MBE Advance Access published March 8, 2012

# Multiple Adaptive Losses of Alanine-Glyoxylate Aminotransferase Mitochondrial Targeting in Fruit-Eating Bats

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**Associate editor:** Michael Nachman

## Abstract

The enzyme alanine-glyoxylate aminotransferase 1 (AGT) functions to detoxify glyoxylate before it is converted into harmful oxalate. In mammals, mitochondrial targeting of AGT in carnivorous species versus peroxisomal targeting in herbivores is controlled by two signal peptides that correspond to these respective organelles. Differential expression of the mitochondrial targeting sequence (MTS) is considered an adaptation to diet-specific subcellular localization of glyoxylate precursors. Bats are an excellent group in which to study adaptive changes in dietary enzymes; they show unparalleled mammalian dietary diversification as well as independent origins of carnivory, frugivory, and nectarivory. We studied the AGT gene in bats and other mammals with diverse diets and found that the MTS has been lost in unrelated lineages of frugivorous bats. Conversely, species exhibiting piscivory, carnivory, insectivory, and sanguinivory possessed intact MTSs. Detected positive selection in the AGT of ancestral fruit bats further supports adaptations related to evolutionary changes in diet.

**Key words:** Chiroptera, alanine-glyoxylate aminotransferase, mitochondrial targeting sequence, diet, adaptive evolution.

The liver enzyme alanine-glyoxylate aminotransferase 1 (AGT) has an important role in detoxifying glyoxylate, an intermediate metabolite that would otherwise be converted to the harmful oxalate (Ichiyama 2011). In carnivorous mammals, the precursor of glyoxylate is hydroxyproline, which occurs in the mitochondria (Takayama et al. 2003), whereas in herbivores, the main precursor is glycolate, which accumulates in peroxisomes (Noguchi 1987). As an adaptive consequence of the need to detoxify glyoxylate rapidly, AGT has evolved organelle-specific targeting among taxa according to dietary specializations. In carnivores, AGT is mostly mitochondrial; in herbivores, it is mostly peroxisomal; and in omnivores, it targets both organelles equally (Danpure 1997). Mistargeting of AGT in humans is linked to the disease primary hyperoxaluria type 1, whereby insoluble oxalate forms kidney stones (Danpure et al. 1989).

AGT compartmentalization has been attributed both to a mitochondrial targeting sequence (MTS) in the N-terminus and a peroxisomal targeting sequence type 1 (PTS1) in the three C-terminal amino acids (Danpure 1997). Expression of the MTS, which overrides the function of PTS1, is controlled by an upstream 5′ transcription start site, followed by a 5′ translation start site and second 3′ transcription start site contained within the signal peptide itself, and a downstream 3′ translation start site adjacent to the MTS (Oatey et al. 1996). Most omnivorous mammals express AGT isoforms with and without the MTS, with associated targeting of both organelles (Oda et al. 1990). However, in herbivorous species, such as the rabbit, the first translation start site has been lost, which means that the MTS is not expressed and only peroxisomes are targeted (Purdue et al. 1992). In contrast, in the cat, all AGT proteins contain the MTS due to a loss of the 3′ transcription start site, so that targeting is predominantly mitochondrial (Lumb et al. 1994).

Ancestral bats are thought to have been insectivorous (Gunnell and Simmons 2005); however, modern bats collectively exhibit the widest range of diets of any mammalian order. Different species specialize on insects, large arthropods, small vertebrates, blood, nectar, fruits, and leaves (Kunz and Fenton 2003), and frugivory, nectarivory, and carnivory have all evolved independently several times (Kunz and Fenton 2003). This convergence in diet is best exemplified by nectar and fruit eating members of the two families, Pteropodidae (Old World fruit bats) and Phyllostomidae (New World fruit bats), which in turn belong to the suborders Yinpterochiroptera and Yangochiroptera, respectively (Springer et al. 2001).

Here, we obtained and analyzed new AGT gene sequences from a taxonomically wide range of bats with varied diets and compared these with published data from other mammals. Our examination of the MTS portion of the AGT gene indicated that all focal bat species that prey exclusively on either invertebrates or vertebrates (including the sanguinivorous vampire bat) have retained MTS. In contrast, mitochondrial targeting appears to have been lost at least twice in fruit-eating bats (fig. 1). In the Phyllostomidae, substitutions in the 5′ translation start sites were







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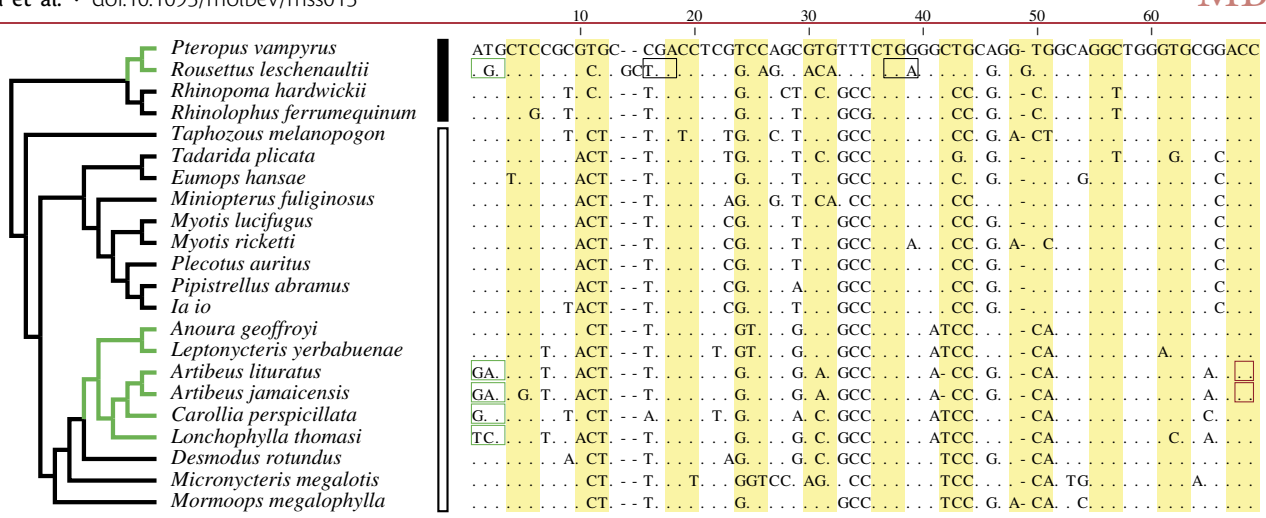


**FIG. 1.** Sequence alignment of bat MTSs. Indicated are nonfunctional MTSs caused by mutations in 5′ translation start sites (green boxes), stop codons in *Rousettus leschenaultii* (black boxes), and frame shifts in *Artibeus* (brown boxes). Phylogenetic relationships are shown with frugivorous lineages in green. Note: *Micronycteris* is insect-dominated omnivorous. The two suborders Yinpterochiroptera (black bar) and Yangochiroptera (white bar) are also indicated.

observed in species of the genera *Lonchophylla*, *Carollia*, and *Artibeus*. In the latter, a 1 bp deletion was also detected at position 42. More dramatic mutations were detected in the MTS of the Old World fruit bat *Rousettus leschenaultii*, with a substitution in the 5′ translation start site and two insertions at positions 14 (2 bp) and 49 (1 bp), the former causing two downstream stop codons. From these results, we conclude that unrelated frugivorous bats have undergone parallel losses of mitochondrial targeting of AGT and that the resulting peroxisomal switch is likely to be an adaptive response to these species' diets (also see Purdue et al. 1992; Holbrook et al. 2000; Birdsey et al. 2004). Perhaps surprisingly, however, not all frugivorous bats showed the altered 5′ translation start sites; based on data available, some members of the main groups of fruit bats appeared to possess functional MTSs (fig. 1). This finding is interesting given some reports of insectivory in Old World (Barclay et al. 2006; Clulow and Blundell 2011) and New World fruit bats (Rex et al. 2010; Monteiro and Nogueira 2011), though peroxisomal AGT seen in *Pteropus* (Birdsey et al. 2005) casts doubt on the extent of mitochondrial targeting.

Across all mammals, we found evidence of nine separate losses of the 5′ translation start site in MTS, mostly in herbivorous species, including previously unreported losses in the hyrax and pika (fig. 2). As described before (Holbrook et al. 2000), primates appear to buck this trend, with losses of the translated MTS in large omnivorous species, including humans and chimpanzees, yet retention in others such as the orangutan. To obtain additional information on the targeting efficiency of putative intact MTS domains in bats, we undertook computational prediction of AGT mitochondrial subcellular localization and compared our results with published values from carnivores (Birdsey et al. 2004). We found that the level of mitochondrial targeting is likely to differ widely among bats (supplementary table S1, Supplementary Material online). In fact, the lowest recorded value for bats, the nectarivorous *Leptonycteris yerbabuenae* (−1.94), was below that of the giant panda (Birdsey et al. 2004), consistent with strong peroxisomal targeting in a diet derived predominantly from plants. At the other extreme, the highest value for a bat (2.46) was inferred for the piscivorous species *Myotis ricketti*. To date, experimental data on the subcellular distribution of AGT in bats and other mammals are limited; nevertheless, peroxisomal localization in three frugivorous bat species (*Carollia perspicillata* and the genera *Pteropus* and *Cynopterus*) (Birdsey et al. 2005) supports our findings (fig. 2).

By sequencing the mature peptide region (non-MTS data set) in a subset of taxa, we were able to test whether the AGT gene has undergone adaptive evolution in bats. Two branch wise episodes of positive selection, in which $\omega$ values (nonsynonymous substitution rate/synonymous substitution rate) exceed one significantly, were detected in bats: in the ancestral branch of the Old World fruit bats and the ancestral branch of the suborder Yangochiroptera (table 1 and supplementary fig. S1, Supplementary Material online). Different sets of amino acids appear to have been involved in these episodes, suggesting that each case might have altered enzymatic activity to meet metabolic demands associated with respective diets based on fruits and insects. Selection tests undertaken for other key herbivorous mammals revealed significant positive selection only on the branch leading to the guinea pig (table 1 and supplementary fig. S1, Supplementary Material online). Finally, a phylogenetic network reconstructed from non-MTS data set showed a clear split grouping Old World fruit bats with rodents and the rabbit (supplementary fig. S2*A*, Supplementary Material online), differing from the species tree (supplementary fig. S2*B*, Supplementary Material online). Given that both modern Glires and their fossil relatives are associated with herbivory (Meng et al. 2003), this result lends further support for a link between AGT sequence evolution and diet.

## Materials and Methods

Bat AGT genes were amplified by polymerase chain reaction (PCR) from both genomic DNA and cDNA

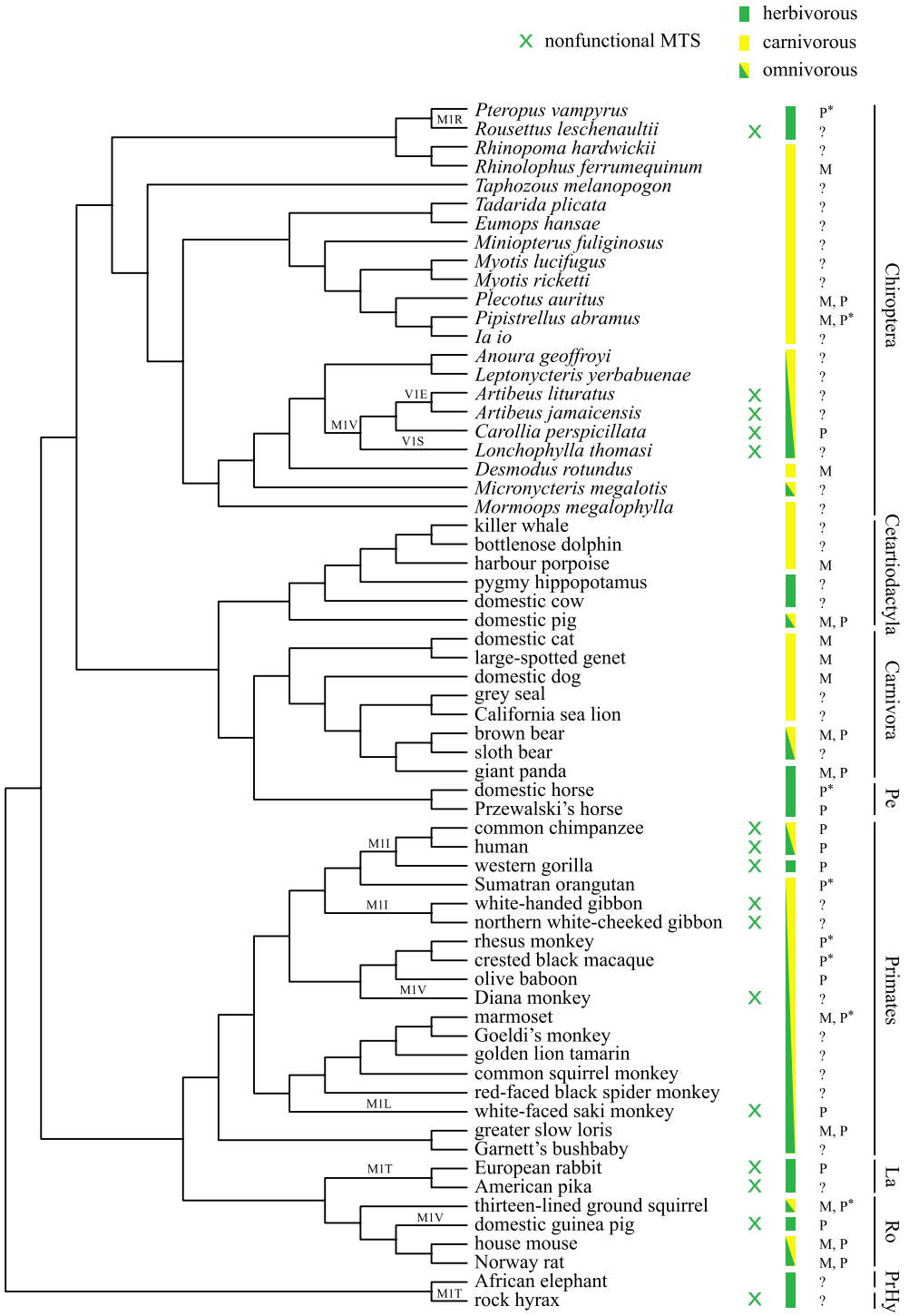


**FIG. 2.** MTS evolution in mammals. Species tree showing amino acid substitutions (position 1 of the 5′ translation start site) on the branches. Also shown are mammalian diets (Nowak and Paradiso 1983) and empirical subcellular distributions of AGT (M = mitochondrial, P = peroxisomal) (Birdsey et al. 2005). For the latter, * = data based on congeneric species and ? = unknown targeting. Taxonomic orders with abbreviations include: Perissodactyla (Pe), Lagomorpha (La), Rodentia (Ro), Proboscidea (Pr), and Hyracoidea (Hy).

samples. Detailed methods and PCR primers (supplementary table S2, Supplementary Material online) are given in supplementary material, Supplementary Material online. Additional mammalian AGT gene sequences were obtained from NCBI (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org). For details of taxa studied, see supplementary tables S3 and S4, Supplementary Material online. GenBank accession numbers of new sequences are JQ302758–JQ302790.

To study AGT sequence evolution, we undertook separate alignments of mammalian MTS and non-MTS sequences using ClustalW (Thompson et al. 1994) within MEGA 5 (Tamura et al. 2011). We reconstructed the evolutionary history of the MTS using maximum-likelihood estimation

**Table 1.** Parameter Estimations from Lineages under Positive Selection.

| Focal Branch | Branch-Site Model A (Test 2) | Log-Likelihood | *P* Value | Parameters of Focal Branch | Sites under Positive Selection[a] |
|---|---|---|---|---|---|
| Ancestor of Old World fruit bats | Alternative hypothesis | −9,631.585 | 0.017 | $p_0 = 0.876$, $p_1 = 0.098$, $p_{2a} = 0.023$, $p_{2b} = 0.003$<br>$\omega_0 = 0.094$, $\omega_1 = 1$, $\omega_{2a} = 6.992$, $\omega_{2b} = 6.992$ | 16, 84, 133, 257, 276, 290 |
| | Null hypothesis | −9,634.438 | | $p_0 = 0.841$, $p_1 = 0.095$, $p_{2a} = 0.057$, $p_{2b} = 0.007$<br>$\omega_0 = 0.093$, $\omega_1 = 1$, $\omega_{2a} = 1$, $\omega_{2b} = 1$ | Not allowed |
| Ancestor of Yangochiroptera | Alternative hypothesis | −9,632.927 | 0.01 | $p_0 = 0.89$, $p_1 = 0.1$, $p_{2a} = 0.009$, $p_{2b} = 0.001$<br>$\omega_0 = 0.095$, $\omega_1 = 1$, $\omega_{2a} = 261.269$, $\omega_{2b} = 261.269$ | 123, 177, 184, 291, 340 |
| | Null hypothesis | −9,636.237 | | $p_0 = 0.831$, $p_1 = 0.094$, $p_{2a} = 0.067$, $p_{2b} = 0.008$<br>$\omega_0 = 0.094$, $\omega_1 = 1$, $\omega_{2a} = 1$, $\omega_{2b} = 1$ | Not allowed |
| Guinea pig | Alternative hypothesis | −9,630.793 | 0.004 | $p_0 = 0.88$, $p_1 = 0.103$, $p_{2a} = 0.015$, $p_{2b} = 0.002$<br>$\omega_0 = 0.093$, $\omega_1 = 1$, $\omega_{2a} = 47.258$, $\omega_{2b} = 47.258$ | 7, 107, 241 |
| | Null hypothesis | −9,634.927 | | $p_0 = 0.86$, $p_1 = 0.1$, $p_{2a} = 0.037$, $p_{2b} = 0.004$<br>$\omega_0 = 0.093$, $\omega_1 = 1$, $\omega_{2a} = 1$, $\omega_{2b} = 1$ | Not allowed |

[a] The underlines represent the sites with a posterior probability higher than 95%.

by CODEML in PAML 4 (Yang 2007) based on the species tree (see supplementary material, Supplementary Material online). Subcellular targeting of functional bat MTS was predicted using PSORT II (Nakai and Horton 1999). To test for positive selection associated with herbivorous dietary specialization in major lineages of bats and other mammals, we analyzed the non-MTS data set using a modified branch-site model A (test 2) implemented in CODEML (Zhang et al. 2005). All branches tested are shown in supplementary fig. S2*B*, Supplementary Material online. To assess further phylogenetic relationship of mammalian AGT sequences, we undertook a phylogenetic network reconstruction (neighbor-net method) based on amino acid distances (uncorrected P) using SplitsTree 4 (Huson and Bryant 2006).

## Supplementary Material

Supplementary methods, tables S1–S4, and figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

## Acknowledgments

We thank Fanxing Meng and Naijian Han for technical help in the lab. Our work was supported by the Key Construction Program of the National "985 Project" and "211 Project" to S.Z., a Ministry Reward for Excellent Doctors in Academics (MXRZZ2010010) to Y.L., and a Royal Society Fellowship and a Biotechnology and Biological Sciences Research Council partnering grant to S.J.R.

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