Mechanistic Characterization of N-Formimino-L-glutamate Iminohydrolase from *Pseudomonas aeruginosa*

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Abstract: N-Formimino-L-glutamate iminohydrolase (HutF) from *Pseudomonas aeruginosa* catalyzes the deamination of N-formimino-L-glutamate in the histidine degradation pathway. An amino acid sequence alignment between HutF and members of the amidohydrolase superfamily containing mononuclear metal centers indicated that residues Glu-235, His-269, and Asp-320 are involved in substrate binding and activation of the nucleophilic water molecule. The purified enzyme contained up to one equivalent of zinc. The metal was removed by dialysis against the metal chelator dipicolinate with the complete loss of catalytic activity. Enzymatic activity was restored by incubation of the apoprotein with Zn\(^{2+}\), Cd\(^{2+}\), Ni\(^{2+}\), or Cu\(^{2+}\). The mutation of Glu-235, His-269, or Asp-320 resulted in the diminution of catalytic activity by two to six orders of magnitude. Bell-shaped profiles were observed for \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) as a function of pH. The \(pK_a\) of the group that must be unprotonated for catalytic activity was consistent with the ionization of His-269. This residue is proposed to function as a general base in the abstraction of a proton from the metal-bound water molecule. In the proposed catalytic mechanism, the reaction is initiated by the abstraction of a proton from the metal-bound water molecule by the side chain imidazole of His-269 to generate a tetrahedral intermediate of the substrate. The collapse of the tetrahedral intermediate commences with the abstraction of a second proton via the side chain carboxylate of Asp-320. The C–N bond of the substrate is subsequently cleaved with proton transfer from His-269 to form ammonia and the N-formyl product. The postulated role of the invariant Glu-235 is to ion pair with the positively charged formimino group of the substrate.

N-Formimino-L-glutamate iminohydrolase (HutF) catalyzes the formation of ammonia and N-formyl-L-glutamate from N-formimino-L-glutamate (1) as shown in Scheme 1. This reaction sequence is the penultimate step in one of the three known pathways for the degradation of L-histidine to L-glutamate (1). In one of the bacterial catabolic pathways, L-histidine is initially converted to urocanate and ammonia by histidine ammonia-lyase (HutH\(^\delta\)). Urocanase (HutU) then transforms urocanate to L-5-imidazolone-4-propionate, which is subsequently hydrolyzed to N-formimino-L-glutamate by imidazolone propionate amidohydrolase (HutI). HutF catalyzes the formation of N-formyl-L-glutamate, which is then hydrolyzed by HutG to generate formate and L-glutamate. In *Pseudomonas aeruginosa* we have demonstrated that Pa5106 (gi: 15600299) is HutF and that Pa5091 is HutG (2). *P. aeruginosa* can also hydrolyze N-formimino-L-glutamate directly to L-glutamate and formamide by the action of Pa3175 (2).

HutF has been identified as a member of the amidohydrolase superfamily (AHS). This superfamily was first recognized from the three-dimensional structural similarities found within the active sites and protein folds of urease (URE), phosphotriesterase (PTE), and adenosine deaminase (ADA) (3). Members of this functionally diverse superfamily are found in every organism sequenced to date (4, 5) and are characterized by a mono- or binuclear metal center embedded within the C-terminal end of a \((\beta\alpha)_8\) barrel structural fold (3). HutF most closely resembles the sequences possessed by enzymes that catalyze the deamination of the heterocyclic base, cytosine (2), and the herbicide, atrazine (3). The structural similarity within these three substrates is shown in Scheme 2.

Relatively little is known about the reaction mechanism for the deamination of N-formimino-L-glutamate by HutF. Based upon the sequence alignment of HutF with the two most closely structurally characterized analogues, it is anticipated that this enzyme will possess a mononuclear metal center in the active site. The single divalent cation will be ligated to His-56 and His-58 from the end of \(\beta\)-strand

![Scheme 1](image-url)

**Scheme 1**

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1, His-232 from β-strand 5, and Asp-320 from β-strand 8. A water molecule will function as the fifth ligand to the lone divalent cation. This water molecule will be hydrogen bonded to His-269 from the end of strand 6 and to the carboxylate of Asp-320 that also interacts with the divalent cation. The remaining invariant residue within the active site of HutF is Glu-235. This residue is located at the end of strand 5 within an HxxE motif that is found in members of the AHS that catalyze the deamination of aromatic bases such as adenosine, guanine, and cytosine. The purported function of the analogous residue in adenosine and cytosine deaminase is for proton donation to the aromatic base upon nucleophilic attack by water. A homology model for the active site of HutF based upon these considerations is presented in Figure 1.

The current investigation presents the biochemical characterization of the mechanism of reaction of HutF from *P. aeruginosa*. This reaction mechanism was probed to enhance our current understanding of the breadth of the reactions catalyzed by members of the amidohydrolase superfamily. The active site core of HutF most closely resembles those enzymes that catalyze a nucleophilic aromatic substitution. How these highly conserved residues are marshaled together within the active site of HutF to catalyze the deamination of an N-formimino group will aid in our attempt to correctly annotate enzymes of unknown function within this diverse superfamily of enzymes. The functional roles of those residues anticipated to be directly involved in catalysis and substrate specificity were addressed by the construction and characterization of site-directed mutant enzymes. The proton-transfer steps in the catalytic transformation were probed by the measurement of the kinetic constants as a function of pH. The role of the active site metal was interrogated by formation and reconstitution of apo-HutF with a variety of divalent cations.

**MATERIALS AND METHODS**

*Materials.* All chemicals were obtained from Sigma-Aldrich, unless otherwise stated. The genomic DNA from *P. aeruginosa* was purchased from the American Type Culture Collection (ATCC). The oligonucleotide synthesis and DNA sequencing reactions were performed by the Gene Technology Laboratory of Texas A&M University. The pET30a(+) expression vector and the BL21(DE3)star cells were acquired from Novagen. The Quick Change site-directed mutagenesis kit was purchased from Stratagene.

*Cloning and Site Directed Mutagenesis.* The gene encoding for Pa5106 (HutF) was cloned from *P. aeruginosa* PA01 into a pET30a(+) expression vector as previously described (2). Site directed mutagenesis of HutF at residues Glu-235, His-269, and Asp-320 was accomplished using the Quick Change site directed mutagenesis kit. The wild-type and mutant forms of HutF were expressed in BL21(DE3)star cells.

*Specific Activity Determination.* The specific activity of HutF toward the deamination of N-formimino-1-glutamate was followed by coupling the production of ammonia to the oxidation of NADH. The decrease in the concentration of NADH was followed spectrophotometrically at 340 nm using...
a SPECTRAmax-340 microplate reader (Molecular Devices Inc.). The standard assay was modified from that reported by Muszbek et al. (6) and contained 100 mM HEPES, pH 7.5, 100 mM KCl, 7.4 mM α-ketoglutarate, 0.4 mM NADH, 6 units of glutamate dehydrogenase, 20 nM of HutF, and N-formimino-L-glutamate in a final volume of 250 μL at 30 °C.

**Metal Analysis.** Metal-free HutF (apo-HutF) was prepared and reconstituted with different divalent metal cations, as previously described (2). Purified HutF was treated with 3 mM dipicolinate, pH 5.6, at 4 °C for 48 h. The chelator was removed by loading the protein/chelator mixture onto a PD10 column (GE Health Care) and eluting with metal-free buffer A. The apo-HutF was reconstituted with 1.0 equiv of the desired divalent metal (Zn²⁺, Cu²⁺, Ni²⁺, Cd²⁺, and Mn²⁺) in 50 mM HEPES at pH 7.5. The metal content of the apo-HutF and reconstituted enzymes was verified using a Perkin-Elmer AAAnalyst 700 atomic absorption spectrometer and inductively coupled plasma emission-mass spectrometry (ICP-MS).

**pH–Rate Profiles.** The pH dependence of the kinetic parameters was determined for the Zn, Cd, Cu, and Ni forms of the wild-type HutF using N-formimino-L-glutamate as the substrate. The pH–rate profiles were obtained for the Zn-substituted forms of the E235Q and E235A mutants. The pH dependence of the kinetic parameters was determined at either high or low pH, respectively. The kinetic parameters for the metal-reconstituted HutF and active site mutants are presented in Table 1.

**RESULTS**

**Metal Substituted Variants of HutF.** The functional significance of a divalent cation in the reaction catalyzed by HutF was probed by formation of the apoenzyme and reconstitution of this protein with a variety of other metal ions. The isolated recombinant protein expressed in *Escherichia coli* was found to contain 0.5 equiv of Zn²⁺ per enzyme subunit and a specific activity of 5.2 s⁻¹. Incubation of HutF with the metal chelator dipicolinate for 3 h at pH 5.6 was effective in the removal of zinc from the active site. A metal analysis of the apoenzyme found 0.03 equiv of zinc per subunit and a specific activity of less than 0.1 s⁻¹. More than 98% of the original catalytic activity was lost after removal of the zinc from the enzyme.

The metal center was reconstituted in about 10 min after the addition of 1 equiv of Cd²⁺, Cu²⁺, Ni²⁺, or Zn²⁺ to the apoenzyme. The reconstitution of apo-HutF with these metal ions resulted in catalytically active enzyme with Cd²⁺ > Ni²⁺ > Zn²⁺ > Cu²⁺ with respect to *kₐcat*. The reconstitution of apo-HutF with Co²⁺ and Mn²⁺ could not be accomplished even after 48 h of incubation with up to 10 equiv of metal per subunit of protein. The average metal content per subunit of the reconstituted proteins was 1.2, 0.8, 1.2, and 1.7 for the Zn-, Ni-, Cd-, and Cu-substituted forms of HutF, respectively. The kinetic parameters for the metal-reconstituted variants of HutF are presented in Table 1.

**Site-Directed Mutagenesis.** Sequence alignments of HutF with the structurally characterized members of the amidohydrolase superfamily indicate that this enzyme is most closely related to those proteins that catalyze the deamination of exocyclic amino groups from nitrogen heterocyclic substrates (2). The X-ray crystal structures of adenosine deaminase (8) and cytosine deaminase (9), in conjunction with these sequence alignments, suggest critical roles for Glu-235, His-269, and Asp-320 in the catalytic activity of HutF. The functional significance of His-269 and Asp-320 in the active site of HutF was assessed by mutation of these residues to alanine, asparagine, and cysteine. The role of Glu-235 was

**Table 1: Kinetic Parameters for Metal-Reconstituted HutF and Active Site Mutants**

<table>
<thead>
<tr>
<th>HutF (M)</th>
<th>M²⁺/subunit</th>
<th><em>Kₐ</em> (mM)</th>
<th><em>kₐ</em> (s⁻¹)</th>
<th><em>kₐ/Kₐ</em> (M⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Zn)</td>
<td>1.2</td>
<td>0.22 ± 0.03</td>
<td>13.2 ± 0.4</td>
<td>(6.0 ± 0.1) × 10⁴</td>
</tr>
<tr>
<td>WT (Ni)</td>
<td>0.81</td>
<td>2.7 ± 0.1</td>
<td>21.3 ± 0.3</td>
<td>(8.0 ± 0.3) × 10³</td>
</tr>
<tr>
<td>WT (Cd)</td>
<td>1.2</td>
<td>2.1 ± 0.3</td>
<td>31.0 ± 0.1</td>
<td>(1.5 ± 0.2) × 10⁴</td>
</tr>
<tr>
<td>WT (Cu)</td>
<td>1.7</td>
<td>1.2 ± 0.1</td>
<td>8.5 ± 0.1</td>
<td>(7.3 ± 0.4) × 10³</td>
</tr>
<tr>
<td>E235A (Zn)</td>
<td>0.62</td>
<td>2.2 ± 0.1</td>
<td>(1.0 ± 0.2) × 10⁻¹</td>
<td>(4.7 ± 0.6) × 10⁻³</td>
</tr>
<tr>
<td>E235D (Zn)</td>
<td>0.51</td>
<td>0.66 ± 0.05</td>
<td>(5.0 ± 0.1) × 10⁻³</td>
<td>7.9 ± 0.6</td>
</tr>
<tr>
<td>E235Q (Zn)</td>
<td>0.77</td>
<td>19 ± 2</td>
<td>(1.2 ± 0.1) × 10⁻¹</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>H269A (Zn)</td>
<td>0.12</td>
<td>1</td>
<td>(1.6 ± 0.1) × 10⁻¹</td>
<td>(7.3 ± 1.0) × 10⁻¹</td>
</tr>
<tr>
<td>H269C (Zn)</td>
<td>0.95</td>
<td>1</td>
<td>(1.8 ± 0.1) × 10⁻¹</td>
<td>&lt;2.0 × 10⁻²</td>
</tr>
<tr>
<td>H269N (Zn)</td>
<td>0.17</td>
<td>1</td>
<td>&lt;2.0 × 10⁻²</td>
<td>&lt;2.0 × 10⁻²</td>
</tr>
<tr>
<td>D320A (Zn)</td>
<td>0.50</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D320C (Zn)</td>
<td>1.0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The kinetic parameters were determined with N-formimino-L-glutamate as the substrate at pH 8.0, 30 °C, from a fit of the data to eq 1.

\[ \log y = \log \left( \frac{c(1 + (H/K_a) + (K_e/H))}{(K_e)} \right) \]  (2)

\[ \log y = \log \left( \frac{c(1 + (H/K_a) + (K_e/H))}{(K_e)} \right) \]  (3)

\[ \log y = \log \left( \frac{c(1 + (H/K_a) + (K_e/H) + (H^2/(K_e)^2))}{(K_e)} \right) \]  (4)

\[ \frac{v}{E_i} = k_{cat} A/(K_m + A) \]  (1)
addressed by mutation to alanine, aspartate, and glutamine. The mutant proteins were purified to homogeneity, and the zinc content of the purified proteins varied from 0.17 to 1.0 equiv of Zn\(^{2+}\) per protein subunit. The effects of these mutations on the kinetic parameters of HutF are presented in Table 1.

There was a substantial drop in the catalytic activity as a result of the mutations at each of the three residues. For the three characterized mutations at His-269 the catalytic activity is approximately 5 orders of magnitude lower than that of the wild type enzyme. The values of \(k_{\text{cat}}\) for these mutants could not be determined since the velocity of the catalyzed reaction was not saturated at the highest concentration of substrate (10 mM). The metal content of the H269A and H269N mutants was significantly lower than that of the H269C mutant. Site-directed mutagenesis of Asp-320 to asparagine produced an insoluble protein, whereas mutation to alanine and cysteine resulted in soluble protein with undetectable catalytic activity. However, these two mutants could not be still able to bind a significant amount of zinc. The mutation of Glu-235 was less disruptive than the modifications to either His-269 or Asp-320. Reductions of 2–4 orders of magnitude were found for \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) relative to the wild type enzyme. However, the Michaelis constants were in an experimentally accessible range.

**pH–Rate Profiles.** The kinetic constants for the enzymatic hydrolysis of \(N\)-formimino-\(L\)-glutamate by HutF were determined as a function of pH. The plots of \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) versus pH for the zinc substituted enzyme are presented in Figure 2. The shape of these two pH–rate profiles indicates that two groups are required to be in the proper state of ionization for maximum catalytic activity; one of these groups must be ionized while the other must be protonated. When the native zinc in the active site is replaced with other divalent cations, the pK values for the groups that ionize within this pH range change slightly (Figure 3). For Cd-HutF, the two ionizations are very close to one another in the pH–rate profile of \(k_{\text{cat}}\), and it is not possible to accurately obtain the values for pK\(_a\) and pK\(_b\) when the difference between them is less than 0.6 pK units (7). The fitting routine therefore assumes that the two ionizations have the same pK value (7). A similar situation applies to the pH rate profile of \(k_{\text{cat}}/K_m\) for Ni-HutF. For Cd-HutF, the pH–rate profile for \(k_{\text{cat}}/K_m\) decreases with a slope of −2 for the group(s) that must be protonated for activity. These data were fit to eq 3, which assumes that both ionizations have the same value for pK\(_b\). The pK values from the kinetic measurements are presented in Table 2.

The pH–rate profiles were determined for the E235Q and E235A mutants. The pH–rate profiles for \(k_{\text{cat}}\) are bell-shaped with activity decreasing at high and low values of pH. For
mutants are presented in Figure 4, and the \( pK_a \) deaminase. All of these nucleotides contain structural motifs.

**DISCUSSION**

A number of enzymes within the amidohydrolase superfamily catalyze the deamination of purine and pyrimidine nucleotides, including cytosine, adenosine, and guanine deaminase. All of these nucleotides contain structural motifs that are homologous to the formimino substituent in \( N\)-formimino-\( L \)-glutamate. The chemical reaction mechanism for adenosine deaminase has been elaborated through a comprehensive array of biochemical, computational, and crystallographic experiments (8, 10–13). In these investigations several residues within the active site of ADA have been recognized to be directly involved in substrate binding and catalysis. The most salient residues for adenosine deaminase from mouse include Glu-217, His-238, and Asp-295. A homologous trio of amino acids is predicted to function in an analogous fashion in cytosine deaminase (9), guanine deaminase (5), and AMP deaminase (5). In these enzymes a water molecule is coordinated to the single divalent cation localized to the \( M_\alpha \)-position in the active site. The water molecule is furthered hydrogen bonded to a histidine from strand 6 and an aspartate from strand 8. The glutamic acid from strand 5 functions as a general acid through proton transfer to the aromatic leaving group. Although HutF does not catalyze the deamination of an aromatic amine, it does catalyze the analogous deamination of an \( N\)-formimino group to an \( N\)-formamide derivative.

A working model for the chemical mechanism of the reaction catalyzed by HutF is presented in Scheme 3. In this mechanism the single divalent cation is bound to His-56 and His-58 from strand 1, His-232 from strand 5, and Asp-320 from strand 8. The final ligand to this metal ion is a water molecule that is in turn hydrogen bonded to His-269 from strand 6 and Asp-320 from strand 8 (see also Figure 1). The substrate binds into the active site in an orientation such that the metal bound water molecule is poised to attack the formimino functional group. The \( pK_a \) value of the formimino group is approximately 11.2 (16), and thus the substrate likely binds with the \( N\)-formimino group protonated, but it is unclear as to the relative distribution of the positive charge over the two nitrogen atoms. Once bound, the side chain carboxyate of Glu-235 makes an electrostatic interaction with the positively charged formimino group of the substrate. In this proposed mechanism the reaction is initiated by the abstraction of a proton from the bound water molecule by the side chain imidazole of His-269 to generate a tetrahedral intermediate. The collapse of the tetrahedral intermediate commences with the abstraction of the second proton via the side chain carboxyate of Asp-320. The \( \text{C} = \text{N} \) bond is then cleaved concomitantly with proton transfer from His-269 to liberate ammonia and generate the \( N\)-formyl product. After product release the resting state of the enzyme is regenerated after the binding of another water molecule.

A single divalent metal ion is essential for the catalytic activity of HutF. The most active enzyme was found to contain one equivalent of Zn per protein subunit. The metal ion can be removed by metal chelators, and the apoenzyme is essentially inactive. The apoenzyme can be reconstituted with one equivalent of Zn, Cd, Ni, or Cu with a modest variation in the final catalytic activity. It is assumed that Zn is the native divalent cation. The loss of activity after removal of the divalent cation from the active site of HutF is consistent with the role of the metal ion as a binding site for the nucleophilic water molecule. Similar results have been found for other members of the amidohydrolase superfamily (8, 9, 14, 15). The loss of activity exhibited by HutF at low pH for the \( \text{Zn}^{2+} \)-substituted enzyme indicates the protonation of a single group with a \( pK_a \) of approximately 7.3. Since the substrate does not ionize at this pH (16), it is assumed that this ionization must originate with the enzyme. Therefore, the most likely candidate for the group that must be deprotonated for catalytic activity is the histidine from strand 6 (His-269) that is hydrogen bonded to the water molecule that is ligated to the lone divalent cation in the active site. This conclusion is supported by the catastrophic loss of activity when this residue is mutated to alanine, cysteine, or asparagine. It should be noted, however, that the H269A and H269N mutants have a somewhat lower metal occupancy than the wild type HutF.

Activity is also lost at high pH when a functional group is deprotonated. The decrease in catalytic activity at high pH for the \( k_{\text{cat}}/K_m \) profile suggests the deprotonation of a group with a \( pK_a \) value of around 8.5. Based on the crystal structures of cytosine deaminase (9) and adenosine deaminase (8, 13) and the comparison of their amino acid sequences with the amino acid sequence of HutF, there is no obvious residue within the active site of HutF that might be responsible for the decrease in activity at high pH. The \( pK_a \) value for the formimino group of \( N\)-formimino-\( L \)-glutamate has been reported to be 11.2 (16), and thus the ionization of the substrate does not appear to be responsible for the...
diminution of catalytic activity at high pH. It is unclear as to the group that is responsible for the loss of activity at high pH.

In the mechanism proposed in Scheme 3 the other residue that serves to activate the water in conjunction with His-269 is Asp-320 from strand 8. When this residue is mutated to either alanine or cysteine, the catalytic activity of HutF is reduced by over 6 orders of magnitude. Even though this residue also ligates to the divalent cation in the active site, these two mutants are still able to bind a significant amount of zinc. The substantial loss of catalytic activity is consistent with the role proposed by this residue during the deimination of the substrate.

In the mechanisms of action proposed for the deamination of adenosine and cytosine, the invariant glutamate in the HxxE motif at the end of strand 5 serves to donate a proton to the ring nitrogen during the course of the reaction. In the reaction catalyzed by HutF, the role of this highly conserved residue must be slightly different. Since the formimino group of the substrate is protonated, there is no reason why this residue must function as a general acid. It can, however, function as an electrostatic bridge from the protein to the substrate. The importance of this interaction is reflected in the loss of activity when this residue (E235) is mutated to either alanine, aspartate, or glutamine. The E235A mutant is the most active of the three modifications made to this site, but it is reduced in catalytic activity by 3 orders of magnitude.

ACKNOWLEDGMENT

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REFERENCES


