Iron(II)-dependent dioxygenase and N-formylamide deformylase catalyze the reactions from 5-hydroxy-2-pyridone to maleamate

Yuxiang Yao, Hongzhi Tang, Huixue Ren, Hao Yu, Lijuan Wang, Wei Zhang, Edward J. Behrman, & Ping Xu

1State Key Laboratory of Microbial Metabolism, and School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, PR China, 2School of Municipal and Environmental Engineering, Shandong Jian Zhu University, Jinan 250101, P.R. China, 3Department of Chemistry and Biochemistry, The Ohio State University, Columbus, Ohio 43210, USA.

5-Hydroxy-2-pyridone (2,5-DHP) is a central metabolic intermediate in catabolism of many pyridine derivatives, and has been suggested as a potential carcinogen. 2,5-DHP is frequently transformed to N-formylmaleamic acid (NFM) by a 2,5-DHP dioxygenase. Three hypotheses were formerly discussed for conversion of 2,5-DHP to maleamate. Based on enzymatic reactions of dioxygenase (Hpo) and N-formylamide deformylase (Nfo), we demonstrated that the dioxygenase does not catalyze the hydrolysis of NFM but rather that this activity is brought about by a separate deformylase. We report that the deformylase acts both on NFM and its trans-isomer, N-formylfumaramic acid (NFF), but the catalytic efficiency of Nfo for NFM is about 1,400 times greater than that for NFF. In addition, we uncover catalytic and structural characteristics of the new family that the Hpo belongs to, and support a potential 2-His-1-carboxylate motif (HX52HXD) by three-dimensional modeling and site-directed mutagenesis. This study provides a better understanding of 2,5-DHP catabolism.

Large quantities of wastes containing the pyridine ring are produced from coal and shale oil processing, pharmaceutical, food, dye and tobacco industries, and agriculture. They are classified as priority pollutants by USEPA (United States Environmental Protection Agency) because of their toxicity, carcinogenicity, and environmental hazards. Many pyridine derivatives such as 2- and 3-hydroxypyridine, nicotinic acid, and nicotine are degraded by aerobic microorganisms to generate 5-hydroxy-2-pyridone (2,5-dihydroxypyridine, 2,5-DHP) as an intermediate. 2,5-DHP was shown to cause DNA strand scission and suggested as a potential carcinogen. 2,5-DHP is subsequently oxidized to form N-formylmaleamic acid (NFM) and then hydrolyzed to maleamate and formate by some Pseudomonas species (shown in the red oval of Fig. 1). This pathway was first shown by Behrman and Stanier in Pseudomonas putida N-9 (formerly known as P. fluorescens). 2,5-DHP dioxygenase, termed NicX or Hpo for the enzyme from P. putida strain KT2440 or strain S16, respectively, catalyzes the dioxygenation of 2,5-DHP; however, it has been disputed for about 40 years whether or not 2,5-DHP dioxygenases also catalyze NFM hydrolysis. Investigation of the further transformation of NFM has not been straightforward for several reasons. Gauthier and Rittenberg studied the enzymatic hydrolysis of what they thought was NFM, and they reported that the purified 2,5-DHP oxygenase had both dioxygenase and deformylase activities. Behrman showed that the compound synthesized by Gauthier and Rittenberg was the trans isomer, N-formylfumaramic acid (NFF), and that both of these N-formylamides were rapidly hydrolyzed to the corresponding amides non-enzymatically. Maleamic acid is the next intermediate in the bacterial pathway whereas fumaramic acid is not metabolized by the cells. Further work was hampered by the lack of a convenient synthetic source of NFM, as the photochemical isomerization of NFF gives a mixture of geometric isomers. Jimenez et al. prepared a homogenous sample by cleavage of 2,5-DHP with a highly purified oxygenase (NicX), demonstrated that NicX did not catalyze the hydrolysis of NFM, and identified a separate deformylase (NicD) which did so in strain KT2440. NFM formation and hydrolysis were studied in strain S16, but NFF was
mistakenly used as the deformylase substrate by following the method of Gauthier and Rittenberg. Two syntheses of authentic NFM were reported in 2008. NicX was characterized and shown to belong to a new non-heme iron(II) ring-cleavage dioxygenase family. However, it is still unclear whether these properties are the general characteristics of the family or specific to NicX. The similarities and differences between the new family and other dioxygenases are also unknown.

A 2-His-1-carboxylate motif is a nearly universal platform for other non-heme iron(II) dioxygenases, but this architecture has not yet been confirmed in the new family.

Recently, we found a 2,5-DHP dioxygenase gene in a nicotine-degrading strain P. putida S16. Some basic catalytic characteristics of C-terminal His_{6}-tagged 2,5-DHP dioxygenase (His-Hpo), such as temperature effects, optimal pH, $K_m$ value, and $k_{cat}$ value, were characterized. In this study, two 2,5-DHP dioxygenases (Hpo and NicX) and a NFM deformylase (Nfo) were heterologously expressed and purified. Using a genuine sample of NFM, we now conclude that neither NicX nor Hpo could further hydrolyze NFM to maleamate while the deformylase isolated from the nicotine-degrading organism is capable of catalyzing the hydrolysis of both NFM and NFF, but at different rates. Kinetic studies show that the catalytic efficiency of Nfo for NFM is about 1,400 times greater than that for NFF which is consistent with the semi-quantitative results of Hillenbrand, E. L. (M.S. thesis, the Ohio State University, 1980). Comparing the catalytic characteristics of Hpo and NicX, we found some similarities and differences.
differences between them. Using structure prediction and site-specific mutagenesis analysis, we obtained data that support the 2-His1-carboxylate motif of the new non-heme iron(II) dioxygenases family. The motif HX52HXD is different from the sequence motif of other non-heme iron(II) enzymes.

Results

Characterization of Hpo. 2,5-DHP dioxygenase activity was purified by following the ability to decrease the absorption of 2,5-DHP at 320 nm. After three steps of anion-exchange column chromatography, the recombinant Hpo was purified about 16-fold with an overall yield of 24% (Table 1). The molecular mass of the purified protein as it existed in solution was about 119 ± 5 kDa, which implied a trimer (Fig. 2A, 2B, 2C). The UV-visible spectrum (data not shown) showed a maximum at 280 nm associated with the protein, and no evidence for heme, flavin or bound ferric ion was found for the dioxygenase purified by Gauthier and Rittenberg17.

The purified enzyme was stored at –80°C in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT), under which the enzyme retained more than 90% of its activity for 30 days. Contrastingly, the enzyme lost all of its activity after being stored at 0°C in 20 mM Tris-HCl buffer (pH 7.5) for 1 day. The addition of 1 mM DTT could not regenerate the activity (data not shown).

To compare the steady-state kinetic data between Hpo and NicX, NicX was purified from E. coli BL21(DE3) harboring plasmid pET24a-nicX (Table 2). The calculated Km of Hpo for 2,5-DHP was 0.138 ± 0.013 mM, more than that of NicX (Km = 0.074 ± 0.003 mM), and the values of Vmax and kcat of Hpo were 20.5 ± 1.0 U mg\(^{-1}\) and 12.9 ± 0.6 s\(^{-1}\), higher than the values of NicX (2.00 ± 0.04 U mg\(^{-1}\) and 1.28 ± 0.03 s\(^{-1}\) (Fig. S1).

O\(_2\) consumption and \(^{18}\)O labeling. During the first 15 s of the enzymatic reaction, the rates of decrease for O\(_2\) and 2,5-DHP were approximately linear, 0.88 ± 0.01 \(\mu\)mol L\(^{-1}\) s\(^{-1}\) for O\(_2\) and 0.90 ± 0.02 \(\mu\)mol L\(^{-1}\) s\(^{-1}\) for 2,5-DHP (Fig. 2D). Therefore, the ratio of O\(_2\) used per 2,5-DHP oxidation was approximately 1 : 1.

Reactions were carried out using O-18 labeled water or oxygen, and the products were analyzed by liquid chromatography–mass spectrometry (LC-MS) to assess the source of the added oxygen. The substrate 2,5-DHP was completely transformed in the presence of O\(_2\), while it was partially transformed in the presence of \(^{18}\)O probably due to an insufficient \(^{18}\)O concentration (Fig. 2E, 2F, 2G, 2H). The single product molecular ion peak detected in the presence of H\(_2\)O was at m/z 142.0146, corresponding to NFM lacking \(^{18}\)O (theoretical m/z = 142.0146) (Fig. 2F, 2G). In contrast, the molecular ion peak of NFM with two \(^{18}\)O atoms (theoretical m/z = 146.0231) was found for sample produced in the presence of \(^{18}\)O (Fig. 2H). Thus the source of both oxygen atoms in the product is dioxygen, not water.

Dioxygenase substrate specificity. Many pyridine and benzene derivatives, including 2,5-DHP, 2,3-DHP, 2,4-DHP, 2,6-DHP, 2-hydroxyxypyrine, 3-hydroxyxypyrine, 4-hydroxyxypyrine, 2,3-pyridinedicarboxylic acid, 2,4-pyridinedicarboxylic acid, 2,5-pyridinedicarboxylic acid, 2,6-pyridinedicarboxylic acid, 3,4-pyridinedicarboxylic acid, 3,5-pyridinedicarboxylic acid, 2-picolinic acid, 4-picolinic acid, nicotinic acid, 6-hydroxynicotinic acid, pyridoxal hydrochloride, pyridoxamine hydrochloride, catechol, resorcinol, hydroquinone, pyrogallol, protocatechuic, \(\beta\)-hydroxybenzoic acid, gentisate and gallate, were tested as potential substrates of Hpo by monitoring substrates decrease and O\(_2\) consumption. Only 2,5-DHP was oxidized, showing that the substrate specificity of Hpo is very strict.

Aminopeptidase activity of 2,5-DHP dioxygenase. The sequence of Hpo, like that of NicX, contains an aminopeptidase (AmpS)-like conserved domain according to blastp analysis24. Many proteins homologous to Hpo are predicted to belong to the leucine aminopeptidase (LAP) or M29 peptidase families (Fig. 3A), and such a sequence could reasonably account for the previously proposed deformylase activity of the protein. To ascertain the LAP activity of the new dioxygenase family, Hpo and NicX were used to incubate with \(\alpha\)-Leu-\(\beta\)-nitroaniline under many conditions. No activity was detected for either sample (data not shown).

Phylogenetic and secondary structure analyses. Phylogenetic analysis showed that Hpo and NicX do not belong to any known non-heme iron(II) ring-cleavage dioxygenase family (Fig. 3B). Structure prediction indicated that the secondary structures of Hpo and NicX are similar, although the amino acid sequence identity is only 43% (Fig. 3C). The sequence alignment shows that Nfo is highly similar with those of the \(\alpha/\beta\)-hydrolase-fold enzyme superfamily (Fig. S2)13,25. The predicted secondary structure of Nfo shows remarkable similarity to NicD (Fig. S2)13.

Three-dimensional structure prediction and directed mutagenesis. Since the PSI-BLAST programs revealed no known 3D structure that was homologous to Hpo, the protein sequence was submitted to the mGenThreader server for fold assignment. Protein structures with significant hits were chosen as templates for further modeling (Table S1). Five models were generated for Hpo and the one with the lowest DOPE score was used for visualization (Table S2, Fig. 4A). The protein is predicted to contain two global domains. One is constituted mainly by residues from the N-terminal region (residues 1–126 and 173–182) and shows a Rossmann fold structure. It contains three parallel \(\beta\) strands (\(\beta2\)-\(\beta3\)-\(\beta1\)) linked to two pairs of \(\alpha\) helices (\(\alpha1\–\alpha2\) and \(\alpha3\–\alpha4\)). The other domain is mainly constituted by two layers of \(\beta\) sheets (\(\beta4\–\beta5\–\beta8\–\beta11\–\beta12\–\beta16\–\beta17\–\beta18\) and \(\beta6\–\beta7\–\beta9\–\beta10\–\beta13\–\beta14\–\beta1\)) that are flanked by \(\alpha\) helices (\(\alpha7\–\alpha8\)) at each side (Fig. 4A).

Despite the phylogenetic diversity of non-heme iron(II) ring-cleavage dioxygenases (Fig. 3B), each is thought to contain a 2-His1-carboxylate facial triad for coordinating the metal ion; significantly, a similar motif was predicted for Hpo (consisting of H257, H310, and D312) (Fig. 4B). To test the significance of this motif, E. coli BL21(DE3) strains harboring pET28a-hisH257R, pET28a-hisH310K or pET28a-hisD312E plasmid were separately constructed (Table 2). The His\(_6\)-tagged variant proteins of His-H257R, His-H310K and His-D312E were purified (Fig. 4C). In these

| Table 1 | Hpo purification from E. coli BL21(DE3) harboring pET28a-hpo |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Step            | Total volume    | Total protein   | Total activity  | Specific activity | Yield |
| Crude extract   | 30.2 ml         | 86.0 mg         | 34.4 U          | 0.40 U mg\(^{-1}\) | 100 %        |
| Q Sepharose XL  | 28.5 mg         | 57.7 mg         | 29.8 U          | 0.52 U mg\(^{-1}\) | 86.5 %       |
| Source 3QQ      | 19.6 mg         | 5.1 mg          | 18.8 U          | 3.68 U mg\(^{-1}\) | 54.6 %       |
| Mono Q          | 1.4 mg          | 1.2 mg          | 8.1 U           | 6.56 U mg\(^{-1}\) | 23.6 %       |
|                  |                 |                 |                 |                  | 16.4 Fold     |

References:
1. Rittenberg, 17.
2. www.nature.com/scientificreports
variants, the amino acid H257, H310 or D312 was replaced by R257, K310 or E312. The far-UV circular dichroism analysis showed that the secondary structures of the mutation variants were not changed (Fig. 4D, Table S3), and these variants lost almost all of their 2,5-DHP dioxygenase activities (Fig. 4E). This result implies that the amino acids H257, H310 and D312 are important or crucial for the activity of Hpo.

The predicted tertiary structure of Nfo shows a β-sheet enwrapped by a series of α helices (Fig. S3). The conserved catalytic triad characterized by a nucleophilic residue at the end of β5 (Ser94), an acidic residue at the end of β6 (Asp118), as well as a histidine located in a loop followed β8 (His238) was predicted (Fig. S3).

Deformylase activity. LC-MS analysis showed that both Hpo and NicX catalyze the formation of NFM from 2,5-DHP (Fig. S4). There was no significant hydrolysis of standard NFM after incubation at pH 7, 25°C, for 15 min (Fig. 5A, 5B). Neither Hpo nor NicX promoted the process (Fig. 5B, 5C, 5D), while Nfo catalyzed the process (Fig. 5E, 5F, 5G, 5H).
and...

A recent study indicated that NFM deformylation has been in dispute. Gauthier showed that Nfo catalyzed the hydrolysis of NFM much faster than NFM hydrolysis is catalyzed by NFM deformylase, which can also catalyze NFM hydrolysis but at a reduced rate.

**Discussion**

The mechanism of NFM deformylation has been in dispute. Gauthier and Rittenberg deduced that it was catalyzed by 2,5-DHP oxygenase, while Behrman showed that the spontaneous hydrolysis of NFM was undoubtedly important under the conditions that Gauthier and Rittenberg used. The mechanism of NFM deformylation has been in dispute. Gauthier showed that Nfo catalyzed the hydrolysis of NFM much faster than NFM hydrolysis is catalyzed by NFM deformylase, which can also catalyze NFM hydrolysis but at a reduced rate.

Some differences exist between the two dioxygenases. Identities of the amino acid sequences between the two dioxygenases are only 43%. These may lead to higher efficient binding pockets of Hpo than those of NicX, which may cause their differences in kinetics: the apparent affinity of Hpo for 2,5-DHP is less than that of NicX while the catalytic efficiency of Hpo is 5 times greater than that of NicX (Fig. S1). The predicted 3D structures of NicX and of Hpo are quite different. The N-terminal domain of NicX was predicted to contain six β strands (β2-β1-β3-β4-β9-β8) flanked by six α helices (α1-α2-α3-α4-α5-α6), while the N-terminal domain of Hpo was predicted to contain only 3 β strands (β2-β1-β3) and 5 α helices, with 3 at one side (α1, α2, and α5) and another 2 (α3 and α4) at the other side (Fig. 4A).

The 2-His-1-carboxylate metal-binding motif, consisting of two His residues and an Asp/Glu residue, that exists in a wide array of non-heme iron(II) enzymes is also predicted to be present in Hpo (composed of H257, H310, D312). Site-directed mutations led to variants at these positions, suggesting that they were all necessary for the activity (Fig. 4D). Site-directed mutations led to variants at these positions, suggesting that they were all necessary for the activity (Fig. 4D, E, Table S3). Different from other known non-heme iron(II) ring-cleavage dioxygenases, the carboxylate donor of the 2-His-1-carboxylate motif in the new family might be an Asp residue rather than Glu. The D312 residue cannot be replaced by E312 residue in Hpo (Fig. 4E). The sequence of 2-His-1-carboxylate motif in the new family (HX33HXD) is distinct from other non-heme iron(II) enzymes.

Sequence alignment and phylogenetic analysis indicates that Hpo is the second member of a new ring-cleavage dioxygenase family, whose founding member is NicX. The predicted secondary structures and 3D models of the two members of the new ring-cleavage dioxygenase family are similar. They are both predicted to contain two anti-parallel β sheets (β18-β17-β16-β4-β3-β8-β11-β12 and β6-β7-β13-β14-β15-β9-β10 in Hpo, β21-β20-β19-β5-β6-β11-β14-β15 and β7-β10-β16-β17-β18-β12-β13 in NicX) flanked by α helices (α6 and α7 in Hpo, α7 and α8 in NicX) at each side of the C terminal domain (Fig. 3C, Fig. 4A). These similarities are probably the reasons for the similar properties of this new family. Hpo is a trimmer in solution (Fig. 2A, 2B, 2C) and NicX is a hexamer assembly composed of 2 cyclic trimers suggesting a sort of “trimmer-module” is conserved in both NicX and Hpo. Hpo and NicX catalyze 5,6-dioxygenation reactions that convert 1 mole of 2,5-DHP to NFM consuming 1 mole O2; are unstable at 0°C without DTT; are totally inhibited by metal-chelators or oxidants; depend on iron(II) and exhibit strict substrate specificity for 2,5-DHP. Although AmpS-like conserved domains are present in each protein, neither Hpo nor NicX has LAP activity.

**Table 2** | Primers used for amplification and mutagenesis

| Primer     | Sequence (5'-3') | Use                                           | Recombinant plasmid   |
|------------|------------------|------------------------------------------------|-----------------------|
| hishpo-f   | 5’ cggcccaatacgacattgagtttcaccgagat3’ | Amplifying the 1043-bp fragment containing hpo, which was digested with Ncol/Xhol and cloned into pET28a | pET28a-hishpo         |
| hishpo-r   | 5’ cggctcgactaattgctagtcgtaatttc3’   | Mutagenesis to obtain the hpoH257R mutant, which was digested with Ncol/Xhol and cloned into pET28a | pET28a-hishH257R      |
| hpo+H257K-f | 5’ eactgactcgtaggtggga3’              | Mutagenesis to obtain the hpoH310K mutant, which was digested with Ncol/Xhol and cloned into pET28a | pET28a-hishH310K      |
| hpo+H257K-r | 5’ eacttaccacgggaggatatta3’          | Mutagenesis to obtain the hpoD312E mutant, which was digested with Ncol/Xhol and cloned into pET28a | pET28a-hisdD312E      |
| hpo+H310K-f | 5’ ecgcgtcgatccttgattttttacg3’       | Amplifying the 1065-bp fragment containing nicX, which was digested with Ndel/HindIII and cloned into pET24a | pET24a-nicX          |

Bold front, the native gene sequence; Italic front, the site of directed mutation; ccatgg, NcoI site; ctcgag, XhoI site; catatg, NdeI site; aagctt, HindIII site.
Figure 3 | Amino acid sequence alignment analysis of Hpo. Details of all sequences are included in supplementary information. The sequence alignments were performed with CLUSTAL W. The same residues are highlighted in red and the highly conserved residues are colored in red. (A) Partial sequence alignment of various proteins homologous to Hpo. The probable iron(II) binding sites are indicated by the black circles. The abbreviations of proteins are listed as follows: HYP, hypothetical protein; PLAP, predicted LAP; PPase, predicted M29 family peptidase. (B) Phylogenetic analysis of non-heme iron(II) ring-cleavage dioxygenase. The phylogenetic tree was constructed with the neighbor joining method using MEGA 4.1. Hpo and NicX are indicated by black stars. The abbreviations of dioxygenases are listed as follows: 2,3-CTD, catechol 2,3-dioxygenase; HPCD, homoprotocatechuate 2,3-dioxygenase; DHPAD, 3,4-dihydroxyphenylacetate 2,3-dioxygenase; DHBD, 2,3-dihydroxybiphenyl dioxygenase; BphC, 2,3-dihydroxybiphenyl-1,2-dioxygenase; Ligab, protocatechuate 4,5-dioxygenase; PCD, protocatechuate 4,5-dioxygenase. (C) Secondary structure predictions of Hpo and NicX. The secondary structures were predicted by PSIPRED v3.3 on the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred.html). The blue and orange shapes indicate the secondary structure of Hpo and NicX, respectively: helix, α-helix; arrows, β-strand.
Figure 4 | Predicted three-dimensional model of Hpo and directed mutagenesis of supposed iron(II) binding sites. (A) Predicted three-dimensional structural model of Hpo. (B) Comparison of the iron(II) binding sites. Purple residues, the iron(II) binding sites of BphC from *Bhurkholderia xenovorans* LB400 (PDB_ID, 1HAN); yellow residues, the predicted iron(II) binding sites of Hpo. (C) SDS-PAGE analysis of purified His-H257R, His-H310K and His-D312E variant proteins; M, marker proteins; 1, cell-free extracts of induced *E. coli* BL21(DE3) without plasmid; 2, 4 and 6 cell-free extracts of induced *E. coli* BL21(DE3) harboring plasmid pET28a-hish257R, pET28a-hish310K, and pET28a-hisD312E; 3, 5 and 7, purified His-H257R, His-H310K and His-D312E variants. (D) Circular dichroism spectra of His-Hpo, His-H257R, His-H310K and His-D312E variants. (E) Enzyme activity comparison of His-Hpo and the His-H257R, His-H310K and His-D312E variant proteins; each error bar represents standard deviation of three independent experiments.

Gene isolation, site-directed mutagenesis and plasmid constructions. DNA was isolated, manipulated and transformed according to standard protocols. All primers are listed in Table 2. The hpo gene was PCR amplified from the genome DNA of strain S16 using primers hishpo-f/hishpo-r or hpo-f/hpo-r. The variants of hpoH310K, hpoH310K and hpoD312E were acquired by TaKaRa MutantBest Kit according to the manufacturer's instructions. All the products were digested with Ncol and XhoI and cloned into pET28a, then transformed into *E. coli* DH5x or *E. coli* BL21(DE3). The nicX gene was PCR amplified from the genome DNA of *P. putida* KT2440 using primers nicX-f/nicX-r, digested with NdeI and HindIII, cloned into pET24a, then transformed into *E. coli* DH5x and *E. coli* BL21(DE3).

Activity analyses of proteins. 2,5-DHP dioxygenase activity was assayed by detecting the absorption of 2,5-DHP at 320 nm (ε320 = 5,200 cm⁻¹ mol⁻¹^⁻¹) with a UV-visible 2550 spectrophotometer (Shimadzu, Japan) at 20 °C. The reaction system used 1 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.2 mM 2,5-DHP, 0.025 mM Fe²⁺ and 9.6 mg l⁻¹ protein. For potential substrates, the oxygen consumption of the reaction was calculated by using a micro-respiration system (Unisense, Denmark), and the substrates decrease was monitored by HPLC. At 20 °C, one unit of Hpo in 20 mM Tris-HCl buffer (pH 7.5) catalyzes the oxidation of 1 μmol of 2,5-DHP in 1 min.

LAP activity was assayed by determining the generation of p-nitroaniline at 405 nm (ε405 = 9,920 cm⁻¹ mol⁻¹^⁻¹) utilizing l-Leu-p-nitroaniline as substrate. One unit of enzyme catalyzes the production of 1 μmol of p-nitroaniline in 1 min. The reaction mixture was 5 mM l-Leu-p-nitroaniline, 0.025 mM metal ion, and 9.6 mg l⁻¹ enzyme in 1 ml of 20 mM Tris-HCl buffer (pH 7.5). LAP was used as the positive control.

Deformylase activity was measured directly by following the disappearance of NFM or NFF by mass spectrometry or, more easily, by monitoring the decrease in absorbance at 290 nm attendant upon formation of the corresponding amide 19. The MutantBest Kit was purchased from TaKaRa Biotechnology (China). Q Sepharose XL, Source 30Q, Mono Q 5/50 GL, 5 ml HisTrap FF, and 5 ml HiTrap desalting columns were from GE Healthcare (Uppsala, Sweden).

Methods

Materials. L-(-)-Nicotine (≥ 99% purity) was obtained from Fluka Chemie GmbH (Buchs Corp., Buchs, Switzerland). LAP and maleamic acid were from Sigma–Aldrich (Sigma–Aldrich Chemie GmbH, Buchs, SG, Switzerland). Fumaramic acid was made from fumaric acid by enzymatic hydrolysis of NFF and characterized by its UV spectrum 19,22. 2,5-DHP was purchased from SynChem OHG (Kassel Corp., Kassel, Germany). 18O₂ and 18O were obtained from the Shanghai Research Institute of Chemical Industry.

In summary, Hpo is the second member of a new iron(II)-dependent ring-cleavage dioxygenase family, which works with an independent N-formylamide deformylase Nfo to catalyze the transformation of 2,5-DHP to maleamate. This study provides a better understanding of the catabolism of 2,5-DHP.
Figure 5 | Mass spectra of reaction solutions using NFM or NFF as substrate. (A) Initial sample of 0.1 mM NFM. (B) 0.1 mM NFM incubated at room temperature for 15 min. (C) 0.1 mM NFM incubated with 9.6 mg l$^{-1}$ Hpo and 0.025 mM Fe$^{2+}$ at room temperature for 15 min. (D) 0.1 mM NFM incubated with 9.6 mg l$^{-1}$ NicX and 0.025 mM Fe$^{2+}$ at room temperature for 15 min. (E) 0.1 mM NFM incubated with 9.6 mg l$^{-1}$ Nfo at room temperature for 15 min. (F) Initial sample of 0.1 mM NFF. (G) 0.1 mM NFF incubated at room temperature for 15 min. (H) 0.1 mM NFF incubated with 1 g l$^{-1}$ Hpo and 0.025 mM Fe$^{2+}$ at room temperature for 15 min. (I) 0.1 mM NFF incubated with 1 g l$^{-1}$ NicX and 0.025 mM Fe$^{2+}$ at room temperature for 15 min. (J) 0.1 mM NFF incubated with 1 g l$^{-1}$ Nfo at room temperature for 15 min.
Tris-HCl buffer (pH 7) containing 0.1 mM substrate, 0.025 mM Fe²⁺, and appropriate concentrations of enzymes (9.6 mg l⁻¹ for NFM and 1 g l⁻¹ for NFF) were incubated at room temperature for 15 min. The UV assay used 1 ml of 20 mM Na₃HPO₄-NaH₂PO₄ buffer (pH 6.7) containing 1 mM substrate and appropriate concentrations of Nfo (2 mg l⁻¹ for NFM and 0.1 g l⁻¹ for NFF) that were incubated at 20°C. Because there is about 1% (w/v) sodium iodate in the NFM sample, more sodium iodate (1%, w/w) was added to the NFM catalyzed system to see if iodate affects the activity of Nfo; it does not. All these experiments were performed in a dark room. At 25°C, one unit of Nfo in 20 mM Na₃HPO₄-NaH₂PO₄ buffer (pH 6.7) can catalyze the hydrolysis of 1 μmol of substrate in 1 min.

### Purification of proteins

E. coli BL21(DE3) strains carrying the recombinant plasmids were cultured to OD₆₀₀ 0.6 ~ 0.8 at 37°C, 220 rpm. The cells were induced by addition of isopropyl thiogalactoside (IPTG) to a final concentration of 0.1 mM, followed by growth at 30°C for 10 h, then the cells were harvested by centrifugation. The cell-free supernatants were clarified by centrifugation (12,000 g, 20 min), the supernatant was loaded onto a 0.5-cm Q Sepharose XL column pre-equilibrated with low salt buffer. The active fractions containing 0.45 M NaCl were concentrated and desalted. Finally, the enzyme was purified by ultrafiltration (Millipore Corporation, USA), and the concentrated enzyme was used as a control.

### Analytical techniques

2,5-DHP, 2,5-DHP analogues, NFM and NFF were monitored at 254 nm by HPLC using an Agilent eclipse XDB C-18 (5 μm, 15 cm). The mobile phase was a mixture (90:10, vol/vol) of H₂O containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH, and the flow rate was 0.2 ml min⁻¹. The detection was monitored at 254 nm by HPLC using an Agilent eclipse XDB C-18 (5 μm, 15 cm). The mobile phase was a mixture (90:10, vol/vol) of H₂O containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH and methanol containing 0.1% HCOOH, and the flow rate was 0.2 ml min⁻¹. The detection wavelength was 254 nm.

### Structure predicting

The secondary structures of the proteins were predicted by PSIPRED v3.3 on PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred.html)²⁹. The method of three-dimensional modeling was as previously described³⁰, templates for further structure prediction of Hpo were listed in Table S1. The iron(II) binding sites of Hpo were identified using a 18O₂ labeling reaction. The 18O₂ labeling reaction was performed in a rubber sealed bottle attached to an anaerobic workstation (model AW200GC, Electrotek Ltd, UK) filled with a gas mixture of 10% H₂, 10% CO₂, and 80% N₂. One milliliter of H₂O containing 1 mM DTT and 0.025 mM Fe²⁺ was injected into the bottle, air in the bottle was replaced by the continuous gas mixture, 18O₂ was injected, and 10 μg Hpo was added to the mixture injected at 25°C for 5 min. Another reaction containing 1 ml H₂O, 1 mM 2,5-DHP, 0.025 mM Fe²⁺ and 10 μg ml⁻¹ Hpo in air was used as a control. The supernatants were directly analyzed by LC-MS after ethanol precipitation and centrifugation (12,000 × g, 2 min).

### Size-exclusion chromatography analysis

Size-exclusion chromatography analysis was performed as described by Jimenez et al.³¹. The Superdex 200 10/300 GL Tricorn column (GE Healthcare) was equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT. Thyroglobulin (669 kDa), apoferritin (443 kDa), aldolase (158 kDa), catalase (232 kDa), and ovalbumin (44 kDa) in the same buffer were used to calibrate the column. The Kᵅ values of the proteins were calculated according to the relationship Kᵅ = (Vₑ⁻⁻ Vₑ) / (Vₑ⁻ Vₑ) where Vₑ is the elution volume, Vₑ is the void volume, and Vₑ is the total volume of the column. The size of Hpo was determined by its Kᵅ value times three according to a fitted standard curve of a base-10 logarithm of the molecular weight (MW) of the standard proteins versus their Kᵅ values.

### 18O labeling

H₂O⁻¹⁸O (≥ 98% purity) and H₂O⁻¹⁸O (≥ 98% purity) were used to determine the size of the source of the oxygen atoms in NFM. The H₂O⁻¹⁸O containing 1 mM 2,5-DHP, 0.025 mM Fe²⁺ and 10 μg ml⁻¹ Hpo, incubated at 25°C for 5 min. The 18O₂ labeling reaction was performed in a rubber sealed bottle attached to an anaerobic workstation (model AW200GC, Electrotek Ltd, UK) filled with a gas mixture of 10% H₂, 10% CO₂, and 80% N₂. One milliliter of H₂O containing 1 mM DTT and 0.025 mM Fe²⁺ was injected into the bottle, air in the bottle was replaced by the continuous gas mixture, 18O₂ was injected, and 10 μg Hpo was added to the bottle, with the mixture incubated at 25°C for 5 min. Another reaction containing 1 ml H₂O, 1 mM 2,5-DHP, 0.025 mM Fe²⁺ and 10 μg ml⁻¹ Hpo in air was used as a control. The supernatants were directly analyzed by LC-MS after ethanol precipitation and centrifugation (12,000 × g, 2 min).

### Figure 6

Kinetic studies of Nfo for NFM and NFF. The experiments were performed in 1 ml of 20 mM Na₃HPO₄-NaH₂PO₄ buffer (pH 6.7) at 25°C. Red line, fitted Michaelis-Menten curve of Nfo for NFM; blue line, fitted Michaelis-Menten curve of Nfo for NFF; each error bar represents standard deviation of three independent experiments. The inset table shows the Michaelis-Menten equation and the fitted parameters.
Secondary structure analysis by circular dichroism spectra. The circular dichroism spectra of His Hpo and the variant proteins were obtained by JASCO J-815 at 25 °C. The spectra were 0.1 mg ml⁻¹ His-Hpo, His-H257R, His-H510K and His-D312E in 20 mM NaH₂PO₄-Na₂HPO₄ buffer (pH 7.5), respectively. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. According to the circular dichroism spectra, the secondary structure was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. The measurement range was 190 nm – 250 nm; the cell length was 1.0 mm; 10 spectra were accumulated for each run. 

Acknowledgments

This work was supported by the grants from the Chinese National Natural Science Foundation (31230002 and 31121064). We also acknowledge the Shanghai Rising-Star Program (13QA1401700) and the Chen Xing Project of Shanghai Jiao Tong University. The authors would like to acknowledge Ben Ma, Tingwen Bao, Jun Hao and Rong Zou (Shanghai Jiao Tong University) for their technical assistance on the scientific projects. We thank Prof. Robert P. Hausinger (Michigan State University) for very helpful and scientific comments on the manuscript. We also thank Dr. Dale Zhang (Beijing Institute of Genomics) and Ruoxu Gu (Shanghai Jiao Tong University) for the kind help in three-dimensional structure predictions of Hpo and Nfo.

Author contributions

P.X., Y.Y., H.T. and E.I.B. conceived and designed the project and experiments. E.I.B. synthesized the NFM. Y.Y., H.R., H.Y., L.W. and W.Z. performed the experiments. Y.Y., H.T. and E.I.B. analyzed the data and wrote the paper. All authors reviewed the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports/

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

How to cite this article: Yao, Y.X. et al. Iron(II)-dependent dioxygenase and N-formylmaleimide deformylase catalyze the reactions from 5-hydroxy-2-pyridone to maleamate. Sci. Rep. 3, 3235; DOI:10.1038/srep03235 (2013).

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported license. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0