Discovery of a Cytokinin Deaminase

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Supporting Information

ABSTRACT: An enzyme of unknown function within the amidohydrolase superfamily was discovered to catalyze the hydrolysis of N-6-substituted adenine derivatives, several of which are cytokinins. Cytokinins are a common type of plant hormone and N-6-substituted adenines are also found as modifications to tRNA. Patl2390, from Pseudoalteromonas atlantica T6c, was shown to hydrolytically deaminate N-6-isopentenyladenine to hypoxanthine and isopentenylamine with a $k_{cat}/K_m$ of $1.2 \times 10^7$ M$^{-1}$ s$^{-1}$. Additional substrates include N-6-benzyl adenine, cis- and trans-zeatin, kinetin, O-6-methylguanine, N-6-butyladenine, N-6-methyladenine, N,N-dimethyladenine, 6-methoxypurine, 6-chloropurine, and 6-thiomethylpurine. This enzyme does not catalyze the deamination of adenine or adenosine. A comparative model of Patl2390 was computed using the three-dimensional crystal structure of Pa0148 (PDB code 3PAO) as a structural template, and docking was used to refine the model to accommodate experimentally identified substrates. This is the first identification of an enzyme that will hydrolyze an N-6-substituted side chain larger than methylamine from adenine.

With the rapidly increasing volume of protein sequence data available, a need has been created for a comprehensive strategy for analyzing and annotating newly sequenced genomes. Clusters of orthologous groups (COG) were created to aid in the assignment of function for related groups of proteins. Within the amidohydrolase superfamily, there are 24 clusters of orthologous groups defined by NCBI. Members of the AHS may have either a mononuclear or binuclear metal center embedded within a $\beta/\alpha$-barrel structural fold with metal binding residues found at the C-terminal ends of $\beta$-strands 1, 4, 5, 6, and 8.† The metal center is responsible for activating a hydrolytic water molecule for nucleophilic attack on amino acids, sugars, nucleic acids, and organophosphate esters. Three of these clusters of enzymes are known to catalyze aromatic deamination reactions: cog1001, cog0402, and cog1816. One of these clusters, cog1816, contains nearly 500 bacterial proteins that are currently annotated as adenosine deaminases.‡ The prototypical adenine deaminase (ADE) and N-6-methyladenine deaminase (6-MAD) are members of cog1001.¶ Enzymes that deaminate guanine, cytosine, S-adenosylhomocysteine, thiomethyl adenosine, N-formiminoglutamate, and 6-oxoguanine are found in cog0402.¶

A sequence similarity network for cog1816 from the amidohydrolase superfamily is presented in Figure 1. The node represents a single sequence, and each edge (depicted as lines) represents the pairwise connection between two sequences at a BLAST E-value of better than $1 \times 10^{-70}$. Lengths of edges are not significant, except for tightly clustered groups, which are more closely related than sequences with only a few connections. Group 5 contains the $E. coli$ adenosine deaminase. The yellow dots in Group 3 represent proteins that will deaminate adenine. The proteins represented by the pink dots at the periphery of Group 3 are characterized in this paper.

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Figure 1. Sequence similarity network created using Cytoscape® of cog1816 from the amidohydrolase superfamily. Each node in the network represents a single sequence, and each edge (depicted as lines) represents the pairwise connection between two sequences at a BLAST E-value of better than $1 \times 10^{-70}$. Lengths of edges are not significant, except for tightly clustered groups, which are more closely related than sequences with only a few connections. Group 5 contains the $E. coli$ adenosine deaminase. The yellow dots in Group 3 represent proteins that will deaminate adenine. The proteins represented by the pink dots at the periphery of Group 3 are characterized in this paper.

†010898705; Leum0809 from Leuconostoc mesenteroides, gi|116617920; Lsa0086 from Lactobacillus sakei, gi|81427699; Cja0578 from Cellvibrio japonicas, gi|192359911; MADE1015570 from Alteromonas macleodii, gi|332142506; Jeden_1580 from Jonesia denitrificans DSM 20603, gi|256832804; Glaag_1976 from Glaciecola agarlytica 4H-3-7±YE-5, gi|332173667; ambt_10110 from Alteromonas sp. SN2, gi|332993493; Sros_2140 from Streptosporangium roseum DSM 43021, gi|271963671; Sked_12620 from Sanguibacter keddiae DSM 10542, gi|269794583; and LBU_0899

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from Lactobacillus delbrueckii subsp. Bulgaricus 2038, gi|325125754) are highlighted in pink in Figure 1. These 11 proteins originate from organisms with an authentic adenine deaminase from either cog1001 or Group 3 of cog1816. On the basis of these observations, we predicted that the 11 proteins at the periphery of Group 3 from cog1816 will not deaminate adenine but will deaminate an adenine-like substrate.

**Substrate Discovery.** We established the substrate profile for one of the proteins at the periphery of Group 3 from cog1816, Patl2390 from *P. atlantica* T6c, an agarolytic bacterium.9 The gene for Patl2390 was cloned and expressed, and the resultant protein purified to homogeneity. The purified protein contained 0.6 equiv of Zn2+ per monomer. The substrate profile for Patl2390 was determined by monitoring the changes in absorbance after the addition of enzyme to a small library of modified purines, including adenosine, adenine, N-6-methyladenine, and N-6-isopentenyladenine. On the basis of the results of the initial screen, a larger collection of 6-substituted purines was assayed, including 6-chloropurine, 6-thiomethylpurine, N-trans-6-isopentenyladenine, N-trans-6-benzyladenine, N-methylguanine, and N-trans-zeatin. The structure of Patl2390, along with four proteins predicted to have the same substrate profile as Patl2390, is presented in Supplementary Figure S1. The crystal structure of Patl2390, most residues in the proximity of adenine are conserved relative to 3PAO, except for Asp203 and Phe257. The backbone and side chain conformations of segments near the two variable residues (Cys202 to Gln206, Pro252 to Ser261) were refined in the presence of adenine by Modeler for loops and by PLOP for side chains.11 The high-energy intermediate (HEI) library that contains ~22,500 different intermediate forms of 4207 KEGG14,15 molecules was docked to this refined model of Patl2390 by DOCK 3.5.54.16,17 Six known substrates that were included in the docking library (N-6-benzyladenine, N-trans-zeatin, N-6-methyladenine) were ranked 56, 60, 64, 88, 147, and 328, respectively. The docking poses of these six substrates are similar to the binding mode of adenine in 3PAO. For N-6-benzyladenine, the activated hydroxide attacks C-6 on the re face of the purine ring, forming a tetrahedral intermediate with R-stereochemistry. Asp203 is hydrogenbonded to protonated N-1 of the purine ring.

**Table 1. Kinetic Constants for Patl2390**

<table>
<thead>
<tr>
<th>substrate</th>
<th>(k_{\text{cat}}/K_m) (s(^{-1}))</th>
<th>(K_m) ((\mu)M)</th>
<th>(k_{\text{cat}}/K_m) (M(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.3 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.2 (0.2) × 10(^5)</td>
</tr>
<tr>
<td>2</td>
<td>8.5 ± 0.9</td>
<td>86 ± 7</td>
<td>9.9 (0.3) × 10(^5)</td>
</tr>
<tr>
<td>3</td>
<td>10.1 ± 0.5</td>
<td>9.2 ± 0.9</td>
<td>1.1 (0.1) × 10(^6)</td>
</tr>
<tr>
<td>4</td>
<td>10.2 ± 0.6</td>
<td>9 ± 2</td>
<td>1.1 (0.3) × 10(^6)</td>
</tr>
<tr>
<td>5</td>
<td>5.6 ± 0.5</td>
<td>9 ± 2</td>
<td>6.2 (0.2) × 10(^5)</td>
</tr>
<tr>
<td>6</td>
<td>6.5 ± 0.4</td>
<td>50 ± 6</td>
<td>1.3 (0.2) × 10(^5)</td>
</tr>
<tr>
<td>7</td>
<td>13.1 ± 0.6</td>
<td>10 ± 1</td>
<td>1.3 (0.1) × 10(^6)</td>
</tr>
<tr>
<td>8</td>
<td>11.4 ± 0.6</td>
<td>1.5 ± 0.3</td>
<td>8 (1) × 10(^6)</td>
</tr>
<tr>
<td>9</td>
<td>11.6 ± 0.6</td>
<td>7 ± 1</td>
<td>1.6 (0.3) × 10(^6)</td>
</tr>
<tr>
<td>10</td>
<td>13 ± 1</td>
<td>35 ± 5</td>
<td>3.6 (0.5) × 10(^6)</td>
</tr>
<tr>
<td>11</td>
<td>13 ± 1</td>
<td>38 ± 5</td>
<td>3.2 (0.3) × 10(^6)</td>
</tr>
<tr>
<td>12</td>
<td>13 ± 1</td>
<td>38 ± 5</td>
<td>3.3 (0.4) × 10(^6)</td>
</tr>
</tbody>
</table>
In the comparative model, residues that coordinate the mononuclear metal center (His21, His23, His201, and Asp282) and residues that apparently are involved in substrate recognition (Tyr68, Asp105, Ser174, and Asp283) superimpose with the aligned residues from the structural template. A deletion of two Patl2390 residues in the sequence \( \text{E176Q and E176A} \) E176Q was insoluble, \( \beta \)-strand 4 that is in the vicinity of the active site was mutated to an asparagine. On the basis of these results and the docking of known substrates into the active site of Patl2390, we conclude that Asp203 functions to deliver a proton to N-1 of the substrate during catalysis.

The substrates identified for Patl2390 are known as cytokinins. These compounds promote cell growth and division in plants. They are produced by nearly all plants and have been isolated from red algae. Similar compounds are also found as modified adenosines in serine and phenylalanine tRNA. Naturally occurring cytokinins include N-6-benzyladenine, kinetin, cis-zeatin, trans-zeatin, and N-6-isopentenyladenine. N-6-Isopentenyladenine, as well as cis- and trans-zeatin, are believed to originate from dimethylallyl pyrophosphate transfer to AMP.

The effects of cytokinins on plants have been well-studied, but the metabolism of these compounds has received less attention. The cytokinin oxidase from Zia mays catalyzes the oxidative cleavage of N-6-isopentenyl or zeatin side chains of both the adenine and nucleoside forms.

The bacterium P. atlantica T6c was first isolated in association with marine red algae, which are known to contain agar in their cells walls and to produce cytokinins. P. atlantica T6c is able to degrade the cell walls of red algae through production of an extracellular agarase, which enables the bacteria to acquire nutrients from the algae. The enzyme identified here may function in salvaging of nutrients from algae through the conversion of cytokinins into hypoxanthine, which can then be incorporated into the purine salvage pathway. The reaction catalyzed by Patl2390 with isopentenyl adenine is presented in Scheme 2. The ability to remove a methoxy group from O-6-methylguanine may also indicate a role of this enzyme in DNA repair.

**Strategy for Functional Annotation.** Current estimates put the percent of unknown, uncertain, or incorrect annotations of bacterial genes at more than 30%. For this investigation the NCBI database was used to construct a sequence similarity diagram for cog1816 from the amidohydrolase superfamily, which identified 11 proteins as having a high sequence identity to authentic adenine deaminases of Group 3 but missing some key catalytic residues. The genes for these proteins are located in the vicinity of genes for other enzymes involved in purine metabolism, including the experimentally verified adenine deaminases. Using adenine as a structural scaffold, a small library of compounds was assembled to ultimately identify the substrate profile for Patl2390. We demonstrated that Patl2390 is capable of accepting cytokinins and several modified purines as substrates. No catalytic activity toward adenine or adenosine was observed. The construction of a comparative structural model for Patl2390 allowed for the identification of the structural rational for the preferential hydrolysis of large substituents attached to C-6 of the purine base.

![Scheme 2](image-url)
Materials. All chemicals were purchased from Sigma unless otherwise stated. 6-Methoxypurine was obtained from Tokyo Chemical Industry Co. N-6-Methyladenine was procured from Spectrum. N-6-Butyladenine was bought from Ryan Scientific. cis-Zeatin was ordered from MP Biomedicals, and zeatin riboside was acquired from Carbo-synth. O-6-Methylguanine was obtained from LT Pharmatech.

Cloning and Purification of Patl2390 from Pseudoalteromonas atlantica T6c. Patl2390 was cloned from P. atlantica T6c genomic DNA (ATCC) using restriction sites for BamHI and EcoRI, digested, and ligated into a pET-30a(+) vector. The cloned gene fragment was sequenced to verify fidelity of the PCR amplification. Patl2390 in a pET-30a(+) vector was overexpressed in BL21(DE3) cells (Novagen) and purified by affinity chromatography using a HiFeatp column (GE Healthcare). Additional information is available in Supporting Information.

Activity Screens. Patl2390 (10 nM) was incubated for 16 h with adenine, 2,6-diaminopurine, 6-mercaptopurine, 6-methylthiopurine, 6-chloropurine, 6-methylurine, N-6-isopentyl adenine, isoguanine, N-6-methyladenine, zeatin, zeatin riboside, 4-aminopuridine, 6,4-diamino pyrimidine, 2,4-diamino pyrimidine, 7-methyladenine, toxopyrimidine, cytosine, 5-hydroxymethylcytosine, 2-chloroadenine, and 2-dimethylamino adenine. Substrate concentration was 80 μM for all screens. Activity was monitored by changes in absorbance between 240 and 300 nm on a SpectraMax384Plus spectrophotometer (Molecular Devices).

Measurement of Enzymatic Activity. Assays were conducted with 3–200 μM substrate. Dechloration of 6-chloropurine was monitored by an increase in absorbance at 250 nm. Formation of hypoxanthine from N-6-isopentenyladenine, N-6-methyladenine, trans-zeatin, benzyladenine, 6-methoxypurine, and N-6-butyladenine was monitored at 270 nm. Decreases in absorbance at 275 nm were used to monitor formation of hypoxanthine from cis-zeatin and N6-imidazolyladenine. Conversion of 6-methylthiopurine and kinetin were monitored by changes in absorbance between 240 and 300 nm on a SpectraMax384Plus spectrophotometer (Molecular Devices).

Metal Analysis. Metal content of the proteins was determined by ICP-MS.29 Protein samples for ICP-MS were digested with HNO3 by refluxing for ~45 min to prevent protein precipitation during the measurement. Protein concentration was adjusted to ~1.0 μM with 1% (v/v) HNO3.

Data Analysis. Sequence alignments were created using ClustalW at http://www.compbio.dundee.ac.uk/ jalviewWS/services/ClustalWS. Steady-state kinetic data were analyzed using Softmax Pro version 5.4. Kinetic parameters were determined by fitting the data to eq 1 using the nonlinear least-squares fitting program in SigmaPlot 9.0, where A is the substrate concentration, K_m is the Michaelis constant, v is the velocity of the reaction, and k_cat is the turnover number.

\[
\frac{v}{E_i} = \frac{k_{cat}A}{(K_m + A)}
\] (1)

Model Building and Computational Docking. The sequence alignment between Patl2390 and Pat0148 was computed by MUSCLE (Multiple Sequence Comparison by Log-Expectation).30 The original alignment was manually adjusted, so that the active site residues Asp203 in Patl2390 was aligned to Glu199 in Pat0148. The crystal structure of Pat0148 with adenine bound (PDB id 3PAO) was used as the template. A total of 500 comparative models were generated with the standard “automodel” class in Modeler-9v8. The model with the best DOPE19 score was selected for refinement of backbone and side chain conformations of residues Cys202 to Gln206 and Pro252 to Ser261, using the “loopmodel” class in Modeler. Side chains of residues in these two loops were subsequently refined using the “side chain prediction” protocol in PLOP, in the presence of adenine. This refinement resulted in two representative models of Patl2390, in which (1) residues Cys202 to Gln206 were refined with respect to the original model and (2) residues Cys202 to Gln206 and Pro252 to Ser261 were refined with respect to the original model. The high-energy intermediate (HEI)15,16 library of KEGG15,16 molecules was docked to both refined models of Patl2390 by DOCK 3.5.5.14,16 The second model yielded a higher enrichment for six known substrates (N-6-benzyladenine, cis-zeatin, kinetin, N-6-isopentenyladenine, trans-zeatin, N-6-methyladenine) was selected to represent the binding mode of N-6-benzyladenine in Patl2390 (Figure 2).

ASSOCIATED CONTENT

Supporting Information. Protein sequence alignment of Patl2390 with five other proteins (Figure S1) and additional information on the cloning and purification of Patl2390. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

ADE, adenine deaminase; AHS, amidohydrolase superfamily; ICP-MS, inductively coupled plasma mass spectrometry

REFERENCES