STRUCTURAL
BIOLOGY

ISSN 2059-7983

Biochemical and structural characterization of *Klebsiella pneumoniae* oxamate amidohydrolase in the uric acid degradation pathway

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Received 3 February 2016

Accepted 26 April 2016

Edited by Q. Hao, University of Hong Kong

[‡] Present address: Department of Chemistry, SUNY Cortland, Cortland, NY 13045, USA.**Keywords:** Ntn-hydrolase superfamily; uric acid degradation; glutamyltranspeptidase; selenium SAD phasing.**PDB reference:** HpxW, 5ht**Supporting information:** this article has supporting information at journals.iucr.org/d

HpxW from the ubiquitous pathogen *Klebsiella pneumoniae* is involved in a novel uric acid degradation pathway downstream from the formation of oxalurate. Specifically, HpxW is an oxamate amidohydrolase which catalyzes the conversion of oxamate to oxalate and is a member of the Ntn-hydrolase superfamily. HpxW is autoprocessed from an inactive precursor to form a heterodimer, resulting in a 35.5 kDa α subunit and a 20 kDa β subunit. Here, the structure of HpxW is presented and the substrate complex is modeled. In addition, the steady-state kinetics of this enzyme and two active-site variants were characterized. These structural and biochemical studies provide further insight into this class of enzymes and allow a mechanism for catalysis consistent with other members of the Ntn-hydrolase superfamily to be proposed.

1. Introduction

The final stage of purine catabolism is the degradation of uric acid, which is a key nitrogen source in some bacteria and plants (Vogels & Van der Drift, 1976; Zrenner *et al.*, 2006). A gene cluster in the ubiquitous pathogen *Klebsiella pneumoniae* has been identified and characterized up to the formation of allantoate (Pope *et al.*, 2009; de la Riva *et al.*, 2008). However, there were still a number of genes in the cluster with unknown functions that were presumed to be involved in catabolism downstream of allantoate formation. Recent work has begun to fill in these gaps (French & Ealick, 2010; Werner *et al.*, 2010). Fig. 1 summarizes the currently accepted pathway.

The degradation of allantoate is hypothesized to involve its conversion to ureidoglycine by an allantoate amidohydrolase, HpxK (Serventi *et al.*, 2009). The ureidoglycine aminotransferase HpxJ then converts the resulting ureidoglycine and an α -keto acid to oxalurate and the corresponding amino acid. HpxJ has been biochemically and structurally characterized (French & Ealick, 2010; Ramazzina *et al.*, 2010; Werner *et al.*, 2010). Oxalurate degradation has been characterized in *Streptococcus allantoicus* and Enterobacteriaceae. In these organisms, oxalurate is converted to carbamoyl phosphate and oxamate by oxamate transcarbamoylase (Vogels, 1963; Vogels & Van der Drift, 1976). The resulting oxamate is then converted to the final end products ammonia, carbon dioxide and adenosine triphosphate (ATP). However, in the novel *K. pneumoniae* pathway HpxY is predicted to be an oxalurate amidohydrolase that converts oxalurate to ammonia and oxamate (Pope *et al.*, 2009). The resulting oxamate is then acted on by the putative oxamate

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amidohydrolase HpxW, resulting in the formation of ammonia and oxalate, which is further catabolized.

Sequence analysis indicates that HpxW is a member of the γ -glutamyltranspeptidase (GGT) family. Most members of this family are involved in the cleavage of an α -glutamyl amide linkage and transfer of the resulting α -glutamyl group to other amino acids and peptides (Serventi *et al.*, 2009). The GGT family is a member of the N-terminal nucleophile hydrolase (Ntn) superfamily, which has been well characterized (Brannigan *et al.*, 1995; Oinonen & Rouvinen, 2000). Ntn hydrolases are synthesized in an inactive form, which is autocatalytically cleaved to form an active heterodimer consisting of α and β subunits. A threonine, serine or cysteine residue that is the nucleophile in both the autoproducting and catalytic reactions is located at the N-terminus of the newly formed β subunit (Oinonen & Rouvinen, 2000). This residue corresponds to Thr342 in HpxW.

In this work, we present the first X-ray crystal structure of the oxamate amidohydrolase HpxW. Guided by the similarity of HpxW to other enzymes, we modeled the substrate of the reaction into the active site and constructed active-site variants, which were kinetically characterized. Together, these results were used to propose a mechanism for catalysis that is consistent with our structural and biochemical data and with the proposed mechanisms for other members of the Ntn-hydrolase superfamily.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis

Standard methods were used for DNA restriction endonuclease digestion, ligation, site-directed mutagenesis and transformation of DNA (Sambrook *et al.*, 1989). Automated DNA fluorescence sequencing was performed at the Cornell Life Sciences Core Laboratory Center. Plasmid DNA was purified with a GeneJET Miniprep Kit (Fermentas, Glen Burne, Maryland, USA). DNA fragments were separated by agarose gel electrophoresis, excised and purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange,

California, USA). *Escherichia coli* strain MachI (Invitrogen, Madison, Wisconsin, USA) was used as a recipient for transformations during plasmid construction and for plasmid propagation and storage. An Eppendorf Mastercycler and Phusion DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA) were used for PCR. Site-directed mutagenesis was performed by a standard PCR protocol using *Pfu*Turbo (Agilent) as per the manufacturer's instructions, followed by digestion with DpnI to remove methylated parental DNA. All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, Massachusetts, USA). *E. coli* strain BL21(DE3) and the pET overexpression system were purchased from Novagen (Madison, Wisconsin, USA).

The *hpxW* gene was PCR-amplified in two fragments from *K. pneumoniae* genomic DNA (ATCC No. 700721D) using the following primers: the sense primer 5'-GGG TAG CAT ATG CAC AGT AGC AAC GTT TCG ACC CAC GG-3' (inserts an NdeI site at the start codon of the *hpxW* open reading frame) with the antisense primer 5'-CGC CGC CGA GGC CAT TCA TGT GGG GAT AGA CGA CGG CG-3' (removes an internal NdeI site by silent mutagenesis) and the sense primer 5'-CGC CGT CGT CTA TCC CCA CAT GAA TGG CCT CGG CGG CG-3' (removes an internal NdeI site by silent mutagenesis) with the antisense primer 5'-CCC TAG GAT CCT TAG TAC CCG GCG GCG GCG CCG TTG-3' (inserts a BamHI site after the end of the *hpxW* open reading frame). The purified PCR products were mixed and subjected to another round of PCR using the outmost primers to yield a complete ORF with the internal NdeI site removed by SOE-PCR. This purified PCR product was digested with NdeI and BamHI, purified and ligated into similarly digested pTHT (a pET-28-derived vector which allows attachment of a modified 6 $\times$ His tag followed by a TEV protease cleavage site onto the N-terminus of the expressed protein). Colonies were screened for the presence of the insert and a representative plasmid was designated pKpHpxW.THT. The PCR-derived DNA was sequenced and shown to contain no errors.

To obtain the pKpHpxW T342A.THT plasmid, the following complementary primer pair was used:

Figure 1

Purine catabolic pathway in *K. pneumoniae*. The enzyme names are shown below the arrows. In the HpxJ-catalyzed aminotransfer reaction, the amino group from ureidoglycine modifies an α -keto acid substrate, resulting in formation of the corresponding amino-acid product.

5'-CGG CAA AGG CCC GGG CGA TGC GGT CTG GAT GGG CGT CGT G-3' and 5'-CAC GAC GCC CAT CCA GAC CGC ATC GCC CGG GCC TTT GCC G-3'. Putative variants were screened for the presence of the desired changes by PCR using 5'-GCA AAG GCC CGG GCG ATG CG-3' and a vector-specific primer. The pKpHpxW S360A.THT plasmid was made using the following complementary primer pair: 5'-GGC AGT GTC GTT TAT TCA GGC GAT CTA TCA CGA GTT CGG-3' and 5'-CCG AAC TCG TGA TAG ATC GCC TGA ATA AAC GAC ACT GCC-3'. Putative variants were screened for the presence of the desired changes by PCR using 5'-CAG TGT CGT TTA TTC AGG CG-3' and a vector-specific primer. Variants were verified by sequencing.

2.2. Protein expression and purification

In order to obtain selenomethionyl-incorporated (SeMet) HpxW, *E. coli* B834(DE3) cells were transformed with the pKpHpxW.THT plasmid. Native HpxW was obtained by transforming *E. coli* BL21(DE3) cells with the pKpHpxW.THT plasmid. Overnight cultures were grown by transferring a single colony to 15 ml LB medium supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin at 37°C with shaking for 16 h. The overnight cultures were used to inoculate 1 l cultures.

SeMet protein was obtained by growing the *E. coli* B834(DE3) cells in minimal medium supplemented with 1 $\times$ M9 minimal salts, 20 mg l^{-1} of all amino acids except methionine, 50 mg l^{-1} L-selenomethionine, 1 $\times$ MEM vitamin mix, 0.4% glucose, 2 mM MgSO_4 , 0.1 mM CaCl_2 , 25 mg l^{-1} FeSO_4 and 25 $\mu\text{g ml}^{-1}$ kanamycin. Cells were grown with shaking at 37°C until the OD_{600} reached 0.9. The temperature was then reduced to 15°C and, after 1 h at this temperature, expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The cells were then grown for an additional 18 h, harvested by centrifugation and stored at -80°C until purification. For native protein expression, *E. coli* BL21(DE3) cells transformed with the pKpHpxW.THT plasmid were grown in 1 l LB medium containing 25 $\mu\text{g ml}^{-1}$ kanamycin at 37°C with shaking until the OD_{600} reached 0.7. The temperature was then reduced to 15°C and, after 45 min at this temperature, protein expression was induced with 1 mM IPTG. The cells were then harvested as described above.

Purification of both native and SeMet HpxW followed a similar protocol. Frozen cell pellets were resuspended in 40 ml lysis buffer (20 mM Tris pH 8, 500 mM NaCl, 30 mM imidazole), and one cComplete Mini EDTA-free Protease Inhibitor Cocktail tablet (Roche) was added to the resuspended cells. For the purification of SeMet HpxW, 3 mM β -mercaptoethanol was added to the lysis buffer. The cells were lysed by sonication and the cell lysate was cleared by centrifugation at 40 000g for 30 min at 4°C. The clarified lysate was then passed over a 3 ml Ni-NTA column (Qiagen) pre-equilibrated with lysis buffer. The column was washed with 100 ml lysis buffer and with 10 ml lysis buffer supplemented with 50 mM imidazole. HpxW was eluted from the column by the addition of 10 ml lysis buffer containing 250 mM imidazole. The protein was 95% pure according to SDS-PAGE analysis (results not

Table 1

Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

	SeMet HpxW	Native HpxW
Beamline	24-ID-C	24-ID-E
Resolution ($\text{\AA}$)	2.9	2.7
Wavelength ($\text{\AA}$)	0.9792	0.9792
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters		
a ($\text{\AA}$)	69.6	70.7
b ($\text{\AA}$)	125.0	124.1
c ($\text{\AA}$)	156.4	156.8
Measured reflections	192075	138140
Unique reflections†	29601	39286
Average $I/\sigma(I)$	14.7 (1.9)	12.5 (1.5)
Multiplicity	6.5 (6.6)	3.5 (3.0)
Completeness (%)	99.9 (99.9)	96.0 (83.6)
$R_{\text{merge}}^\ddagger$ (%)	14.1 (73.3)	11.3 (52.2)

† Unique reflections include Bijvoet pairs. $\ddagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of the i reflections with intensities $I_i(hkl)$ and common indices hkl .

shown) and bands were observed corresponding to the full-length protein (55 kDa) and cleaved protein (35.5 kDa α subunit and 20 kDa β subunit). The resulting samples were further purified using gel-filtration chromatography (HiLoad 26/60 Superdex 200 pg, GE Healthcare) with a running buffer consisting of 20 mM Tris pH 8, 50 mM NaCl. For the purification of SeMet HpxW, the running buffer also contained 1 mM dithiothreitol. The protein samples were then concentrated to 9 mg ml^{-1} SeMet HpxW and 8 mg ml^{-1} native HpxW as measured by the Bradford assay. The final protein samples were determined to be greater than 95% pure by SDS-PAGE electrophoresis. Aliquots of protein were flash-frozen after concentration and immediately stored at -80°C for crystallization trials.

2.3. Overexpression and purification of variants

The following HpxW variants were prepared: S360A and T342A. The cells were grown and purified as described above for native HpxW. The protein samples were concentrated to 6 mg ml^{-1} for the S360A variant and 9 mg ml^{-1} for the T342A variant following gel-filtration chromatography. All protein samples were determined to be greater than 95% pure by SDS-PAGE analysis.

2.4. Crystallization, data collection and processing

Initial crystallization conditions were identified using the hanging-drop vapor-diffusion method (Crystal Screen and Crystal Screen 2 from Hampton Research; Wizard Screens 1, 2, 3 and 4 from Emerald Bio) at 18°C. Hanging drops were formed by mixing 1.5 μl reservoir solution with 1.5 μl protein sample. Optimized SeMet and native crystals both required a microseeding step. Briefly, native crystals were transferred to a seed-stabilizing solution consisting of 0.1 M HEPES pH 7.6, 16% PEG 6000, 200 mM NaCl and were then crushed using a Seed Bead (Hampton Research). The freshly prepared seed solution (0.5 μl) was added to a drop consisting of 1.25 μl reservoir solution and 1.25 μl protein sample. The optimized

Table 2
Summary of data-refinement statistics for native HpxW.

Resolution (Å)	2.7
No. of protein atoms	6380
No. of ligand atoms	0
No. of water atoms	0
Reflections in working set	37226
Reflections in test set	1964
<i>R</i> factor† (%)	25.7
<i>R</i> _{free} ‡ (%)	29.8
R.m.s.d. from ideal	
Bond lengths (Å)	0.008
Angles (°)	1.065
Average <i>B</i> factor (Å ²)	47.9
Ramachandran plot	
Favored (%)	94.98
Allowed (%)	4.44
Disallowed (%)	0.58

† *R* factor = $\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are observed and calculated structure factors, respectively. ‡ For R_{free} the sum is extended over a subset of reflections (5%) that were excluded from all stages of refinement.

reservoir solution for SeMet and native HpxW consisted of 12–20% PEG 3350, 0.2–0.7 *M* ammonium nitrate. Rod-shaped crystals grew to 200–400 × 20–40 µm in 3–4 d. The crystallization buffer supplemented with 20% glycerol was used as cryoprotectant for the SeMet HpxW crystals. The cryoprotectant for native HpxW crystals was the crystallization solution with 30% ethylene glycol. After incubation in the cryoprotectant, the samples were flash-cooled by plunging them into liquid nitrogen.

Data from a SeMet HpxW crystal were collected on the Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C of the Advanced Photon Source (APS) at Argonne National Laboratory. The data were collected at a wavelength of 0.9792 Å using one crystal and a total rotation of 180° with a 1° oscillation range. The SeMet crystal diffracted to 2.9 Å resolution. Data from a native HpxW crystal were collected on NE-CAT beamline 24-ID-E. The native crystal diffracted to 2.7 Å resolution and data were collected at a wavelength of 0.9792 Å over a total rotation of 90° with a 1° oscillation range. The data from the SeMet crystal were indexed, integrated and scaled using *RAPD* (<https://rapd.nec.aps.anl.gov/rapd/>). The native data were analyzed using the *HKL-2000* suite of programs (Otwinowski & Minor, 1997). Table 1 summarizes the data-collection statistics.

2.5. Structure determination, model building and refinement

HpxW contains 24 methionine residues in the asymmetric unit. Each asymmetric unit contains two αβ heterodimers. Initial Se-atom positions were located using *AutoSol* (Terwilliger *et al.*, 2009) in the *PHENIX* suite of programs, and 18 of 24 possible SeMet residues were identified. *AutoSol* was used to calculate initial electron-density maps using these heavy-atom sites and also to perform automated model building. The initial model consisted of 642 residues of the 1102 in the asymmetric unit and required extensive manual model building in *Coot* (Emsley *et al.*, 2010). *Bacillus halodurans* cephalosporin acylase (PDB entry 2nlz; New York

SGX Research Center for Structural Genomics, unpublished work) was used for guidance during model building. Initial refinement was carried out using *REFMAC5.0* and the *CCP4i* interface (Winn *et al.*, 2011). Iterative rounds of manual model building were performed with *Coot* (Emsley *et al.*, 2010) and later rounds of refinement were carried out using *PHENIX* (Adams *et al.*, 2010). Tight noncrystallographic symmetry (NCS) restraints were used and were gradually loosened through refinement (Vellieux & Read, 1997). The SeMet-incorporated HpxW structure was used as the starting model for refinement against the native data set. The refinement statistics are summarized in Table 2.

2.6. Steady-state kinetic assays

HpxW activity was measured for wild-type HpxW and for the S360A and T342A variants using a previously described assay that couples ammonia release to nicotinamide adenine dinucleotide (NADH) oxidation (Muratsubaki *et al.*, 2006). All assays were carried out at room temperature in a buffer consisting of 50 mM KH₂PO₄ pH 8.5, 1 mM NADH, 6 mM α-ketoglutarate, 4 units of glutamate dehydrogenase and varying concentrations of oxamate (0–10 mM). The reaction was initiated by the addition of 0.5–1.0 µM HpxW and the resulting decrease in absorbance at 340 nm was monitored for 30–60 min in a Synergy HT multi-mode plate reader (BioTek). The background rate of NADH oxidation was measured in the absence of HpxW at each oxamate concentration and was subtracted from the change in absorbance. The kinetic parameters k_{cat} , $k_{\text{cat}}/K_{\text{m}}$ and K_{m} were determined by fitting the initial velocity as a function of substrate concentration to the Michaelis–Menten equation,

$$v_0 = \frac{V_{\text{max}}[S]}{K_{\text{m}} + [S]}. \quad (1)$$

In this equation, v_0 is the initial velocity, V_{max} represents the maximal velocity, K_{m} is the substrate concentration at half-maximal velocity and $[S]$ is the concentration of oxamate. The first-order rate constant k_{cat} was determined by dividing the V_{max} value by the HpxW concentration. All kinetic data were analyzed using *KaleidaGraph* (Synergy Software).

2.7. Figure preparation and structural homology search

The topology diagram was based on results from the *PDBsum* server (Laskowski, 2009). The *DALI* server (Holm & Rosenström, 2010) was used to search for structural homologues of HpxW. All other figures were prepared using *PyMOL* (DeLano, 2008) and *ChemBioDraw* (Cambridge-Soft).

3. Results and discussion

3.1. Overall structure of HpxW

The initial model of HpxW was determined using single-wavelength anomalous diffraction to 2.9 Å resolution. Final refinement was performed using data from native crystals that diffracted to 2.7 Å resolution. The space group for both SeMet

and native HpxW crystals was $P2_12_12_1$, with average unit-cell parameters $a = 70$, $b = 124$, $c = 156$ Å. The asymmetric unit consisted of two chains, corresponding to a Matthews coefficient of $3.1 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 60% (Matthews, 1968). The HpxW protomer is shown in Fig. 2(a). HpxW is autoprocessed into an $\alpha\beta$ heterodimer that consists of a 35.5 kDa α subunit and a 20 kDa β subunit. The site of autoprocessing is Thr342, which becomes the N-terminal residue of the β subunit. The C-terminus of the α subunit is located in a disordered loop region consisting of residues 325–341. The two heterodimers are nearly identical, with a root-mean-square deviation of 0.45 Å for the main-chain atoms. Despite clear electron density, one residue, Asp301, in the first heterodimer and four residues, Ala43, Ala88, Glu158 and Val259, in the second heterodimer are in the disallowed region based on *PROCHECK* (Laskowski *et al.*, 1993). As all of these residues, except Ala43, are present on the protein surface, differential crystal packing could explain why the conformation of the corresponding residues in the other heterodimer are in an allowed region. At this resolution (2.7 Å), water molecules are not visible and are not in the final model.

HpxW is a member of the Ntn-hydrolase superfamily that is characterized by a four-layer $\alpha\beta\alpha$ sandwich (Brannigan *et al.*, 1995; Oinonen & Rouvinen, 2000). The core of the enzyme consists of one antiparallel seven-stranded β -sheet ($\beta7\uparrow\beta8\downarrow\beta11\uparrow\beta12\downarrow\beta15\uparrow\beta16\downarrow\beta1\uparrow$) surrounded by five α -helices ($\alpha12$, $\alpha13$, $\alpha14$, $\alpha15$ and $\alpha16$) and one small parallel β -sheet ($\beta13$ and $\beta14$), and one six-stranded antiparallel β -sheet ($\beta17\uparrow\beta3\downarrow\beta9\uparrow\beta10\downarrow\beta3\uparrow\beta4\downarrow$) flanked by a small antiparallel β -sheet ($\beta5$ and $\beta6$) and a bundle of α -helices consisting of $\alpha1$, $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\alpha8$, $\alpha9$, $\alpha10$ and $\alpha11$ (Fig. 2b). Similar to the *E. coli* and *Helicobacter pylori* GGT structures, the C-terminus of the α subunit (Ser324 in both heterodimers) is $>36 \text{ Å}$ away from the N-terminus of the β subunit (Thr342). This observation suggests that these enzymes undergo a large conformational change involving the

newly formed C-terminus of the α subunit and N-terminus of the β subunit following the autoprocessing event (Okada *et al.*, 2006).

The HpxW heterodimer ($\alpha\beta$) is shown in Fig. 2(c). Each heterodimer has a surface area of approximately $17\,000 \text{ Å}^2$ and the buried surface area between the α and β subunits in the heterodimer is approximately 4500 Å^2 (Krissinel & Henrick, 2007). The buried surface area between the two heterodimers is 1100 Å^2 , indicating that the biologically relevant form of the enzyme may not be a heterotetramer. This interface consists of 21 residues from the first heterodimer and 23 residues from the second heterodimer (Laskowski, 2009).

Figure 2

Structure of HpxW. (a) Stereoview ribbon diagram of HpxW with secondary-structural elements labelled. α -Helices are shown in blue and β -strands are shown in green with loops colored yellow. The position of Thr342 is highlighted. (b) Topology diagram of HpxW. The color scheme is the same as in (a). (c) The HpxW heterodimer ($\alpha\beta$) is shown with α and β subunits in red and blue, respectively.

Table 3

Enzymes identified as structurally similar to HpxW using DALI.

Protein	PDB code	Z-score	R.m.s.d. (Å)	Identity (%)	No. of aligned residues
<i>Thermoplasma acidophilum</i> γ -glutamyltransferase-related protein	2i3o	45.4	2.4	31	444
<i>Escherichia coli</i> γ -glutamyltranspeptidase	2dbu	43.0	2.3	31	438
<i>Bacillus halodurans</i> cephalosporin acylase	2nlz	42.6	2.6	36	462
<i>Helicobacter pylori</i> γ -glutamyltranspeptidase	2nqo	27.7	2.7	27	308
<i>Bacillus subtilis</i> γ -glutamyltranspeptidase	3a75	25.9	3.1	27	307
<i>Bacillus anthracis</i> capsule-biosynthesis protein CapD	3g9k	24.2	2.6	23	275

The interface region is mainly hydrophobic, with more than 100 nonbonded contacts and 11 hydrogen bonds. Based on gel-filtration chromatography, HpxW is a heterodimer in solution (data not shown).

3.2. Comparison of HpxW to other enzymes

Ntn hydrolases are enzymes with diverse functions and low sequence similarity. The unifying characteristic of the superfamily is autoprocessing into a mature form, resulting in a β -subunit with an N-terminal nucleophile (the side chain of serine, threonine or cysteine). Most, but not all, family members cleave amide bonds (Oinonen & Rouvinen, 2000). GGT is a relatively recent addition to the Ntn-hydrolase superfamily (Suzuki & Kumagai, 2002). Many members of the Ntn-hydrolase superfamily have been structurally characterized, including *B. subtilis* glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase (Smith *et al.*, 1994), *E. coli* penicillin G acylases (Duggleby *et al.*, 1995) and the *E. coli*, *H. pylori* and *B. subtilis* GGTs (Boanca *et al.*, 2007; Okada *et al.*, 2006; Wada *et al.*, 2010). BLAST results suggested that HpxW is a member of the GGT family, which has modest sequence identity among its members (~30%; Suzuki *et al.*, 1989). The N-terminal nucleophile in the GGT family is a highly conserved threonine, which corresponds to Thr342 in HpxW (Brannigan *et al.*, 1995). A DALI search (Holm & Rosenström, 2010) performed on the HpxW structure

Figure 3

Putative HpxW active site and glutamate-bound *E. coli* GGT. The putative HpxW active site was determined by manually positioning oxamate (OXM) into HpxW guided by the structure of glutamate-bound *E. coli* GGT (PDB entry 2dbx). (a) Potential substrate-binding site between HpxW (green C atoms, blue N atoms and red O atoms) and the substrate oxamate (OXM; white C atoms). (b) The *E. coli* GGT active site with bound glutamate (GLU) (PDB entry 2dbx). The color scheme is as in (a).

confirmed that it is a member of the Ntn-hydrolase superfamily and the GGT family. The proteins with the highest similarity to HpxW are outlined in Table 3, where they are sorted by decreasing Z-score.

The members of the Ntn-hydrolase superfamily contain an $\alpha\beta\beta\alpha$ core structure, in which a central two β -sheet sandwich is flanked on each side by α -helices (Oinonen & Rouvinen, 2000).

The HpxW core structure is illustrated in Fig. 2(a). Within the Ntn hydrolases, the N-terminal β -sheet is usually comprised of five to eight β -strands and the second β -sheet usually contains four to ten β -strands (Oinonen & Rouvinen, 2000). HpxW contains seven β -strands in the N-terminal β -sheet and six in the second β -sheet. The orientation of the β -sheets varies among the superfamily members. Typically, the β -sheets are rotated 30° with respect to each other (Brannigan *et al.*, 1995). However, there are exceptions, such as aspartylglucosaminidase, for which the β -sheets are almost parallel, with a rotation of only 5° (Oinonen *et al.*, 1995), while in proteasome and glutamine phosphoribosylpyrophosphate amidotransferase the β -sheets are rotated 35° (Duggleby *et al.*, 1995; Muchmore *et al.*, 1998). In HpxW, the β -sheets are inclined approximately 27° .

3.3. Active site of HpxW

All attempts to determine the liganded structure of HpxW were unsuccessful. These attempts included both the cocrystallization of oxamate with the S360A variant and the soaking of oxamate into crystals of the S360A variant, and similar approaches using oxalate and wild-type HpxW crystals. The S360A variant crystals showed poor diffraction (>3 Å) and the T342A variant did not crystallize. The putative active site was identified through sequence and structural similarity to other members of the Ntn-hydrolase superfamily. The oxamate substrate was positioned in the active site (Figs. 3a and 3b) based on the position of the glutamate in the structure of the *E. coli* GGT–glutamate complex (PDB entry 2dbx; Okada *et al.*, 2006). This active site is located in a solvent-exposed cleft which is approximately 11 Å deep and 10 Å wide. A number of nonpolar amino acids line the binding cleft, including Met423, Gly424 and Gly425. Other amino acids in the active site are Thr342, Ser360, Tyr362 and Leu405. Based on *E. coli* GGT, Gly424, Gly425 and Thr342 are conserved residues. In *E. coli* GGT there is a Thr at the position corresponding to Ser360 in HpxW (Okada *et al.*, 2006).

In the putative active site, an oxyanion hole is formed between the amide carbonyl of the oxamate and the backbone amide N atoms of Gly424 (2.6 Å) and Gly425 (2.6 Å). The catalytic nucleophile and the site of the autoprocessing event is Thr342, which is located at the beginning of the β_9 strand. The hydroxyl group of Thr342 is hydrogen-bonded to the hydroxyl group of Ser360 (3.0 Å). The free α -amino group on

Thr342, which serves as a general base, is located 2.9 Å away from its own hydroxyl group.

3.4. Active-site comparison

The members of the Ntn-hydrolase superfamily have active sites containing a nucleophile, a proton donor and an oxyanion hole (Brannigan *et al.*, 1995). GGTs usually contain a catalytic dyad consisting of either threonine–serine or threonine–threonine (Boanca *et al.*, 2007). The first member of the dyad is located on the N-terminus of the newly formed β subunit and is essential for both the autoprocessing of the proenzyme and for the catalytic reactions. In HpxW this residue corresponds to Thr342, which is located at the beginning of strand β 9. The second member of the dyad is the proton donor, which corresponds to Ser360 in HpxW and is located at the end of strand β 10. Consistent with the *H. pylori* GGT enzyme, mutation of the catalytic threonine to alanine (T342A) results in the formation of a catalytically inactive enzyme. However, T398S *H. pylori* GGT was still able to autoprocess into α and β subunits, although the $t_{1/2}$ for processing was increased 1.4-fold. In contrast, T342A HpxW did not autoprocess (data not shown; Boanca *et al.*, 2007).

A potential model for the binding of the oxamate substrate in the HpxW active site was obtained using the structure of glutamate-bound *E. coli* GGT (PDB entry 2dbx) as a template. Specifically, as shown in Fig. 3, the oxamate was positioned into the active site based on superposition of the corresponding atoms of the glutamate in the *E. coli* GGT structure (PDB entry 2dbx). In addition, the positions of the

putative active-site residues Thr342, Ser360, Gly424 and Gly425 were manually adjusted based on the corresponding residues in *E. coli* GGT. In *E. coli* GGT these residues correspond to Thr391, Thr409, Gly483 and Gly484, respectively.

In *E. coli* GGT, *H. pylori* GGT and HpxW, the catalytic threonine side chain is located >36 Å away from the C-terminus of the α subunit, suggesting that the autoprocessing event is linked to a large conformational change (Okada *et al.*, 2006). Biochemical studies performed on GGTs indicate that the autoprocessing reaction is an intramolecular event in which the catalytic threonine acts as a nucleophile and cleaves the link between itself and its upstream neighbor, which corresponds to Asp341 in HpxW (Suzuki & Kumagai, 2002). This residue is not visible in the HpxW structure as the C-terminus of the β -subunit is largely disordered, similar to observations in both the *E. coli* and *H. pylori* GGT structures (Boanca *et al.*, 2007; Okada *et al.*, 2006).

3.5. Activity of HpxW

HpxW catalyzes the conversion of oxamate to oxalate and ammonia. The kinetics of the reaction were measured using a previously described assay that couples ammonia release to the oxidation of NADH by glutamate dehydrogenase (Muratsubaki *et al.*, 2006). The background rate for NADH oxidation is approximately $1.2 \times 10^{-5} \text{ mM s}^{-1}$, which was subtracted from all measured rates. The k_{cat} for turnover of the wild-type enzyme is $5.5 \pm 0.2 \text{ s}^{-1}$, with a $k_{\text{cat}}/K_{\text{m}}$ of $1159 \pm 96 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 4
Proposed mechanism for the conversion of oxamate (OXM) to oxalate (OXD) by HpxW.

To determine the roles of Thr342 and Ser360 in catalysis, the activity and autoprocessing ability of alanine variants (T342A and S360A) was measured. Based on SDS–PAGE analysis, T342A HpxW did not autoprocess into a mature heterodimer as only a single band at 55 kDa was observed. In addition, no activity was observed for the T342A HpxW variant. S360A HpxW formed a heterodimer (data not shown); however, catalytic activity of the heterodimer was not detectable at the level of sensitivity of the assay.

3.6. Mechanistic implications for HpxW

HpxW is the first example of an oxamate amidohydrolase to be structurally determined; however, the reactions catalyzed by members of the Ntn-hydrolase superfamily have been characterized. These enzymes are involved in a myriad of functions, including protein degradation (Groll *et al.*, 1997) and purine nucleotide biosynthesis (Smith *et al.*, 1994). Based on similarity to other members of the Ntn superfamily and the GGT family, as well as modeling studies, a catalytic mechanism has been proposed (Fig. 4).

In the proposed mechanism, the hydroxyl group of the Thr342 side chain is positioned approximately 3.0 Å away from Ser360 and 2.9 Å away from its newly formed α -amino group (Castonguay *et al.*, 2007). Thus, it is possible that either the hydroxyl side chain of Ser360 or the α -amino group of Thr342 activates the hydroxyl side chain of Thr342. We propose that the α -amino group serves as a general base, consistent with the observation in rat GGT that an imidazolium or primary ammonium ion with a perturbed pK_a is important in catalysis; this mechanism is also consistent with the mechanism proposed for *H. pylori* GGT (Boanca *et al.*, 2007; Ménard *et al.*, 2001). A hydrogen bond between Thr342 and Ser360 increases the nucleophilicity of Thr342, leading to an attack on the amide C atom of oxamate. This attack leads to the formation of a tetrahedral intermediate, the negative charge of which is stabilized by the oxyanion hole consisting of Gly424 and Gly425. Collapse of the tetrahedral intermediate leads to the formation of an acyl intermediate and the release of a molecule of ammonia. We hypothesize that the protonated α -amino group on Thr342 protonates the resulting ammonia leaving group. The α -amino group of Thr342 then deprotonates a water molecule (Oinonen & Rouvinen, 2000), and the resulting hydroxide ion attacks the carbonyl C atom of the intermediate, leading to the formation of the oxalate product.

In conclusion, the structural and kinetic analysis of HpxW presented in this work provides a framework for understanding the reaction catalyzed by the first example of an oxamate amidohydrolase, a recent addition to both the Ntn-hydrolase and GGT superfamilies. These studies also expand our understanding of the novel purine-degradation pathway in the ubiquitous pathogen *K. pneumoniae*.

Acknowledgements

This work was supported by NIH grant GM073220 (SEE) and was based upon research conducted on the Northeastern Collaborative Access Team beamlines at the Advanced

Photon Source, which are supported by award GM103403 from the NIH. Use of the Advanced Photon Source is supported by the US Department of Energy, Office of Basic Energy Sciences under Contract No. DE-AC02-06CH11357. We thank the staff at the NE-CAT beamlines for their assistance in data collection. We acknowledge Dr Cynthia Kinsland of the Cornell Protein Production facility for providing clones of HpxW and the variant enzymes, and Leslie Kinsland for help with manuscript preparation. Lastly, Dr Yang Zhang is acknowledged and thanked for helpful discussions about this work.

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