 550 nm and identified by comparing their UV spectra and retention times with those of authentic standards purchased from Fluka (see Section 2.6.). Mass determinations were made in positive ESI mode (electrospray ionization) in the mass range from 70 to 300.

AaP-catalyzed PY oxidation was also studied in a reaction system where H_2O_2 was continuously generated by the couple glucose (15 mM)/glucose oxidase (0.05 U) at an approximate rate of 50 nmol min^{-1} (calculated according to the specification of the provider). The reaction time was 10 h and the initial concentration of PY 2000 μM.

2.3. Determination of enzyme kinetic data of PY oxidation

Spectral changes in the UV–vis range during the oxidation of PY into PY *N*-oxide are not sufficient to develop a specific photometric assay. Therefore, AaP activity towards PY was directly calculated after HPLC analysis. Reaction conditions were the same as described in Section 2.2 except that the PY concentration was varied between 10 μM and 1000 μM and a smaller amount of AaP (0.29 μM) was used. Reactions were performed in triplicate and stopped after 10 s by addition of 200 mM trifluoroacetic acid (TFA). The concentration of PY and PY *N*-oxide was determined by HPLC using authentic standards. The apparent Michaelis–Menten constants (K_m) and catalytic constants (k_{cat}) were calculated from Lineweaver–Burk and Eadie–Hofstee plots based on the rates obtained at varying substrate concentrations.

The dependence of PY oxidation on pH was tested in the range from pH 2 to 11 using appropriate potassium phosphate buffers (100 mM).

2.4. Experiments with $H_2{}^{18}O_2$

^{18}O-Enriched hydrogen peroxide ($H_2{}^{18}O_2$) was used to prove the origin of oxygen in AaP-catalyzed PY oxidation. Reaction conditions were the same as described in Section 2.2 except that instead of "normal" hydrogen peroxide ($H_2{}^{16}O_2$), ^{18}O-labeled peroxide ($H_2{}^{18}O_2$, >90%); Icon Isotopes, Summit, NJ, USA) was used. Mass detection using the LC–MS system mentioned above enabled us to distinguish between differently O-isotope-labeled PY *N*-oxide molecules.

2.5. Oxidation of substituted PYs

Following PY derivatives were tested as substrates for AaP (1.45 μM): 2-,3-, and 4-monochloropyridines (Cl-PY), 3,5-dichloropyridine (Di-Cl-PY), perchloropyridine (Per-Cl-PY), 3-fluoropyridine (F-PY), 3-bromopyridine (Br-PY), 3-iodopyridine (I-PY), 3-methylpyridine (CH_3-PY; picoline), 3,5-dimethylpyridine (Di-CH_3-PY; lutidine), 3-nitropyridine (NO_2-PY), 3-cyanopyridine (CN-PY), nicotinic acid and nicotine amide (compare Table 2). Reactions were carried out in 1.5-ml glass vials (total reaction volume 1 ml) with 500 μM substrate under the conditions described above. As PY, all substituted pyridines were added dissolved in acetonitrile. H_2O_2 (500 μM) was continuously supplied by a syringe pump (KDS 100, KD Scientific, Holliston, MA, USA) at a rate of 25 nmol min^{-1}. Reaction products were analyzed by HPLC with DAD and MS detection as described above; mixing ratios of methanol and ammonium formate buffer (20 mM, pH 9.4) were varied from 5% to 20% methanol depending on the particular PY derivative analyzed.

2.6. Chemicals

PY all halogenated and methylated pyridines, nicotinic acid, amide, alcohol and aldehyde as well as, pyridine *N*-oxides and veratryl alcohol were purchased from Fluka and Sigma–Aldrich (Schnelldorf, Germany) and served as authentic standards and/or substrates. Organic solvents (at least HPLC grade) and all other chemicals were purchased from Merck (Darmstadt, Germany).

Chloroperoxidase (CPO) from *Caldariomyces fumago* was purchased from Fluka via Sigma–Aldrich in BioChemica quality (22371 U ml^{-1}). Glucose oxidase from *Aspergillus niger* (low in catalase; 296 U mg^{-1}) was obtained from the same provider.

3. Results and discussion

3.1. Oxidation of PY by AaP

AaP efficiently converted PY, and the selective oxidation of the *N*-position in the PY ring was confirmed by HPLC analysis. In the respective elution profiles (Fig. 1A), solely one product appeared that accumulated in the further course of the reaction while the concentration of PY concomitantly decreased. PY *N*-oxide was identified by means of its UV spectrum and retention time compared with authentic PY *N*-oxide

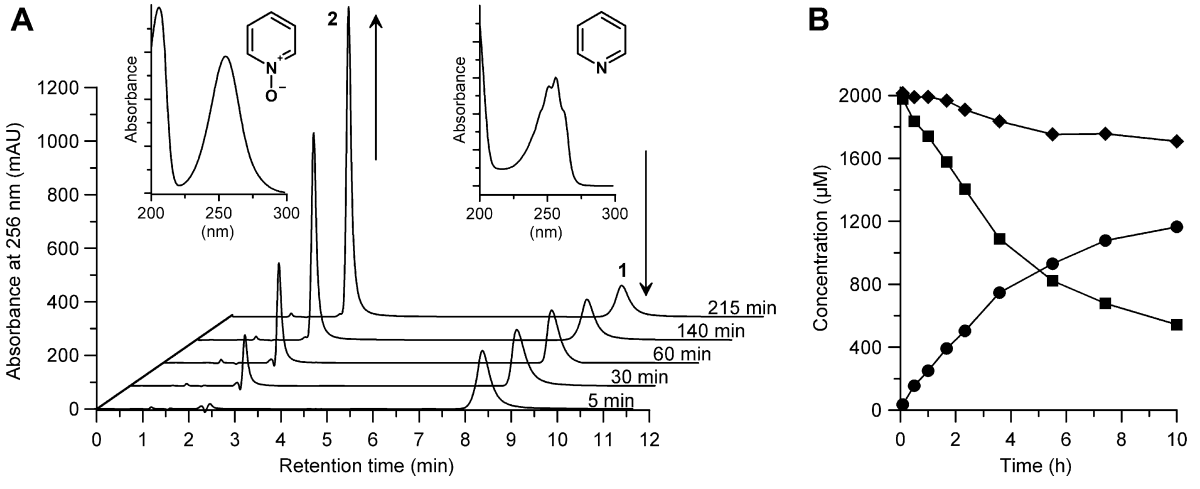


Fig. 1. (A) HPLC elution profiles of PY oxidation by AaP. The insets show the UV spectra of PY (1) and PY *N*-oxide (2). (B) Time course of PY (■) conversion and formation of PY *N*-oxide (●) (◆ sum of both) over a period of 10 hours. The reaction mixture contained PY (2000 μM) and AaP (0.45 μM); H_2O_2 was continuously supplied by glucose oxidase at a rate of 50 nmol min^{-1} ml^{-1}.

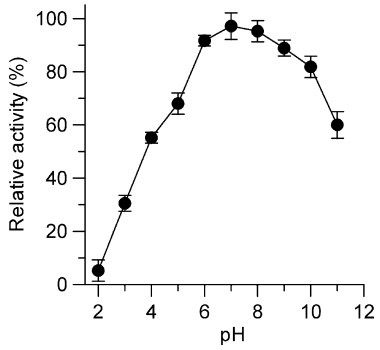


Fig. 2. Influence of pH on the oxidation of PY by AaP. Enzymatic activity was determined in appropriate potassium phosphate buffers. Data points represent mean values of three parallel measurements with standard deviation.

as well as by its mass spectrum (96 *m/z* H^+, compare also Fig. 3). Formation of other oxygenation products was not observed. CPO was not able to oxidize PY and neither any PY conversion nor the appearance of any metabolite were observed in reaction mixtures when AaP was replaced by CPO (data not shown).

Fig. 1B shows the time course of PY conversion and the formation of PY *N*-oxide over a longer period. In the reaction system, H_2O_2 was continuously supplied by glucose oxidase at a relatively low but constant level. Within 10 h, 1.4 mM of PY "disappeared", of which 86% (1.2 mM) was oxygenated by AaP to PY *N*-oxide. The lacking 14% may represent volatilized PY that has a relatively high vapor pressure (20.5 hPa at 20 °C); thus a control without AaP showed a similar PY loss (16%) over the incubation time. PY oxidation, that was linear within the first 4 hours of enzymatic reaction (260 μM h^{-1}), occurred in a broad pH range (between pH 2 and 11) with the maximum activity at neutral pH (Fig. 2).

There are only a few reports in the literature describing the microbial hydroxylation of unsubstituted PY. Though the formation of 2- and 3-hydroxy-PY (but not of PY *N*-oxide) was observed in an earlier study on the PY-utilizing bacteria *Arthrobacter crytallopoietes* and *Rhodococcus opocus* [10], recent reports on the bacterial PY degradation have an agreement that the possible pathway of PY conversion under aerobic conditions involves ring reduction and fission and not hydroxylation as the first step of metabolism [11]. Whether the hydroxy-PYs observed in bacterial cultures are secondary products of primarily reduced PY or co-metabolically formed hydroxylation products of unknown enzymes remains unclear [4].

MMO is a non-heme diiron oxygenase of methylotrophic bacteria with a broad substrate spectrum that includes methane, methanol, other *n*-alkanes and alkenes, aromatic hydrocarbons and heterocycles including PY [24]. The enzyme needs NAD(P)H for function and is associated with a flavin reductase and a specific regulator protein [25]. The specific activity (29 nmol min^{-1} mg^{-1}) reported for the *N*-oxidation of PY was comparatively low (half of the value reported for benzene and 10 times lower than that for methanol) [14,24].

There are no reports on microbial P450s oxidizing PY into PY *N*-oxide [11], but human/animal P450 2E1 (CYP2E1), that is an inducible, microsomal oxygenase in the liver, was reported to catalyze this reaction [26,27]. As MMO, P450 2E1 needs NADPH and auxiliary proteins for function, shows a low catalytic efficiency and is unstable in isolated form. Therefore, most reports on the biological formation of PY *N*-oxide have come from studies on test animals (e.g. rats, rabbits) which were treated with PY. PY *N*-oxide was then detected at varying concentrations in the urine of the test animals and microsomal preparations from respective liver cells showed increased levels of P450 2E1 and PY *N*-oxygenase activity in the presence of NADPH [15,28–30]. *N*-oxygenation of the methoxylated PY ring of omeprazole (a drug that suppresses gastric

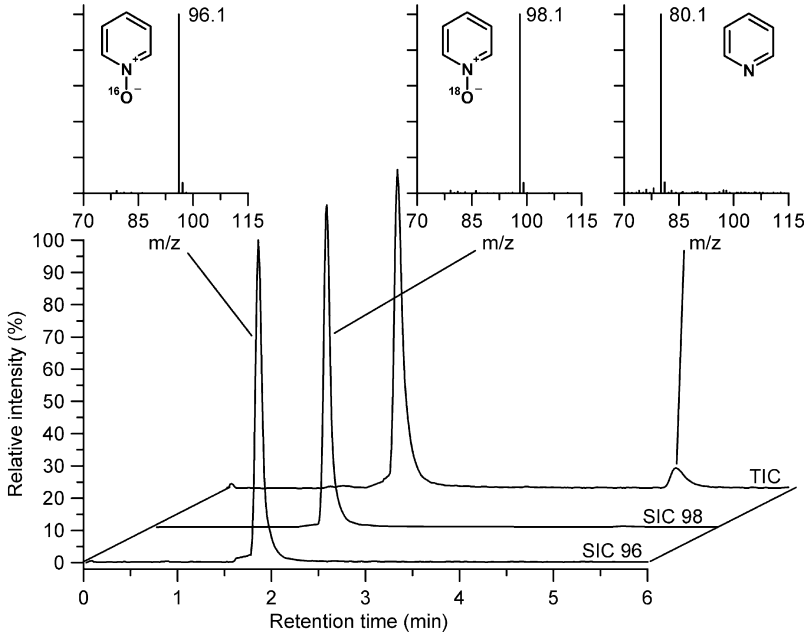


Fig. 3. HPLC elution profiles and mass spectra of a PY *N*-oxide standard (SIC 96 = selected ion chromatogram *m/z* 96) and the product of AaP-catalyzed PY oxidation in the presence of $H_2{}^{18}O_2$ (SIC 98). TIC – total ion chromatogram of PY oxidation showing the peaks of PY (right peak, *m/z* 80) and PY *N*-oxide (left peak, *m/z* 98).

acid secretion) was reported as a side activity for microsomal CYP3A from human liver [31].

3.2. Origin of oxygen in AaP-catalyzed PY N-oxidation

In order to get insight into the mechanism of heterocyclic *N*-oxidation and identify the source of the oxygen atom incorporated into PY, experiments with ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$) were performed and the reaction product, PY *N*-oxide, was analyzed by an LC–MS method. The peak of PY *N*-oxide in the HPLC elution profile (Fig. 3) has the same retention time as an authentic standard but its mass spectrum has a molecular ion peak of 98 [*m*/*z* H]$^+$ that means an *m*/*z* shift of +2 compared to ordinary PY *N*-oxide (96 [*m*/*z* H]$^+$). This finding proves the origin of the transferred oxygen atom from hydrogen peroxide. Therefore, the AaP-catalyzed oxidation of PY has to be regarded as a true peroxygenase reaction.

Peroxygenase-like activities have also been reported for P450s (peroxide "shunt" pathway) and CPO but they are either less efficient side reactions (P450s) or restricted to activated, non-aromatic substrates (CPO) [23,32]. AaP was already found to oxygenate simple aromatic substrates in previous studies. Thus, naphthalene and toluene were epoxidized and hydroxylated resulting in the formation of naphthalene 1,2-oxide, 1-naphthol and traces of 2-naphthol as well as of *p*-cresol and benzyl alcohol (side chain hydroxylation), respectively [16–18]. The present findings demonstrate that the oxygenation of the unactivated heterocycle PY is even more selective than these reactions and yields the corresponding *N*-oxide as sole product.

The term peroxygenase is also used for special plant heme proteins which neither belong to the P450s nor to the peroxidases but show sequence similarity with calcium-binding proteins [33]. Unlike fungal aromatic peroxygenases, plant peroxygenases are integral membrane proteins which are strictly dependent on organic hydroperoxides and preferably oxygenate unsaturated fatty acids [33,34]. In this context, it is interesting to mention that an unknown peroxide-consuming enzyme from plant tissue cultures was proposed to be responsible for PY hydroxylation at the *meta*-position [35]. Finally, also the oxygenation of *p*-substituted phenols by fungal tyrosinase (EC 1.14.18.1, EC. 1.10.3.1; a copper-containing oxidase) in the presence of H_2O_2 has been referred to as a peroxygenase-like activity [36].

3.3. Kinetic data of PY N-oxidation

An HPLC-based method was used to determine the kinetic data of AaP-catalyzed PY *N*-oxidation, which then could be compared with the data of other AaP substrates. The results are summarized in Table 1 and a representative Lineweaver–Burk plot is given in Fig. 4. They show that the catalytic efficiency (k_{cat}/K_m) of PY *N*-oxidation is by two magnitudes lower than that of the oxidation of 2,6-dimethoxyphenol (a typical peroxidase substrate) and aryl alcohols or polycyclic aromatic hydrocarbons such as naphthalene. This may be attributed to the specific properties of the aromatic ring of PY that is less activated than the other molecules and thus the k_{cat} is considerably lower (0.21 s^{-1} vs. 80–250 s^{-1}). On the other hand, the AaP K_m for PY (69 μM) is lower than those for the other aromatic substrates (150–2300 μM) indicating a higher affinity of AaP to PY [21].

K_m Values and specific activities of the *N*-oxygenation of PY by liver P450s were reported to vary from 80 to 1700 μM and from 2 to 4 nmol min^{-1} mg^{-1} protein, respectively [29]. In case of omeprazole *N*-oxygenation by CYP3A, it was not possible to determine specific activities or V_{max} but the authors estimated an apparent K_m of 600 μM [31]. All these K_m values are higher or at best in the same range as the K_m of AaP for PY (69 μM), however, the specific activity of AaP is about 100-fold higher.

In summary, the kinetic data for the heterocyclic *N*-oxygenation by AaP indicate that this reaction may be not just a random side activity of the enzyme but could have physiological relevance.

3.4. Oxidation of substituted PYs

In addition to PY, several PY derivatives were tested with respect to their conversion by AaP. The results are summarized in Table 2. All monohalogenated PYs were oxygenated by AaP into the corresponding *N*-oxides as sole product. Di- and perchlorinated PYs, however, were not converted. A drastic decrease of substrate turnover of higher chlorinated aromatics has also been described for other fungal oxygenases. Thus, NADPH dependent phenol hydoxylases (EC 1.14.13.7) from yeasts (*Candida* spp.) or molds (*Penicillium* spp.) were reported to hydroxylate monochlorinated and monofluorinated phenols with 50–95% of the activity of unsubstituted phenol while they showed only less than 20% activity for higher halogenated derivatives (e.g. 2,4-dichloro- or 2,4,6-trichloro/fluorophenols) [37–39].

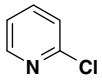
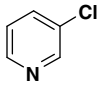
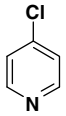
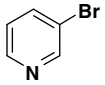
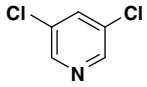
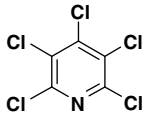
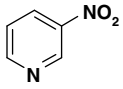
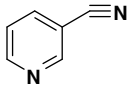
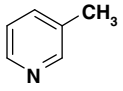
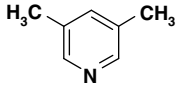
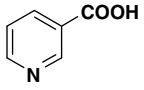
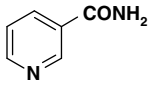
4-Cl-PY and 3-I-PY were even slightly better oxidized than unsubstituted PY, and PY derivatives halogenated at the *meta*-position by different halogens followed the order: I > Br > Cl > F, concerning oxidizibility (Table 2). That means halogenated PYs were oxidized in reverse order to their electronegativity indicating a quite open pocket at AaP's active site since the atom radius of large halogen substituents (I, Br) did not negatively influence oxidation. Comparing the conversion of differently chlorinated PYs by AaP the following order of conversion was observed: *para* > *meta* > *ortho*, i.e. in this case, a larger intramolecular distance of the halogen from the nitrogen favored *N*-oxidation.

Whereas 3-NO_2-PY and 3-CN-PY were just moderate substrates of AaP, 3-CH_3-PY was rapidly converted (Table 2). The formation of two products with the same mass

Table 1
Kinetic constants of PY oxidation by AaP in comparison to data obtained for other AaP substrates. PY conversion was followed by HPLC and data were calculated by Lineweaver–Burk and Eddie–Hofstee plots; an example is given in Fig. 4.

Substrate	Product	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Pyridine (PY)	PY *N*-oxide	69	0.21	3.04×10^3
Naphthalene [17]	1-Naphthol	320	166	5.17×10^5
2,6-Dimethoxyphenol [21]	Coerulignone	298	108	3.61×10^5
Benzyl alcohol [21]	Benzaldehyde	1001	269	2.69×10^5

Table 2
Oxidation of different PY derivatives by AaP. Conversion is given in relation to the oxidation of unsubstituted pyridine (100%). All reactions were performed under identical conditions using 500 μM substrate and 1.45 μM AaP; H_2O_2 was continuously supplied by a syringe pump (25 nmol min^{-1} ml^{-1}).

Substrate	Formula	Relative conversion (%)	Number of products	*m/z* [H^+]	Identified products
PY		100 ± 1.1	1	96	PY *N*-oxide
2-Cl-PY		26.1 ± 2.1	1	130 / 132	2-Cl-PY *N*-oxide
3-Cl-PY		47.2 ± 2.4	1	130 / 132	3-Cl-PY *N*-oxide
4-Cl-PY		102.9 ± 4.5	1	130 / 132	4-Cl-PY *N*-oxide
3-F-PY		39.4 ± 3.9	1	114	3-F-PY *N*-oxide
3-Br-PY		61.8 ± 2.7	1	175 / 177	3-Br-PY *N*-oxide
3-I-PY		102.2 ± 0.9	1	222	3-I-PY *N*-oxide
3,5-Di-Cl-PY		0	0	–	–
Per-Cl-PY		0	0	–	–
3-NO_2-PY		5.4 ± 1.5	1	141	3-Nitro-PY *N*-oxide
3-CN-PY		25.7 ± 0.4	1	121	3-Cyano-PY *N*-oxide
3-CH_3-PY		98.4 ± 0.8	4	110 110 108 (126; 140) 124	3-CH_3-PY *N*-oxide Nicotinic alcohol Nicotinic aldehyde Nicotinic acid
3,5-Di-CH_3-PY		143.4 ± 0.6	3	124 124 122 (140; 154)	3,5-Di-CH_3-PY *N*-oxide* 5-CH_3-Nicotinic alcohol 5-CH_3-Nicotinic aldehyde
Nicotinic acid		0	0	–	–
Nicotine amide		0	0	–	–

*Traces (<1% of the converted substrate).

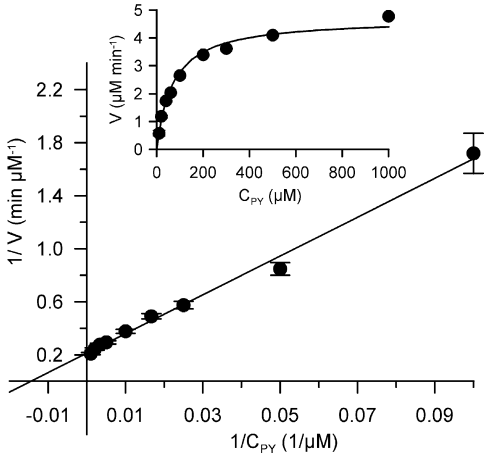


Fig. 4. Lineweaver–Burk plot of PY *N*-oxidation by AaP. Enzymatic activity was determined by HPLC at substrate concentrations between 10 and 1000 µM in the presence of 1000 µM H_2O_2. Data points represent mean values of three parallel measurements with standard deviation.

(110 *m*/*z* H^+, respectively) suggests that in addition to the respective *N*-oxide also an alcohol derivative (nicotinic alcohol) was formed, resulting from the hydroxylation of the methyl side chain. In addition, the MS data indicate the formation of further oxidation products of the methyl group, nicotinic aldehyde and acid. These findings were confirmed by appropriate standards and the formation of individual oxidation products was quantified (Fig. 5). About 30% of 3-CH_3-PY was converted into the corresponding *N*-oxide and 70% was oxidized at the methyl group leading to alcohol, aldehyde and carboxy derivatives. The product pattern resembles that of toluene oxidation by AaP yielding *p*-cresol as well as benzyl alcohol, benzaldehyde and benzoic acid [16].

In contrast to non-oxidizable 3,5-Di-Cl-PY, 3,5-Di-CH_3-PY (picoline) was a good AaP substrate, which was even 1.4-fold faster oxidized than PY again indicating a stronger influence of the substituents electronic effects vs. their size [40]. The favored oxidation site of 3,5-Di-CH_3-PY, however, was one of the methyl side chains and only traces of the corresponding *N*-oxide were detected. Surprisingly, neither nicotinic acid (PY 3-carboxylic acid) nor the corresponding amide was found to be a substrate of AaP (Table 2). A number of intracellular monooxygenases is known to specifically hydroxylate these compounds to form the corresponding 6-hydroxy derivatives

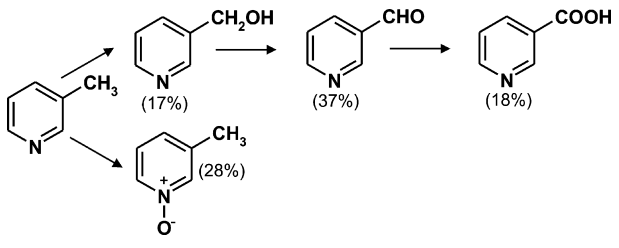


Fig. 5. Reaction scheme for the AaP-catalyzed oxidation of 3-methylpyridine (3-CH_3-PY). Either the heterocyclic N is oxidized (reaction below) or the methyl group is stepwise oxygenated (reaction above) leading to the corresponding alcohol, aldehyde and carboxy derivatives. Oxygenation at both positions in one molecule was not observed.

[1,11]. They belong to a special type of hydroxylase (EC 1.17.1.5) containing non-heme iron, sulphur and molybdenum in the active site [41,42]. The oxygen of the hydroxyl group of 6-hydroxynicotinate was shown to derive from water (nucleophilic attack by OH^-) and does not come from O_2 or H_2O_2 (electrophilic attack by Compound I) as in case of P450s or aromatic peroxygenases (APO) [11,23,43]. Furthermore, the specific hydroxylation of nicotinate at the position C_2 was described for *Ralstonia*/*Burkholderia* strain DSM 6920 [44]. In all these cases, however, nicotinate *N*-oxide was not detected as microbial or enzymatic oxygenation product. Why AaP is not capable of oxidizing nicotinate is still unclear; one explanation could be the polarity of the carboxylic group that may prevent correct substrate binding.

3.5. Conclusions

Our results demonstrate that the peroxidase of *A. aegerita* (AaP) can act as a true PY peroxygenase that regioselectively transfers an oxygen atom from hydrogen peroxide to the heterocyclic nitrogen of the PY molecule. Since neither PY nor naphthalene [16] are substrates of other peroxidases (including CPO), aromatic peroxygenases such as AaP could represent a new type of this group of enzymes (EC 1.11.1.x) or even a new sub-subclass of oxidoreductases [45]. Current molecular studies seem to confirm this assumption.

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