Characterization of Pseudooxynicotine Amine Oxidase of *Pseudomonas putida* S16 that Is Crucial for Nicotine Degradation

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Pseudooxynicotine amine oxidase (Pnao) is essential to the pyrrolidine pathway of nicotine degradation of *Pseudomonas putida* strain S16, which is significant for the detoxification of nicotine, through removing the CH\(_3\)NH\(_2\) group. However, little is known about biochemical mechanism of this enzyme. Here, we characterized its properties and biochemical mechanism. Isotope labeling experiments provided direct evidence that the newly introduced oxygen atom in 3-succinoylsemialdehyde-pyridine is derived from H\(_2\)O, but not from O\(_2\). Pnao was very stable at temperatures below 50 °C; below this temperature, the enzyme activity increased as temperature rose. Site-directed mutagenesis studies showed that residue 180 is important for its thermal stability. In addition, tungstate may enhance the enzyme activity, which has rarely been reported before. Our findings make a further understanding of the crucial Pnao in nicotine degradation.

Tobacco consumption is not only an important pillar of the national economy of China, but also a leading preventable cause of diseases such as lung cancer\(^1\)\(^–\)\(^3\). Nicotine is the major toxic component of tobacco, and it is capable of crossing biological membranes. When tobacco is burned, nicotine is transformed into tobacco-specific nitrosamines (TSNAs)\(^4\)\(^–\)\(^7\). One of the most toxic TSNAs, 4-methylnitrosamino-l-(3-pyridyl)-l-butanone (NNK), can be generated from pseudooxynicotine (PN) through nitrosation\(^8\)\(^–\)\(^9\). PN is an intermediate product in the nicotine degradation pathway of *Pseudomonas putida* strain S16. Our previous studies showed that strain S16 is a nicotine-metabolizing microorganism that transforms nicotine to 2,5-dihydroxypyridine through N-methyl-myosmine, pseudooxynicotine, and 3-succinoylpyridine (SP)\(^10\)\(^–\)\(^13\). A pseudooxynicotine amine oxidase (Pnao) gene has been identified, and the encoded protein could detoxify PN by removing the CH\(_3\)NH\(_2\) group;\(^14\)\(^,\)\(^15\) when the encoded gene *pnao* was deleted, the pathway of nicotine degradation was blocked, showing this is a critical step for nicotine detoxification by *Pseudomonas*\(^15\). In previous studies, only the product and the prosthetic group of Pnao were identified\(^14\). Thus, little is known about Pnao and its mechanism(s) of action.

In this study, His\(_6\)-tagged Pnao was heterologously expressed and purified from *Escherichia coli*, and then characterized. The oxygen atom added to 3-succinoylsemialdehyde-pyridine (SAP) originates from H\(_2\)O; O\(_2\) is involved in the oxidation of FADH\(_2\). We also found that Pnao possesses some special properties, such as thermal stability below 50 °C, promotion of enzyme activity by Na\(_2\)WO\(_4\), and inhibition by Na\(_2\)MoO\(_4\) and FeCl\(_3\). The work described here provides a basis for future studies aimed at determining the enzymatic mechanisms of nicotine detoxification.

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Results

Expression and purification of Pnao. A 1-mL solution containing 10 mg/mL Pnao was purified from a 3-L overnight culture of *E. coli* cells. SDS-PAGE and Superdex200 column analysis (Fig. 1A) showed that the purified Pnao has a molecular mass of approximately 54 kDa, which corresponds to the

![Figure 1. Biochemical characterization by enzymatic assays of Pnao.](image)

(A) Spectrum curve of Pnao on gel filtration. The column (superdex 200) was marked by a standard protein (Ovalbumin, 48.1 kDa). (B) Kinetic curve of Pnao at 30 °C. The apparent $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values for PN at 30 °C are $0.073 \pm 0.018$ mM, $0.790 \pm 0.074$ s$^{-1}$, and $10.822$ L mol$^{-1}$ s$^{-1}$, respectively. (C) Effect of pH on Pnao activity (with immediate detection). (D) Effect of pH on Pnao stability (with overnight incubation). The enzyme was incubated in buffers overnight. (E,F) Effects of metal salts on Pnao activity (with immediate detection) and Pnao stability (with overnight incubation). Metal salts: NaCl, NiCl$_2$, BaCl$_2$, CoCl$_2$, CaCl$_2$, CuCl$_2$, MnCl$_2$, ZnCl$_2$, KCl, Na$_2$MoO$_4$, Na$_2$WO$_4$, CdCl$_2$, BdCl$_2$, FeCl$_3$, and MgCl$_2$. CK, without metal salts. The final concentration of metal salts was 2 mM.
molecular mass deduced from the protein sequence. The apparent \( K_m \), \( k_{cat} \) and \( k_{cat}/K_m \) values for PN at 30 °C were 0.073 ± 0.018 mM, 0.790 ± 0.074 s\(^{-1}\), 10.822 L mol\(^{-1}\) s\(^{-1}\), respectively (Fig. 1B).

**Measurement of Pnao activity.** (a) Effect of pH on Pnao activity. Pnao showed the highest specific enzyme activity at pH 8.5 in Na\(_2\)HPO₃-NaH₂PO₃ buffer, and the enzyme activity in this buffer was much higher than that in the other tested buffers. Thus, in subsequent experiments, Pnao enzyme activity was measured in 25 mM Na\(_2\)HPO₃-NaH₂PO₃ buffer at pH 8.5 (Fig. 1C).

(b) Effects of metal salts on Pnao activity. Na\(_2\)MoO₄ and FeCl₃ strongly inhibited the enzyme activity. In contrast, the enzyme activity was nearly twice as high in the presence of Na\(_2\)WO₄ as that in its absence (Fig. 1E). However, according to the ICP results, there was no difference between the protein solution (0.5938 ppb) and the control group (0.2619 ppb), indicating that Na\(_2\)WO₄ is not a prosthetic group. Therefore, the function of Na\(_2\)WO₄ during the reaction remains to be determined.

The stability of Pnao. (a) Effect of pH on Pnao stability. Pnao enzyme activity was measured after incubation in buffers at 25 °C overnight. Maximal activity was observed after incubation at pH 7.5 (Fig. 1D). Thus Pnao was stored in Tris-HCl buffer pH 7.5.

(b) Effects of metal salts on Pnao stability. The enzyme was incubated in the solutions at 25 °C overnight. Most of the tested metal salts inhibited enzyme activity to different degrees, and Pnao showed very low activity in the presence of Na\(_2\)MoO₄ or FeCl₃. In the presence of Na\(_2\)WO₄, Pnao showed the same activity as that reported in the activity assay (Fig. 1F).

(c) Effect of temperature on Pnao stability. To confirm the critical degeneration temperature, Pnao stability was monitored by circular dichroism spectroscopy (CDS). The analysis showed that Pnao began to degenerate at temperatures above 45 °C (Fig. 2A). At temperatures above 50 °C, Pnao quickly denatured. However, at temperatures from 30 °C to 60 °C, Pnao enzyme activity increased (Fig. S1). Therefore, according to these results, Pnao was stable below 45 °C.

Identification of prosthetic groups. The purified protein appeared yellow, indicating that it is bound to FAD or FMN as a cofactor. The retention time of a compound from the supernatant from a boiled protein solution in HPLC was approximately 6 min, which is similar to that of a standard solution of FAD and different from that of a standard solution of FMN. The maximum UV-Vis absorbance peaks of the supernatant were at 376 nm and 460 nm, which was the same as that of a standard solution of FAD (Fig. S2). These results indicated that FAD was the cofactor associated with Pnao. However, Pnao activity did not show an evident increase after adding additional FAD, which indicates that Pnao was already saturated with FAD.

Site-directed mutagenesis of Pnao. Mutants of Pnao were purified by the same way of wildtype (Fig. 3A). When the enzyme activity of the mutants was assessed in the standard system at 45 °C, the Pro180Ser mutant was the only mutant that was extremely unstable. Thus, we analyzed Pro180Ser by CDS. The curve indicated that the structure of the Pro180Ser mutant changed at approximately 20 °C (Fig. 2B). Thus, we predict that Pro180Ser site is a very important residue for Pnao thermal stability.
Reaction mechanism of Pnao.

In the LC-MS analysis, a molecular peak was observed at m/z 164 in the control, which indicated that the product was SAP. A similar peak was observed for the 18O2 group (Fig. 4A,B). In contrast, a molecular peak at m/z 166 and a small peak at m/z 164 were observed for the H218O group (Fig. 4C), suggesting that the oxygen added to SAP is derived from H2O not from O2. (D) A general view of the reaction process of Pnao. In the first step, PN is activated via the reduction of FAD and a carbon-nitrogen double bond is formed. Then, H2O attaches the carbon-nitrogen double bond and a hypothetic, extremely unstable intermediate is generated. This intermediate is transformed to SAP and CH3NH2 quickly. Finally, FAD is regenerated via the oxidation to make preparation for the next reaction.
Discussion

The mechanism of nicotine degradation in Pseudomonas provides a basis for the disposal of tobacco wastes. In our previous study, we identified a novel gene in P. putida strain S16 whose product is involved in the transformation of PN into SAP during nicotine degradation. The encoded enzyme Pnao can attack the CH-NH bond and remove the CH$_3$NH$_2$ group from PN. The generated intermediates are less toxic and cannot easily be transformed into carcinogenic TNSAs through nitrosation. However, little is known about the characteristics of this enzyme.

Amine oxidases play important roles in the metabolism of various organisms. The amine oxidases are an enormous group of enzymes that could show variations in their features, such as thermal stability and inhibitors. Thus, we performed experiments to determine the specific features of Pnao. Pnao showed thermal stability below 50°C, which is very unique among the enzymes involved in the nicotine degradation. Thus, we analyzed the sequence of pnao and the encoded protein, and found that a 58-amino acid fragment displayed a higher amount of Pro-Glu residues (27.6%) than other regions. Pro and Glu residues are very important for the structural stability of proteins at high temperature. Sixteen residues (Pro or Glu) in this region were changed to Ser or Gly by site-directed mutagenesis, and the mutant proteins were not stable at high temperatures. In fact, one mutant, Pro180Ser, became extremely unstable at high temperature. The absorbance of the α-helices and β-folds started to change at temperatures above 25°C, which was very different from that observed with the wild-type protein. However, Pro180Ser was still activated and the specific enzyme activity of Pro180Ser did not decrease significantly. Thus, we predict that residue 180 is extremely important for maintaining structural stability in different thermal environments as changing this amino acid had no evident effects on the enzyme activity.

In this study, approximately 0.3 mol of FAD/mol of wild-type Pnao was obtained, which was lower than the 0.5 mol/mol of enzyme reported previously. Furthermore, there was no evident loss or increase of FAD regardless of the severity of the mutation (Fig. 3B). Enzyme activity did not increase in the presence of excess FAD, suggesting that the enzyme without FAD is unable to capture FAD. When the excessive FAD was added into the culture, the FAD content didn't increase. These observations suggest that this uncommon FAD to enzyme ratio is not due to insufficient FAD synthesis. In pH assays, W, Mo, and Fe were added into the culture, respectively; however, there was no increase or loss of the FAD content (Fig. 3B). In this case, we infer that W, Mo, and Fe do not work by affecting the content of FAD.

Some mechanistic studies of amine oxidases, which produce NH$_4^+$, described a model in which the substrate was oxidized by O$_2$ to form H$_2$O$_2$ and a carbon-nitrogen double bond, which was subsequently hydrolyzed. When we added PN to a solution containing 15 mg/mL Pnao, the yellow solution turned colorless very quickly, and then returned to yellow after all the PN was degraded. When we performed the same assay in an anaerobic environment, the solution still turned colorless; however, it never returned to yellow, which suggests that O$_2$, and not PN, is involved into the oxidation of FADH$_2$. In addition, we inferred that FAD was reduced first, which then activated PN to form an intermediate.

Based on our results, we believe that PN is activated via FAD reduction and that a carbon-nitrogen double bond is formed in the first step. In the next step, H$_2$O binds to the carbon-nitrogen double bond, and a hypothetical, extremely unstable intermediate is generated. This intermediate is quickly transformed to SAP and CH$_3$NH$_2$. At last, FAD is regenerated via oxidation for the next round of catalysis (Fig. 4D).

In summary, we determined the role of H$_2$O and O$_2$ in the conversion of PN to SAP by Pnao and inferred the mechanism of Pnao, showing unique features, such as unsaturated FAD content, and thermal stability that is related to site-directed mutagenesis with which residue 180 is important for thermal stability. In addition, Pnao shows significant increase of enzyme activity in presence of tungstate, which is rarely found in the literature.

Methods

Materials. L-(-)-Nicotine (≥99% purity) was gained from Fluka Chemie GmbH (Buchs Corp., Buchs, Switzerland). Flavin adenine dinucleotide (FAD) was obtained from Sigma-Aldrich (St. Louis, MO, USA). $^{18}$O$_2$ and H$_2^{18}$O were from Shanghai Research Institute of Chemical Industry. PN (98%) and 3-succinylsemialdehyde-pyridine (SAP) were from Toronto Research Chemicals (Canada). All other reagents and solvents applied in this study were analytical grade and easily available.

Bacterial strains, plasmids and culture conditions. The bacterial strains, vectors, and recombinant plasmids applied in this study are listed in Table 1. P. putida S16 and their derivatives were cultured at 30°C in Lysogeny broth (LB) medium containing 1 g L$^{-1}$ nicotine. E. coli strains were grown at 37°C in LB medium. Kanamycin and ampicillin were used for selection at appropriate concentrations.

Expression and purification of His$_6$-Pnao in vitro. The full-length DNA fragment of pnao was amplified by PCR (primers pnaoEF-Ncol/ pnaoEF-XhoI), and inserted into the Ncol-XhoI sites of the expression vector pET28a to reconstruct pET28a-pnao. Plasmid pET28a-pnao was transferred into E. coli C43(DE3) for heterologous expression. E. coli C43(DE3) with the reconstructed plasmids were cultured in LB containing 100 mg L$^{-1}$ ampicillin, at 37°C to an optical density at 600 nm 0.8 to 1. Isopropyl-$eta$-D-thiogalactopyranoside (IPTG) (200 μmol) was subsequently added to induce the culture at 16°C for 20h to 24h. The harvested cells were re-suspended with 20 mM Tris-HCl (pH 7.5) buffer and...
disrupted by sonication in an ice-water bath. Cell debris and insoluble proteins were removed by centrifugation (12,000 \( \times \) g for 30 min). The crude enzyme was loaded onto a His-bind resin. The His\textsubscript{6}-tagged Pnao was eluted by 200 mM imidazole after elution of the no target proteins with 20 and 50 mM imidazole. The eluted sample was loaded onto a Superdex 200 column which had been pre-equilibrated with 10 mM Tris-HCl (pH 7.5) buffer. The protein concentration was quantified by the Bradford method using bovine serum albumin as the standard. All those steps were performed at 4 °C and the collected His\textsubscript{6}-tagged protein Pnao was preserved at \(-70 \) °C for further study.

Determination of the Pnao cofactor. The purified Pnao was boiled for 5 min to release the cofactor and precipitate the protein. The solution was filtered by 0.22 \( \mu \)m membrane after centrifugation. The filtered supernatant was detected by high-performance liquid chromatography (HPLC) (Agilent 1200 series, Hewlett-Packard Corp., Santa Clara, CA, U.S.A.) with a C-18 column (4.6 by 250 nm; 5 \( \mu \)m). The mobile phase was 10 mM ammonium acetate containing 30% methanol (v v\textsuperscript{-1}). The flow rate was 0.5 mL min\textsuperscript{-1}. The contents were monitored by determining the absorbance at 265 nm. The standard curve was drawn in the range of 0 to 0.3 mM to analyze the concentration of FAD.

Biochemical analysis of Pnao. Buffer, Pnao and PN were all freeze-dried in advance. In the \( ^{2}H_{2}^{18}O \) assay, powdered buffer, PN and Pnao were added into 500 \( \mu \)L \( ^{2}H_{2}^{18}O \), and the whole system was incubated at 45 °C for 1.5 h. The \( ^{18}O_{2} \) labeling reaction and anaerobic assay were performed in a rubber sealed bottle attached to an anaerobic workstation (AW200SG, Electrotek Ltd, UK). All the liquid was exposed in \( N_{2} \) atmosphere for 1 h to remove the \( O_{2} \). The mixture of buffer, powdered PN and Pnao was transferred into a tube filled with \( ^{18}O_{2} \) and sealed again. The reaction was performed in room temperature for 4 h.

Enzymatic reaction \textit{in vitro}. The Pnao activity was determined by measuring the formation of SAP on UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). SAP showed an absorption peak at 230 nm which can be measured by UV-2550 spectrophotometer. The activity could also be quantified based on the peak area of PN on HPLC. The common reaction mixture contained 2 mM PN, and 25 mM \( NaH_{2}PO_{4}-Na_{2}HPO_{4} \) (pH 8.5) in a final volume of 0.5 mL. The reaction was started after the addition of Pnao (50 \( \mu \)g), and the machine would detect the slope within 30 s at 230 nm. The reaction was started after the addition of Pnao (50 \( \mu \)g), and ended by 1 M \( H_{2}SO_{4} \) (10 \( \mu \)L) at 60 s.

Buffers from pH 5.0–7.5 (citric acid/sodium citrate), 7.5–8.5 (Tris-HCl), 8.5–9.0 (monosodium orthophosphate/disodium hydrogen phosphate) and 9.0–10 (sodium carbonate/sodium hydrogen carbonate) were used. The protein was incubated in buffers overnight at 25 °C for the stability assay.

In metal salt assay, \( NaCl, NiCl_{2}, BaCl_{2}, CoCl_{2}, CaCl_{2}, CuCl_{2}, MnCl_{2}, ZnCl_{2}, KCl, Na_{2}MoO_{4}, Na_{2}WO_{4}, CdCl_{2}, BdCl_{2}, FeCl_{3} \) and \( MgCl_{2} \) were used to prepare the 2 mM metal solution. The protein was incubated in 2 mM metal solution overnight at 25 °C for the stability assay.

Electrophoresis of Pnao. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% gel in a MiniProtean III electrophoresis cell (Bio-Rad, Hemel Hempstead Corp., UK). The native page assay (Native-PAGE) was performed using a 7–12% gel in the ice-bath.

**Table 1. Bacterial strains and plasmids used in this study.**
Detection of tungsten in Pnao.  Pnao was added into 30% nitrite acid solution to 10 mL and incubated at 100 °C for 1 h. The product was detected on inductively coupled plasma-atomic (optical) emission spectrometry (ICP-OES, iCAP 6000 Radial, Thermo, America).

Site-directed mutagenesis of Pnao.  The site-directed mutagenesis was performed by pEASY-Uni Seamless Cloning and Assembly Kit (TransGen, China). The primers are listed in Table 2. The mutants of pnao genes were inserted into pET28a and transferred into E. coli C43(DE3). The specific enzyme activity was detected by adding excessive FAD at 230 nm. The concentration of FAD which attached to the protein was determined by HPLC as previously described.

Analytical techniques.  PN and SAP were detected by HPLC, and the mobile phase of HPLC was a mixture of 10% methanol and 90% 1 mM H2SO4. The flow rate was 0.6 mL min⁻¹, and the column (C-18, 4.6 × 250 mm) was at 30 °C. The products of enzymatic reaction were performed on an Agilent 6230 time of flight-MS equipped with ESI sources using C 18 column (4.6 by 150 nm, 5 μm). The mobile phase was an acetonitrile-H2O (0.01% HCOOH [v v⁻¹]; flow rate 0.4 mL min⁻¹). The column temperature was at 30 °C. The samples were ionized by electrospray with a positive polarity. PN and SAP could be easily detected with thin layer chromatography (TLC). The mobile phase consists of chloroform, methanol, ethanol and H2O (12 : 0.8 : 6 : 0.6; [vol/vol/vol/vol]).

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| Primers    | Sequence (5’-3’)                              |
|------------|-----------------------------------------------|
| Wildtype Pnao | F: ATACCATGGTGACAAAAGATGGTGATGAAGGCAGC        |
|            | R: GTGCTGAGGCGATTTGCTCATTTTCTTTTTTAG          |
| PnaoE131G  | F: ATGCACTATGGCGCTAGGGGTGGAGGAGGCGGT          |
|            | R: ACGGCTCTCGCCACCCCTAGGGCAATAGTGCA          |
| PnaoE159G  | F: GAGCCACTGGCGAGGCGGTGTTGGAATTTTCTGTGC     |
|            | R: AAAATTTCAGGCGCCCTGCTGCGAGGTTGT          |
| PnaoE162G  | F: GCAGGAGGGCGGTGTTGGAATTTTCTGTGC          |
|            | R: GCAGGAGGGCAAAATCCACAGGCGCTGTGC          |
| PnaoE174G  | F: ACGAATACCAAGAGGAGACGGAGAATTTTA           |
|            | R: TAAATATTCCGTGCTTCTTTGATATTGC            |
| PnaoP149S  | F: GTCCGTCCTCGAATTCCAGAGAAGGATT            |
|            | R: AATGACAGGTCTTGCAATTCCCAAGGAGGACT        |
| PnaoP156S  | F: AATGTCAAAAGGACATCTGGCAGAGAGGGCT         |
|            | R: ACGCCTCTGCTGAGATGCTCTTTTGTACATT         |
| PnaoP180S  | F: GCAGGGAATTTTATTCCGCGCGCGTTTGAAC         |
|            | R: GTTCAACGGCAGCGGAAATATTTCCGTGC           |

Table 2. Primers.
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Acknowledgements
This work was supported in part by grants from the Chinese National Science Foundation for Excellent Young Scholars (31422004). This work was supported in part by grants from the Chinese National Natural Science Foundation (31230002). We also acknowledge the “Shanghai Rising-Star Program” (13QA1401700) and the “Chen Xing” project from Shanghai Jiaotong University.

Author Contributions
PX. and H.T. conceived and designed the project and experiments. H.H. and W.W. performed the experiments. H.H. and H.T. wrote the paper. H.H., W.W., PX. and H.T. reviewed the manuscript. All authors reviewed the paper.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hu, H. et al. Characterization of Pseudooxynicotine Amine Oxidase of Pseudomonas putida S16 that Is Crucial for Nicotine Degradation. Sci. Rep. 5, 17770; doi: 10.1038/srep17770 (2015).

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