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## A New Class of Arylamine Oxygenases: Evidence that *p*-Aminobenzoate *N*-Oxygenase (AurF) is a Di-iron Enzyme and Further Mechanistic Studies

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Arylamine oxygenases are enzymes that catalyze the unusual oxidation of an aryl amine group to an aryl nitro group. Although arylamine oxygenases seem to be widespread and used in diverse metabolic reactions,<sup>[1,2]</sup> aminopyrrolnitrin oxygenase (PrnD)<sup>[2]</sup> and *p*-aminobenzoate oxygenase (AurF)<sup>[3]</sup> are the only two known examples. Recently, PrnD was biochemically and mechanistically characterized.<sup>[2,4]</sup> AurF, however, has had limited characterization, mainly due to the difficulty of obtaining functional enzymes in vitro.<sup>[1,5]</sup> In addition, previous database searches (BLAST, PROSITE) failed to identify any known oxidoreductase cofactor binding sites or motifs in the AurF sequence,<sup>[1,3]</sup> this indicated that AurF could be a novel class of arylamine oxygenases.

We performed our own BLAST search on AurF using Biology Workbench (<http://workbench.sdsc.edu>) and identified four putative proteins that share low sequence homology with AurF (15–17% identity). Further sequence alignment by Clustal W revealed several conserved aspartic acid, glutamic acid, and histidine residues, and the conservation of two copies of an EX<sub>28-37</sub>DEXXH motif. That this motif is well conserved in several di-iron oxygenases,<sup>[6-13]</sup> suggests that AurF might be a dinuclear non-heme iron oxygenase.

To test this hypothesis, we cloned the *aurF* gene from *Streptomyces thioluteus* (ATCC 12310) into pTrc99A and heterologously expressed it in *Escherichia coli* BL21 (DE3). In vivo AurF activity in *E. coli* could not be observed when using the phosphate–saline solution reaction medium, as reported previously in *Streptomyces* experiments.<sup>[5,14]</sup> However, addition of a carbon source such as glucose, glycerol, acetate, or succinate to the reaction media restored in vivo AurF activity in *E. coli*; this suggests that a certain cellular process is needed for enzyme function. This result is different from what was observed in experiments with *Streptomyces*. In our laboratory as well as in other

laboratories,<sup>[1,5]</sup> *p*-aminobenzoic acid (pABA) conversion to *p*-nitrobenzoic acid (pNBA) was observed in *Streptomyces* in a reaction medium lacking a carbon source. Unfortunately, similar to previous studies,<sup>[1,5]</sup> the AurF activity was completely abolished after lysis of the *E. coli* cells.

To probe the presence of a di-iron cluster in AurF, each of the eight conserved residues in the EX<sub>28-37</sub>DEXXH motif was individually mutated to alanine by site-directed mutagenesis. As expected, the activity of each mutant was significantly lowered. No mutant showed greater than 1% of the wild-type AurF activity; this suggests that each residue is important for enzyme function. Note that each mutant enzyme was detected in at least some soluble form by Western blot; this indicates that lack of solubility was not the reason for complete loss of activity.

The presence of a di-iron cluster in AurF was further determined by inductively coupled plasma (ICP) atomic emission spectrometry and electron paramagnetic resonance (EPR). For ICP studies, an average iron-to-enzyme ratio was determined to be 2.18, which is in good agreement with the expected value of two iron atoms/enzyme. For EPR studies, according to previous reports, a di-iron cluster is usually stable in three states: oxidized (Fe<sup>III</sup>–Fe<sup>III</sup>), mixed-valent (Fe<sup>III</sup>–Fe<sup>II</sup>), and reduced (Fe<sup>II</sup>–Fe<sup>II</sup>).<sup>[15]</sup> AurF purified from *E. coli* cells grown in Luria–Bertani medium generated an oxidized-form EPR spectra (Figure 1A) that resembled a mononuclear manganese(IV) species.<sup>[16]</sup> This spectrum is also similar to the spectrum generated by a ribonucleotide reductase enzyme containing a Fe<sup>III</sup>–Mn<sup>III</sup> center.<sup>[17]</sup> Based on the ICP data, it is unlikely that AurF naturally contains manganese atom(s). Therefore, the enzyme was expressed in *E. coli* by using minimal medium with an overabundance of iron. The resulting enzyme generated an EPR-silent oxidized spectrum, and a mixed valence spectrum containing  $g_x$ ,  $g_y$ , and  $g_z$  values of 1.94, 1.79, and 1.70, respectively (Figure 1B). These mixed-valence  $g$  values are similar to those obtained for methane monooxygenase and ribonucleotide reductase.<sup>[15,18]</sup> In addition, the fully reduced form of AurF generated a silent EPR spectrum, which is similar to that which is observed with the di-iron enzyme acyl-carrier protein  $\Delta^9$  desaturase.<sup>[15]</sup>

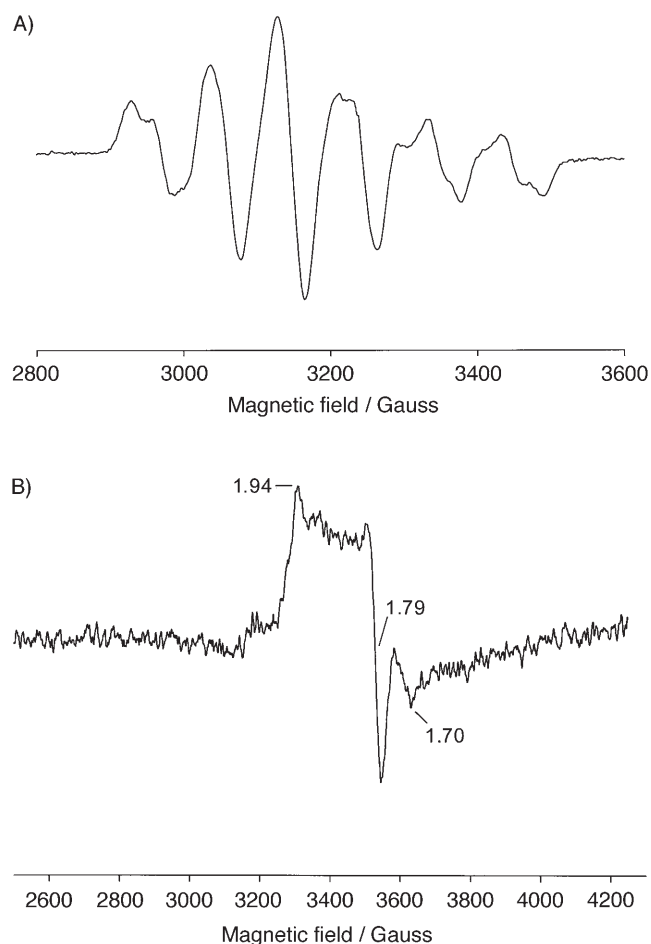
It is not surprising that the EPR spectrum generated by AurF prepared in *E. coli* by using Luria–Bertani medium contained a signal from a manganese atom. It has been shown previously that di-iron enzymes can often take up manganese atoms in place of iron atoms.<sup>[17,19]</sup> This idea was further tested by producing AurF in *E. coli* by using minimal medium with an overabundance of manganese. The resulting AurF did not exhibit in vivo activity. Its EPR spectrum was somewhat similar to that of mononuclear manganese; this does not agree with ICP data showing 2.04 manganese atoms/enzyme (see the Supporting Information).

A catalytic mechanism was previously proposed for AurF based on in vivo assay data (Scheme 1 a).<sup>[1]</sup> In this mechanism, the first step involves the addition of an oxygen atom to produce *p*-hydroxylaminobenzoate (pHABA) from pABA; this was confirmed by the isolation of pHABA. The later steps involve the addition of another oxygen atom, followed by a dehydra-

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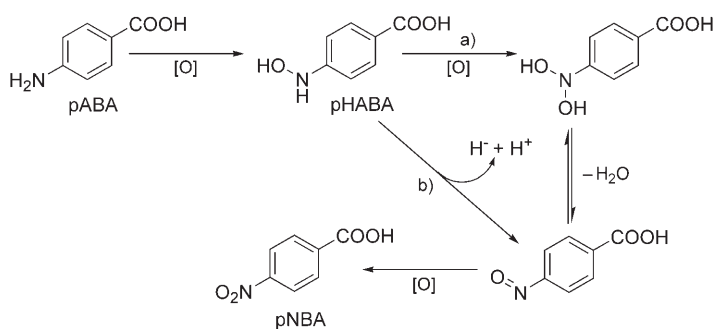
Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.



**Figure 1.** A) EPR spectrum of oxidized AurF prepared in Luria-Bertani medium. The spin concentration of the manganese atom was 23  $\mu\text{M}$ . B) EPR spectrum of mixed-valence AurF. Numbers indicate the  $g$  value of each peak. The spin concentration of the di-iron center was estimated to be between 10 and 40  $\mu\text{M}$ .

tion step to a nitroso compound, and subsequently the addition of a third oxygen atom to produce the nitro compound; these have not been experimentally confirmed yet. In addition, it was speculated that the oxygen atoms used by this enzyme are derived from molecular oxygen.<sup>[1,5]</sup>

We wondered if the catalytic mechanism previously proposed for PrnD could also apply to AurF, even though these



**Scheme 1.** Proposed mechanisms for bioconversion of arylamines into aryl nitro compounds by AurF in a) a previous study<sup>[1]</sup> and b) this study.

two enzymes are completely different.<sup>[4]</sup> In this new mechanism (Scheme 1 b), the hydroxylamine is formed by the addition of an oxygen atom. The second step directly forms the nitroso compound by dehydrogenation, while the last step adds an oxygen atom to the nitroso compound to form the nitro compound.

To test this PrnD-like mechanism with AurF, unlabeled pHABA was added to a sealed vial containing degassed reaction medium and AurF-expressing *E. coli* cells, and then  $^{18}\text{O}_2$  was allowed to enter the vial. The resulting nitro compound was purified and analyzed by EI-MS. Surprisingly, only one  $[M]^+$  peak at  $m/z$  169 was observed for pNBA (Figure 2); this indicates that the originally proposed dehydration step is incorrect. If dehydration were possible, one would expect to observe a  $[M]^+$  peak at  $m/z$  169 and one at  $m/z$  171. We therefore propose that the mechanism for the bioconversion of pABA into pNBA by AurF follows the same catalytic mechanism of PrnD (Scheme 1 b).

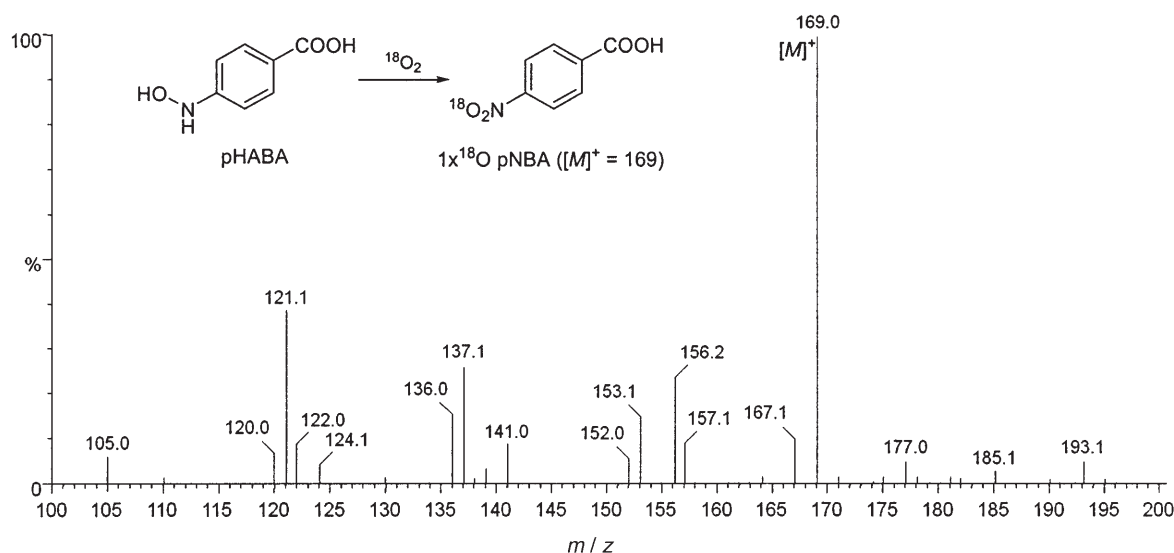
Bioconversions of arylamines to aryl nitro compounds have thus far not been well characterized. In order to learn more about the characteristics and mechanism of AurF, we conducted several biochemical studies. Bioinformatic studies, site-directed mutagenesis, ICP-MS, and EPR experiments all indicated the presence of a di-iron cluster in AurF. Furthermore,  $^{18}\text{O}_2$ -labeling studies suggest that the catalytic mechanism of AurF is similar to that of the only other reported *N*-oxygenase, PrnD. Thus, for the first time, we have demonstrated that AurF is a di-iron non-heme monooxygenase that catalyzes unusual oxidation of arylamine to aryl nitro compounds. This enzyme should represent the first member of a new class of arylamine oxygenases.

## Experimental Section

The *aurF* gene was amplified by PCR from genomic DNA of *Streptomyces thioluteus* (ATCC 12310, ATCC, Manassas, VA) and cloned into pTrc99A (Amersham Biosciences, Piscataway, NJ) using a forward primer (5'-GGATCGGTCCATGGGAGAAGA GCA GCC-3') and a reverse primer (5'-TAATAA GCTTTC AACGCGGCGTGTGGG-3'). A His<sub>6</sub>-tag and a thrombin site were subsequently added to the forward AurF PCR primer for recloning into pTrc99A for expression of His-tagged AurF. This plasmid was also used as a template for site-directed mutants by the mega-primer PCR method.<sup>[20]</sup> Plasmids were transformed into *E. coli* BL21(DE3) cells. For AurF and site-directed mutant expression, cells were grown at 37 °C to OD<sub>600</sub> = 0.6.

Then protein expression was induced with isopropyl- $\beta$ -D-1-thiogalactopyranoside (0.25 mM), and the cells were agitated at 250 rpm and 30 °C for 5 h.

For in vivo assays, cells containing AurF (or mutants; ca. 100 mg) were washed twice and resuspended in assay mixture (25 mL) which contained MOPS (25 mM; pH 7.2), NaCl (8 g L<sup>-1</sup>), KCl (1 g L<sup>-1</sup>), glucose (2 g L<sup>-1</sup>), and pABA (1 mM). Samples were taken every 30 min for 4 h and analyzed on an Agilent 1100 Series HPLC column. The sample was eluted on a ZORBAX SB-C18 column (3  $\times$  150 mm<sup>2</sup>, Agilent). HPLC parameters were as follows: 25 °C; solvent A: 1% acetic acid in water; solvent B: methanol; gradient: 100% A for 1 min; then from 30% B to 70% B over 20 min; flow rate: 0.6 mL min<sup>-1</sup>; detection by UV absorbance at 268 nm.



**Figure 2.** Isotope-labeling experiments of AurF with  $^{18}\text{O}_2$ . The molecular ion is the reaction product of AurF with unlabeled pHABA under  $^{18}\text{O}_2$ -enriched atmospheres.

AurF was overexpressed in *E. coli* as described above, and resuspended in lysis buffer containing  $\text{KH}_2\text{PO}_4$  (50 mM, pH 8), NaCl (300 mM), and lysozyme (1 mg mL $^{-1}$ ). Cells underwent one cycle of freeze–thaw, followed by two passages at 137 000 kPa on a French Pressure Cell. AurF was then purified by nickel affinity chromatography, and resuspended in either  $\text{KH}_2\text{PO}_4$  (50 mM, pH 7) for ICP studies or in HEPES (20 mM, pH 8) for EPR studies.

For the ICP studies, purified AurF was dialyzed several times with a potassium phosphate buffer solution (50 mM, pH 7). The protein concentration was analyzed by the bicinchoninic acid assay. Samples were then lyophilized to remove all water. Lyophilized protein was then analyzed on a Perkin–Elmer ICP (OES Optima 2000 DV, Perkin–Elmer, Norwalk, CT) at the University of Illinois Microanalysis Laboratory (Urbana, IL).

EPR was carried out by using purified protein dialyzed several times with HEPES buffer (20 mM, pH 8). The protein was then concentrated to 15–20 mg mL $^{-1}$  by using Millipore Ultra centrifuge concentrators (MWCO 10000). All reduction experiments were conducted in an anaerobic chamber (100% Ar), and reduction was performed by adding sodium dithionite to the protein samples. Protein samples and buffers were first degassed with several vacuum/ $\text{N}_2$  cycles (–20 mmHg/68.9 kPa) prior to conducting reduction experiments. Samples were allowed to reduce for 30 min in the anaerobic chamber at room temperature. After reduction, 10% glycerol was added to the protein samples, and each sample was placed into a ~23 cm quartz EPR tube (3 mm i.d., 4 mm o.d.). The EPR tubes were capped, removed from the anaerobic chamber, and immediately frozen in liquid nitrogen. Oxidized samples were prepared by the same method, excluding the anaerobic chamber and sodium dithionite. Samples stored in liquid nitrogen were brought to the EPR center at the University of Illinois for analysis. EPR measurements were performed at 15 K on a Varian E-122 X-band spectrometer equipped with an Air Products Helitran cryostat. Other parameters for EPR measurements include 2 mW microwave power, 5-G modulation amplitude, and 9.06 GHz microwave frequency. Spin concentrations were determined by double integrating baseline-corrected spectra. A sample containing  $\text{CuSO}_4$  (1 mM) in a 20% glycerol solution was used for calibration.

For labeling studies with  $^{18}\text{O}_2$ , two vials, one containing substrate and one containing AurF-expressing *E. coli* cells in reaction medium were degassed by application of a vacuum and flushed with argon three times. The anaerobic substrate solution was transferred to the whole-cell enzyme vial. The argon was removed by application of a vacuum, and finally  $^{18}\text{O}_2$  was allowed to enter into the vial. After incubation for 6 h at 30 °C, the reaction mixture was purified by HPLC and analyzed by EI-MS (Micromass Quattro, Micromass, Manchester, UK).

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