Evolution of two alanine glyoxylate aminotransferases in mosquito

Qian HAN*†, Seong Ryul KIM*, Haizhen DING*† and Jianyong LI*†
*Department of Pathobiology, University of Illinois, Urbana, IL 61802, U.S.A., and †Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, U.S.A.

INTRODUCTION

Mosquitoes transmit malaria, dengue fever and West Nile virus, which are major threats to human health and well being in the World. Among them, malaria is considered to be the most prevalent life-threatening disease, with estimates of new cases ranging from 300 millions to 660 millions per year [1]. In mosquitoes, transamination of 3-HK (3-hydroxykynurenine) to XA (xanthurenic acid) is the major branching pathway of tryptophan metabolism [2,3]. XA was identified in 1998 as a natural chemical that catalyses the transamination of 3-HK to XA in mosquitoes, and the other is a typical dipteran insect AGT. We cloned the second AGT from Aedes aegypti mosquitoes [AeAGT (Ae. aegypti AGT)], overexpressed the enzyme in baculovirus/insect cells and determined its biochemical characteristics. We also expressed hAGT for a comparative study. The new cloned AeAGT is highly substrate-specific when compared with hAGT and the previously reported AeHKT and Drosophila AGT, and is translated mainly in pupae and adults, which contrasts with AeHKT that is expressed primarily in larvae. Our results suggest that the physiological requirements of mosquitoes and the interaction between the mosquito and its host appear to be the driving force in mosquito AGT evolution.

Key words: Aedes aegypti, alanine glyoxylate aminotransferase, Drosophila melanogaster, 3-hydroxykynurenine, 3-hydroxykynurenine transaminase, mosquito.

Abbreviations used: AGT, alanine glyoxylate aminotransferase; AeAGT, Aedes aegypti AGT; DrAGT, Drosophila melanogaster AGT; hAGT, human AGT; 3-HK, 3-hydroxykynurenine; HKT, 3-HK transaminase; AeHKT, Aedes aegypti HKT; AnHKT, Anopheles gambiae HKT; HTS, high-titre viral stock; LC–MS/MS, liquid chromatography tandem MS; ORF, open reading frame; PLP, pyridoxal 5-phosphate; RACE, rapid amplification of cDNA ends; XA, xanthurenic acid.

To whom correspondence should be addressed (email lij@vt.edu).

The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number DQ182329.
hAGT and Drosophila AGT, we propose the possible functional differentiation and evolution of AeHKT and AeAGT.

EXPERIMENTAL

cDNA cloning and sequencing

The Ae. aegypti EST (expressed sequence tag) (aest_4508, http://www.tigr.org/db/db2k1/aabe/) was used to design a forward primer and a reverse primer. PCR amplification was then performed using a template of first-strand cDNA synthesized from total RNA of Ae. aegypti larvae and pupae (Clontech) and a 730 bp fragment was obtained and sequenced. To clone the full-length AeAGT cDNA, a 5'-RACE (5'-rapid amplification of cDNA ends) reaction was performed using an available kit (Invitrogen), which resulted in the cloning of a full-length cDNA.

Preparation of polyclonal antibodies and Western-blot analysis

To produce antibodies against AeAGT, its recombinant protein was expressed in a bacterial expression system, pET21b (Novagen). A pair of gene-specific primers (forward: 5'-CATATG-TGTCCTCAGTGCCGGTCTG-3'; reverse: 5'-AAGCTTCTGGACTCGTTTCCAC-3') containing specific restriction sites (underlined nucleotides) was used to amplify a full-length AeAGT cDNA that was subsequently ligated into the pET21b vector. The recombinant pET21b was transformed into Escherichia coli BL21 (DE3) cells and the recombinant protein that was produced in inclusion bodies was solubilized with a denaturant buffer [20 mM phosphate containing 8 M urea, 500 mM NaCl, 20 mM imidazole, pH 7.4, and TBST (25 mM Tris/HCl, 125 mM NaCl and 0.1 % Tween 20, pH 8.0)] and purified using an Ni²⁺-charged His-Bind resin column (1.5 cm × 25 cm; Novagen) according to the manufacturer’s instructions. The affinity-purified protein was further separated by SDS/PAGE and the recombinant protein was excised, eluted from the gel slices, dialysed against 20 mM Tris/HCl and used as antigen to generate anti-AeAGT polyclonal antibodies in a rabbit (Immunological Resource Center, University of Illinois).

For Western-blot analysis, crude proteins from larvae at 1–5 days after hatching, pupae at 0.5 and 12 h after pupation, and adults at 3 days after emergence, were extracted by homogenization in 20 mM potassium phosphate buffer (pH 7.0) containing 1 µM PMSF, 1 mM 1-phenyl-2-thiourea and 5 mM EDTA using a Polytron homogenizer. The homogenates were centrifuged at 13000 g for 10 min at 4°C to obtain soluble proteins that were analysed using SDS/PAGE. Protein concentration was determined by a Bio-Rad protein assay kit using BSA as a standard, and equal amounts of crude proteins were separated on the same gel. After electrophoresis, the proteins were transferred on to a nitrocellulose membrane with a transfer buffer [25 mM Tris and 192 mM glycine in 20 % (v/v) methanol]. The membrane then was blocked by incubation in 1 % BSA solution for 2 h at room temperature (22°C) and then incubated with rabbit anti-recom-
0.8 M formic acid. Supernatant of the reaction mixture, obtained by centrifugation at 15 000 g for 10 min at 4°C, was analysed by HPLC with UV detection at 330 and 340 nm for kynurenic acid and XA respectively.

For other aminotransferase activity assays, an assay mixture, containing 10 mM specific amino acids, 5 mM glyoxylate or pyruvate (to test glycine), 40 µM PLP, 100 mM potassium phosphate buffer (pH 7.5), and 1 µg of enzyme in a total volume of 50 µl, was made and it was incubated for 5 min at 45°C. Using HPLC with electrochemical detection, the product was quantified based on the detection of OPT (o-phthalaldehyde thiol)–amino acid product conjugate after the corresponding reaction mixtures were derivatized by OPT reagent [20]. The kinetic parameters of the recombinant enzymes towards the amino acid or α-oxo acid were calculated by fitting the Michaelis–Menten equation to the experimental data using the Enzyme Kinetics Module in SigmaPlot 8.2 (SPSS Science).

Protein concentration was determined using a Bio-Rad protein assay kit with BSA as a standard.

To determine the effect of pH and temperature on AeAGT and hAGT activity, a buffer mixture consisting of 50 mM phosphate and 50 mM boric acid was prepared and the pH of the buffer was adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0 respectively. The buffer mixture was selected to maintain a consistent buffer composition and ionic strength, yet have sufficient buffering capacity for a relatively broad pH range. To test the enzyme activity change at different temperatures, AGT activity was assayed under the same conditions except for the change in temperatures.

RESULTS

Cloning and analysis of AeAGT cDNA

The AeAGT homologous fragment (Ae. aegypti EST sequence, aaest_804) was identified by a BLAST search of the TIGR (The Institute for Genomics Research; Rockville, MD, U.S.A.) Ae. aegypti BAC (bacterial artificial chromosome) ends sequence database with hAGT and DrAGT (Drosophila melanogaster AGT). This AGT homologous fragment matched the C-terminal region of hAGT and DrAGT. A partial 970 bp cDNA fragment was amplified from a first-strand larval/pupal cDNA using specific primers based on the C-terminal sequence of the Ae. aegypti EST sequence and then sequenced. Extension of the 970 bp fragment using a 5’-RACE method led eventually to the isolation of its full-length cDNA that contained 1369 nt with an ORF of 1179 nt coding for 393 amino acids (GenBank® accession no. DQ182329) (Figure 1). BLAST analysis of the deduced sequence revealed a 48% identity with AeHKT [3], 58% identity with DrAGT [3,21] and 45–46% identity with AGTs from humans and mammals. By BLAST searching of the NCBI protein databases (http://www.ncbi.nlm.nih.gov:80/BLAST/Blast.cgi), it was determined that An. gambiae also has two AGT isoenzymes, one of which recently has been identified as an An. gambiae HKT (AnHKT) with the same function as AeHKT [22]. Cyanobacteria, archaea, yeast, plants, other insects (fruit fly and honeybee), frog, fish, rat, mouse and human have only one AGT. AeAGT and Anopheles AGT have 79% sequence identity, with both of them in the same group as DrAGT in the phylogenetic tree (Figure 2), suggesting that mosquito AGT might be a typical dipteran insect AGT.

Dr. melanogaster is a tractable system for studying conserved aspects of eukaryotic gene function and, with the production of other insect genome sequences, it is a useful baseline for evolutionary studies of gene organization [23]. Whole-genome sequence is available for Dr. melanogaster, Ae. aegypti and An. gambiae, the latter of which diverged from Dr. melanogaster approx. 250 million years ago [24], but the assembly is only available...
for *Dr. melanogaster* and *An. gambiae* (URL: http://www.ensembl.org/index.html). Therefore we used AGTs from *An. gambiae* for a comparative analysis of chromosomal localization and gene organization in an attempt to determine mosquito AGT evolution and function. The chromosomal localization of the two AGTs from *An. gambiae* is quite different (AnAGT: Chromosome 2R; AeHKT: Chromosome 3L), suggesting that they may function differently in mosquitoes. By analysing the gene organization of AGTs from *An. gambiae*, fruit fly and humans, we observed that both *Anopheles* AGT and DrAGT have three exons, which provides partial support for the hypothesis that mosquito AGT and DrAGT might have a common evolutionary origin.

**Expression profile of AeAGT during development**

Using Western-blot analysis, AeAGT protein was detected only in the pupal and adult stages of *Ae. aegypti* (Figure 3a). Further analysis showed that AeAGT was present in both the head and abdomen of adult females (Figure 3b). AeHKT was present in larvae [3], but no larval protein was positively stained using the anti-AeAGT antibodies in Western blot, suggesting that antibodies against AeAGT do not cross-react with AeHKT.

**Recombinant AeAGT and hAGT protein production, purification and identification**

When soluble protein samples from Sf9 cells, collected on the third day after viral inoculation, were analysed by SDS/PAGE, a major protein band with a relative molecular mass of 42 kDa was observed in the AeAGT recombinant virus-infected cells (results not shown). A similar size protein band also was observed in the hAGT recombinant virus-infected cells. Purification of the recombinant AeAGT and hAGT was achieved by DEAE-Sepharose, phenyl-Sepharose, hydroxyapatite and gel-filtration chromatographies. The purified AeAGT was trypsin-digested and then analysed by LC–MS/MS (liquid chromatography tandem MS) with an ion search of the spectral data using ProteinLynx software to verify the AeAGT identity of the recombinant protein (Figure 4). Recombinant hAGT was verified by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) (results not shown).

**Substrate specificity and kinetic parameters**

The recombinant protein was tested for AGT activity using alanine and glyoxylate as the amino donor and acceptor respectively, and it displayed relatively high AGT activity. Under the applied assay conditions, the recombinant AeAGT showed a specific activity of 0.5 μmol·s⁻¹·mg of protein⁻¹. When 3-HK or kynurenine was used as the amino donor and glyoxylate as the amino acceptor, transamination of 3-HK or kynurenine was not detected. The possibility that the enzyme catalysed the transamination of the other 19 proteogenic amino acids, aminoadipate and aminobutyrate in the presence of glyoxylate or pyruvate was also tested. The recombinant protein was only able to catalyse the transamination of serine (0.02 μmol·s⁻¹·mg⁻¹), histidine (0.02 μmol·s⁻¹·mg⁻¹) and glycine (0.02 μmol·s⁻¹·mg⁻¹) with pyruvate as the amino group acceptor. The same method was also applied for the recombinant hAGT protein. The hAGT showed a high specific AGT activity and also displayed considerable activities towards kynurenine, 3-HK, aminobutyrate, methionine and valine. The detailed results are shown in Figure 5. Table 1 lists the kinetic parameters of the AeAGT and hAGT and the previously published results for DrAGT and AeHKT [21]. Both AeHKT and hAGT show activity to 3-HK and kynurenine; in contrast, DrAGT and AeAGT do not have any detectable activity towards them using the same method. The substrate specificity results show that AeAGT is similar to DrAGT, and AeHKT is similar to hAGT, but the former has a much greater efficiency in mediating the 3-HK to XA pathway.

**Effect of pH and temperature on AeAGT and hAGT**

When the phosphate and borate buffer mixture, adjusted to pH 6.0–10.0, was used to prepare the enzyme/alanine/glyoxylate reaction mixtures, both AeAGT and hAGT showed little activity at pH 6.0. However, high activity levels were noted at pH 7.0–10.0, reaching the highest at pH 9.0. Both enzymes showed resistance to heat, but AeAGT, which displayed high activity even at 80°C, was more resistant to heat than hAGT (Figure 6). Comparison of the temperature and pH-related activity profiles of AeAGT, hAGT, DrAGT and AeHKT again show that AeAGT is similar to DrAGT and AeHKT to hAGT.

**DISCUSSION**

AGT has been studied in a number of model species. Disruption of its function affects the conversion of glyoxylate into glycine, which may lead to lethal conditions in humans [15,16], and so AGT studies have emphasized its role in the glyoxylate to glycine pathway. Because concrete evidence for the essential role of AGT in maintaining physiological conditions in living organisms has been demonstrated in some species [14–16], it is reasonable to suggest that AGT is indispensable in other animal species as well. Results obtained in the present study that investigated the molecular regulation and biochemical function of AeAGT, in conjunction with results of our previous experiments studying the biochemical characterization of AeHKT [3,21], suggest that AGT is essential in mosquitoes. It seems apparent that the AeHKT protein, translated during larval development, is involved in the detoxification of 3-HK and glyoxylate metabolism, whereas AeAGT, translated in pupae and adults, is primarily responsible for glyoxylate metabolism.

Characterization of AeHKT was initiated following the determination that the transamination of 3-HK to XA was a major pathway in mosquitoes. In mammals, 3-HK either can be hydrolysed by kynureninase to 3-hydroxanthranilic acid that can be completely oxidized to CO₂ and water through a complicated biochemical pathway or it can be used to synthesize NAD(P)⁺ [25]. Kynurenine to 3-HK is the major pathway in mosquitoes, but...
Mosquito alanine glyoxylate aminotransferase

Figure 4 Verification of recombinant AeAGT by peptide MS

Purified recombinant protein was digested with trypsin, the tryptic peptides were analysed by LC–MS/MS, and collected spectral data were searched against an in-house mosquito protein database. (A) Precursor ions (MS) collected during LC–MS/MS analysis of tryptic-digested protein. Peaks in boldface are those matched to AeAGT sequence. (B) The tandem spectrum (MS/MS) of the doubly charged 714.826 peptide ion from (A) and the derived amino acid sequence. (C) Coverage map shows unambiguous match (boldface) of the spectral data to AeAGT. These data provide evidence that the expressed recombinant protein is AeAGT.

because they do not have kynureninase, the hydrolysis pathway for 3-HK is blocked. 3-HK is a natural metabolite, but it is oxidized easily under physiological conditions, stimulating the production of reactive oxygen species [26–28]. To prevent the accumulation of 3-HK, mosquito larvae efficiently convert the chemically reactive 3-HK into the chemically stable XA [2]. Purification of the protein with 3-HK transamination activity resulted in the isolation of a protein sharing high similarity with mammalian AGT [3], which suggests that the primary function of this mosquito AGT, AeHKT, has expanded to include the detoxification of 3-HK.

In a previous study, we compared substrate specificity between AeHKT and DrAGT and our results showed that DrAGT has high AGT activity and is able to catalyse the transamination of a few other amino acids, but it showed no activity towards 3-HK and kynurenine [21]. The BLAST search analysis clearly indicated that Dr. melanogaster has only one AGT. We interpreted the functional difference between AeHKT and DrAGT based on the difference in their food resources. Mosquito larvae are aquatic and their food resources primarily include protein-rich micro-organisms. In mosquito larvae, a considerable portion of tryptophan, obtained from food supplies, is oxidized to 3-HK [2]. Because mosquitoes cannot dispose of 3-HK through hydrolysis and subsequent oxidation like mammals, the transamination of the chemically reactive 3-HK to the relatively chemically stable XA by AeHKT is considered to be the mechanism by which mosquitoes prevent the over accumulation of 3-HK [3]. In contrast, Dr. melanogaster obtains its food from plant resources that are rich in glycolate, the precursor of glyoxylate; therefore we propose that it is more important to prevent the accumulation
Transamination activity of AeAGT and hAGT towards different amino acids

Glyoxylate was used as the amino group acceptor for all amino acids, except for glycine. When glycine was used as amino group donor, pyruvate was used as an amino group acceptor. Left panel: transamination activity of AeAGT; right panel: transamination activity of hAGT.

Table 1 Kinetic parameters of AeAGT and hAGT towards alanine, kynurenine, 3-HK (DL form) and comparison with previously reported parameters for AeHKT and DrAGT [21]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt; · s&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<tr>
<td>AeAGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>34 ± 10</td>
<td>117 ± 15</td>
<td>3450</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>5.4 ± 1.7</td>
<td>4.3 ± 0.8</td>
<td>800</td>
</tr>
<tr>
<td>3-HK</td>
<td>9.5 ± 3.9</td>
<td>4.4 ± 1</td>
<td>450</td>
</tr>
<tr>
<td>hAGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>15 ± 2</td>
<td>53 ± 3</td>
<td>3483</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>3-HK</td>
<td>4.3 ± 0.8</td>
<td>800</td>
</tr>
<tr>
<td>AeHKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>18 ± 2</td>
<td>20 ± 1</td>
<td>1117</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>3.7 ± 0.2</td>
<td>12 ± 3.7</td>
<td>3250</td>
</tr>
<tr>
<td>3-HK</td>
<td>2.6 ± 0.6</td>
<td>34.2 ± 5.3</td>
<td>13150</td>
</tr>
<tr>
<td>DrAGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>149 ± 44</td>
<td>131 ± 20</td>
<td>879</td>
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</tbody>
</table>

Interestingly, 3-HK is also the initial precursor for the production of ommochromes that are major eye pigments in mosquitoes. Compound eye development and eye pigmentation occur mainly during the pupal and early adult stages. Coincidently, HKT activity is diminished in pupae and adults with the accumulation of high levels of 3-HK in the compound eyes [29]. The down-regulation of AeHKT is likely to be a tactic for mosquitoes to allow 3-HK to be transported and used for eye pigmentation. Although it seems clear that detoxification of 3-HK is a major function of AeHKT in mosquito larvae, the enzyme has high AGT activity and probably plays a critical role in glyoxylate metabolism as well. Consequently, the interruption of AeHKT expression during the pupal and adult stages would affect the metabolism of glyoxylate in pupae and adults and provides an explanation as to why the highly substrate-specific (i.e. highly active for the glyoxylate to glycine pathway without detectable HKT activity) and stage-specific AeAGT has evolved in mosquitoes.

The high sequence identity of AeAGT and AeHKT with the AGTs from cyanobacteria, archaea, yeast, plants, other insects (fruit fly and honeybee), frog, fish, rat, mouse and humans suggests that these enzymes have evolved from a common ancestry. The CLUSTAL W alignment and phylogenetic analysis (Figure 2) show that AeAGT seems to be more closely related to DrAGT.
than AeHKT. Based on substrate specificity, pH and temperature-dependent activity profiles, it seems clear that AeHKT is more closely related to hAGT. The primary function of AeHKT seems to have deviated substantially from the typical dipteran insect AGT [3]. Although AeHKT and AeAGT have significant sequence identity with mammalian AGTs (Figure 7), AeHKT shares similar substrate specificity and other characteristics with hAGT. For example, both hAGT and AeHKT [21] can catalyse the transamination of 3-HK, kynurenine and a number of other amino acids under exactly the same assay conditions, thereby confirming a previous report that showed that native hAGT, purified from human liver, had activity towards 3-HK and kynurenine [30].

Mammalian AGT is localized either in mitochondria or in peroxisomes, which is dictated by the presence of the N-terminal mitochondrial or C-terminal peroxisomal signal peptide in the protein. The localization of AGT provides useful evidence in predicting the metabolic roles of AGT in mammals and other vertebrates. Using online bioinformatic tools, AeAGT and DrAGT are predicted to be peroxisomal proteins (URL: http://mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp) and AeHKT is predicted to be a mitochondrial protein (URL: http://ihg.gsf.de/ihg/mitoprot.html). Based on the respective metabolic roles of mammalian peroxisomal and mitochondrial AGTs, it seems reasonable to suggest that AeAGT and DrAGT may be involved in the metabolism of glycolate-derived glyoxylate, whereas AeHKT is involved in converting hydroxypyrolone-derived glyoxylate into glycine in addition to its function in the transamination of 3-HK to XA.

Differences in enzyme function, in principle, should be reflected in both the primary and three-dimensional structures. The three-dimensional structures for hAGT [31], yeast AGT [32], Nostoc AGT [33] and AnHKT [34] are available. Based on the primary sequences of AeAGT, AeHKT, AnHKT and DrAGT in comparison with hAGT, yeast AGT, Nostoc AGT and AnHKT sequences and their corresponding three-dimensional structures, all residues involved in PLP-binding sites in those sequences are conserved (Figure 7). Other than the PLP-binding sites, there are seven substrate-binding sites and five of them are conserved (Figure 7). The fourth binding residues are serine in hAGT and asparagine in AnHKT and AeHKT, but the binding site is histidine in the other four AGTs (Figure 7). The fifth binding residue is methionine in hAGT, glutamic residue in AnHKT and AeHKT.
but the fifth binding residue becomes serine in AeAGT and DrAGT. The difference in substrate-binding sites may explain the substrate specificities of different AGTs. A three-dimensional structural study is ongoing for DrAGT, AeAGT and AeHKT in our laboratory (Q. Han, H. Robinson, Y. G. Gao and J. Li, unpublished work). We have produced crystals for all three enzymes and diffraction data for AeAGT and AeHKT, as it will be beneficial to have additional evidence to analyse mosquito AGT evolution and to understand the mechanism of catalysis.

Both AeHKT and AeAGT, if determined through random cloning or a genome project, would have been listed as AGTs based on their high sequence identity with mammalian AGTs, and their assigned functional identity also would have been assumed because of the level of their sequence identity with mammalian AGTs (see Figure 7). Primary sequence comparison of AeHKT and AeAGT with mammalian AGTs is insufficient to determine if there is any functional difference between the two Aedes aegypti transaminases and the mammalian AGTs, let alone determine the functional difference between the two mosquito AGTs. However, biochemical characteristics and the molecular regulation of these two enzymes during mosquito development, in conjunction with the physiological requirements of mosquito development, clearly demonstrate the functional differences between AeHKT and AeAGT and provide a reasonable explanation as to why two proteins with AGT functions have evolved in mosquitoes. Based on the substrate specificities of the characterized hAGT, AeAGT, AeHKT and DrAGT, AeHKT has a rather broad substrate specificity, followed in decreasing specificity by hAGT, DrAGT and AeAGT; consequently, this result provides additional insight towards a comprehensive understanding of the functional adaptation and evolution of AGTs in living organisms.

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