A Thermostable Aspartate Aminotransferase from *Aeropyrum pernix* K1

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Aspartate aminotransferase (AAT, EC 2.6.1.1), the best known pyridoxal 5'-phosphate dependent enzyme, is an essential component of nitrogen and carboxylic cellular metabolism, where it catalyzes the reversible transamination between dicarboxylic amino and α-keto acids. Aminotransferases from many species are classified into four subgroups. Family I aminotransferases transfer the amino acids of aspartate, alanine, histidine, and aromatic compounds to other proteins, and have been further subdivided into seven subfamilies (Iα, Iλ, Iβ, IΩ, Iδ, Iγ, and Iφ). The AATs of subfamily Iα include enzymes from *Escherichia coli*, yeasts, plants, and animals, and have been more extensively investigated than other subfamilies to elucidate the structure and functions of the enzymes. Subfamily Iγ includes AATs from *Bacillus* sp. YM-2, *Rhizobium meliloti*, *Sulfolobus solfataricus*, and *Thermus thermophilus* HB8, with sequences that are < 16% identical between the subfamilies. According to the three-dimensional structures of the AATs as determined by X-ray crystallography, both the overall and active site structures are essentially conserved between the AATs of subfamilies Iα and Iγ. Despite the available research, the biochemical properties and functions of the prokaryotic AATs remain to be elucidated.

*Aeropyrum pernix* K1 is an aerobic hyperthermophilic crenarchaeon isolated from a coastal solfataric thermal vent in Kodakara-Jima island of Kagoshima, Japan that grows optimally at 90 ~ 95 °C. Genome sequencing of *Aeropyrum pernix* K1 at the National Institute of Technology and Evaluation in Japan revealed that the open reading frame APE2423, composed of 1149 base pairs, encodes a protein homologous to AATs from *Hyperthermus* butylicus, *Caldicellulosiruptor saccharolyticus*, and *Methanocaldococcus jannaschii* (approximately 37 ~ 59% identity, Fig. 1), as judged by homolog searches in the NCBI database. The amino acid sequences of the APE2423 gene showed ≥ 25% amino acid sequence identity to these thermophilic AATs (results not shown). These results suggest that the product of the APE2423 gene may belong to the Iγ subgroup.

**Figure 1.** Comparison of amino acid sequences of ApAAT and other AATs. Sequence alignment was performed by T-Coffee and Boxshade software (http://www.ch.embnet.org). Dashes indicate gaps, and consensus identical (black) or similar (gray) amino acids are shaded. The following sequences were aligned: ApAAT, AAT from *Aeropyrum pernix* K1 (GenBank accession number, BAE96897); HbAAT, AAT from *Hyperthermus* butylicus (YP_001013233); CsAAT, AAT from *Caldicellulosiruptor saccharolyticus* (YP_001180096); MjAAT, AAT from *Methanocaldococcus jannaschii* D8M2661 (AAB98961); ChHydrogenase, Hydrogenase from *Carboxydothermus hydrogenoformans* (ABB15912).
Table 1. Substrate specificities of ApAAT towards amino acids and α-keto acids

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino donors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>50</td>
<td>0.60</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>50</td>
<td>0.03</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>50</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>α-Keto acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>α-Ketobutyrate</td>
<td>5</td>
<td>0.3</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5</td>
<td>1.2</td>
</tr>
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</table>

In general, it is difficult to express cloned genes from hyperthermophilic bacteria in *E. coli*, because the expressed proteins are insoluble. 11 To effectively overexpress and purify the APE2423 gene product from *Aeropyrum pernix* K1 (ApAAT), a recombinant plasmid was designed using the expression vector pGEX-KG containing glutathione S-transferase (GST) as the fusion partner. The recombinant ApAAT was efficiently purified 10-fold with a yield of 52% by GSH-agarose affinity chromatography and thrombin treatment. Approximately 6 mg of the recombinant ApAAT protein was purified from 1 L of the transformant culture medium. The purified ApAAT yielded a single band on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that indicated an approximate molecular mass of 42 kDa (Figure 2).

Substrate specificities of the recombinant ApAAT were studied with the transamination reaction between amino donors and acceptors. ApAAT was specific for dianionic amino acid, with L-aspartate as an amino donor, but showed very low activity towards L-alanine (Table 1). Aromatic amino acids such as L-phenylalanine and L-tyrosine were negligibly active as amino donors. Substrate specificity for amino acceptors was also

Figure 2. SDS-PAGE analysis of GST-ApAAT (A) and ApAAT (B). Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) in 12.5% gels. The molecular-mass makers were SDS molecular weight standard markers and Coomassie Blue R-250 was used for staining. (A) Lane M, Protein size marker; lane 1, cell-free crude extract; lane 2, flow-through fraction; lane 3, purified GST-ApAAT; (B) Lane M, Protein size marker; lane 1, purified GST-ApAAT; lane 2, thrombin treatment; lane 3, purified ApAAT.
performed with L-aspartate as an amino donor and a variety of α-keto acids (Table 1). ApAAT was specific for α-ketoglutarate as an amino acceptor. Pyruvate and α-ketobutyrate served only slightly as substrates.

To further determine the function of ApAAT, we investigated the kinetic parameters of the enzyme toward L-aspartate and α-ketoglutarate. The \( K_m \) values of ApAAT were 3.5 ± 0.2 mM for L-aspartate and 3.0 ± 0.3 mM for α-ketoglutarate, respectively, which were similar to those of AAT from thermophilic Bacillus sp. Strain YM-2 (3.0 mM for L-aspartate and 2.6 mM for α-ketoglutarate). ApAAT also showed an affinity for L-aspartate that was higher than AAT from Rhizobium meliloti, with a \( K_m \) of 5.3 mM.12

We then determined the optimum pH and temperature for ApAAT with the transamination reaction toward L-aspartate and α-ketoglutarate. The optimum pH for ApAAT was 8.0 (Fig. 3A), with more than 60% of maximum activity in the pH range of 6.5 to 10. These findings were similar to AAT of Rhizobium meliloti (pH 8.0 - 8.5).13,14 At the same time, acidic or neutral ranges of optimum pH have been observed for AATs from Sulfolobus solfataricus (pH 5.8) and Bacillus YM-2 (pH 7.2).13,14 Activity of ApAAT increased steadily in the temperature range from 30 to 85 °C (Fig. 3B), with an optimum temperature of 85 °C. The thermostability of ApAAT was investigated by incubation of the enzyme for 20 min from 60 to 95 °C. The midpoint of the temperature-stability curve was approximately 75 °C (Fig. 3C). The enzyme was fairly stable at temperature up to 65 °C. Moreover, ApAAT was protected against heat activation by the addition of pyridoxal phosphate or α-ketoglutarate, which significantly increased its heat stability by approximately 5~10 °C. In contrast, the addition of L-aspartate decreased the heat stability of ApAAT. This thermostability is comparable with those of AATs purified from other thermoacidic bacteria (2,4,13).

In conclusion, we expressed the hypothetical protein of the APE2423 gene from Aeropyrum pernix K1 and characterized the purified recombinant protein. The hypothetical protein first reported herein is a novel AAT displaying high substrate specificities towards L-aspartate as an amino donor and α-ketoglutarate as an amino acceptor. The recombinant ApAAT was highly stable at high temperatures, making it comparable to other available AATs. Further studies are underway to elucidate the detailed physiological functions of ApAAT and to utilize this recombinant enzyme in medicine and bio-industrial processes.

**Experimental Section**

Cloning of the APE2423 gene and construction of expression plasmid. The cDNA of Aeropyrum pernix K1 was prepared as described by Sako et al.15 The gene APE2423 of A. pernix was amplified by the polymerase chain reaction (PCR) using the cDNA as template. The sequences of the PCR primers were: 5'-cccaagctTTGCTCTGCTGATGAT-3' (upper primer, containing an EcoR I digestion sequence as underlined); 5'-cccaagctTCAAGGCCCTAGGCTAGA-3' (lower primer, containing a Hind III digestion sequence as underlined). The PCR product was cloned into an expression vector pGEX-KG using EcoR I and Hind III sites. The resulting vector was designated as pGEX-ApAAT and used to transform the E. coli strain BL21 (DE3). The colony containing the appropriate insert was identified by DNA sequencing.

Expression and purification of the recombinant enzyme. The E. coli strain BL21 (DE3) harboring each of the constructed plasmids was grown in Luria-Bertani (LB) broth containing ampicillin (25.0 µg/mL) at 37 °C and induced at OD600 = 0.9 - 1.0 with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 8 h.16 The induced cells were harvested by centrifugation at 10,000 g for 10 min at 4 °C and resuspended in 50 mM phosphate-buffered saline (PBS) buffer (pH 7.3) containing 20 mM pyridoxal-5'-phosphate and 20% glycerol. The resuspended cells were subjected to sonication using an ultrasonic processor (Sonic & Materials Inc., Newtown, CT, USA) until lysed. The cell-free extract was obtained by centrifugation at 40,000 g for 60 min, the supernatant was collected and stored at 4 °C.

The supernatant containing the enzyme bearing the glutathione S-transferase (GST)-tag was subjected to affinity column chromatography with glutathione (GSH)-agarose. The column was washed with 50 mM PBS buffer (pH 7.3) containing 50 mM KCl. The fusion protein, GST-ApAAT was then eluted with 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM GSH. Free GSH was removed by dialysis against 50 mM Tris-HCl (pH 8.0) containing 20 mM pyridoxal-5'-phosphate and 5% glycerol. Approximately 2 mg of the fusion protein was incubated with 10 units of thrombin (Amersham pharmacia Biotech, Uppsala, Sweden) at room temperature for 16 h and loaded to GSH-agarose affinity column chromatography. The eluted ApAAT was subjected to dialysis for 24 h at 4 °C with three successive changes against 50 mM Tris-HCl (pH 8.0) containing 20 mM pyridoxal-5'-phosphate and 5% glycerol.

Enzyme assay and kinetic studies. The transamination reaction activities between amino donors of L-aspartate, L-alanine, L-tyrosine, L-phenylalanine and amino acceptors of α-ketoglutarate, α-ketobutyrate, pyruvate were measured spectrophotometrically at 340 nm with a HITACHI U-2000 double-beam spectrophotometer (Hitachi CO., Tokyo, Japan) using cuvettes of 10 mm path-length (Sigma-Aldrich). The reaction mixture contained the following, in a final volume of 1.0 mL: 10 mM Tris-HCl buffer (pH 8.0); 50 mM L-aspartate; 5 mM α-ketoglutarate; 0.1 mM NADH and 2 units of malate dehydrogenase; an appropriate amount of enzyme. The reaction was then initiated by addition of the enzyme preparation. One unit of activity was defined as the amount of enzyme producing 1 µM of oxaloacetate per 1 min. To measure the kinetic parameters, reaction rates were measured at a series of L-aspartate concentrations (0.2 ~ 50 mM) and the rates at various α-ketoglutarate concentrations (0.4 ~ 32 mM) finally fit by Lineweaver-Burk plots. The parameters (with standard deviation) were determined by five separate experiments. The protein concentration was determined by the method of Bradford using γ-globulin as a standard.

Thermostability of the enzyme. The thermostability of the enzyme was measured at 60 ~ 95 °C. The enzyme was incubated in 100 mM Tris-HCl buffer (pH 8.0) for 20 min. After chilling the sample on ice, residual activity was determined under standard conditions.
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References