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A comparative analysis of the evolutionary relationship between diet and enzyme targeting in bats, marsupials and other mammals

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The subcellular distribution of the enzyme alanine : glyoxylate aminotransferase (AGT) in the livers of different mammals appears to be related to their natural diets. Thus, AGT tends to be mitochondrial in carnivores, peroxisomal in herbivores, and both mitochondrial and peroxisomal in omnivores. To what extent this relationship is an incidental consequence of phylogenetic structure or an evolutionarily meaningful adaptive response to changes in dietary selection pressure is unknown. In order to distinguish between these two possibilities, we have determined the subcellular distribution of AGT in the livers of 22 new mammalian species, including members of three orders not studied before. In addition, we have analysed the statistical relationship between AGT distribution and diet in all 77 mammalian species, from 12 different orders, for which the distribution is currently known. Our analysis shows that there is a highly significant correlation between AGT distribution and diet, independent of phylogeny. This finding is compatible with the suggestion that the variable intracellular targeting of AGT is an adaptive response to episodic changes in dietary selection pressure. To our knowledge, this is the first example of such a response being manifested at the molecular and cellular levels across the breadth of Mammalia.

Keywords: molecular adaptation; comparative method; alanine : glyoxylate aminotransferase; dietary selection pressure; mitochondrial protein targeting; peroxisomal protein targeting

1. INTRODUCTION

The liver-specific pyridoxal-phosphate-dependent enzyme alanine : glyoxylate aminotransferase I (AGT, Enzyme Commission (EC) 2.6.1.44) catalyses the transamination of the intermediary metabolite glyoxylate to glycine. This is effectively a detoxification reaction, because glyoxylate, although not necessarily harmful in itself, can be readily oxidized to the metabolic end product oxalate. The effect of too much oxalate can be readily seen in the human autosomal recessive disease primary hyperoxaluria type 1 (PH1), which is caused by a deficiency of AGT. In PH1, the failure to detoxify glyoxylate results in increased oxalate synthesis and urinary excretion and the build up of insoluble calcium oxalate in the kidney and urinary tract. This eventually leads to kidney failure and death (Danpure 2001).

In evolutionary terms, AGT is highly unusual because, unlike almost all other enzymes in the cell, it is targeted to different intracellular compartments in different species. In some mammals AGT is peroxisomal, in others it is mitochondrial, and in yet others it is both peroxisomal and mitochondrial (Danpure *et al.* 1990, 1994). In some species, significant amounts of AGT are also found in the cytosol (Birdsey & Danpure 1998). Analysis of 55 different species indicates that the compartmentalization of AGT in mammals is not random but is instead related to diet. Thus, for example, herbivores tend to have peroxisomal AGT, carnivores mitochondrial AGT and omnivores both peroxisomal and mitochondrial AGT (Danpure *et al.* 1994; Holbrook *et al.* 2000; Birdsey *et al.* 2004).

There is a good metabolic reason why the subcellular distribution of AGT might be related to diet. In order to detoxify glyoxylate efficiently, AGT must be located at the site of glyoxylate synthesis. This site is predicted to be related to diet. In herbivores, the main dietary precursor of glyoxylate is thought to be glycolate (Noguchi 1987), whereas in carnivores it is thought to be hydroxyproline (Ichiyama *et al.* 2000). Glycolate is converted to glyoxylate in the peroxisomes, catalysed by glycolate oxidase. On the other hand, the last step in the conversion of hydroxyproline to glyoxylate, catalysed by 4-hydroxy-2-ketoglutarate aldolase (Takayama *et al.* 2003), occurs in the mitochondria. Thus, the best place for AGT to be is in the peroxisomes in herbivores and mitochondria in carnivores.

In all of the mammals studied so far, AGT is encoded by a single copy gene that can encode a polypeptide varying from 392 to 414 amino acids (Oda *et al.* 1987; Takada *et al.* 1990; Purdue *et al.* 1991; Mori *et al.* 1992; Purdue *et al.* 1992; Lumb *et al.* 1994; Birdsey & Danpure 1998; Holbrook & Danpure 2002). The archetypal AGT gene, as found in the rat or marmoset, for example, has the potential to encode an N-terminal mitochondrial targeting sequence (MTS) of 22 amino acids, and an atypical C-terminal type 1 peroxisomal targeting

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sequence (PTS) of three amino acids. There appears to be a hierarchical dominance of the MTS over the PTS, so that if present together, the former predominates (Oatey *et al.* 1996). The eventual localization of AGT appears to be dependent on whether or not the MTS is contained within the open reading frame and, therefore, present in the nascent polypeptide. Expression of the MTS appears to be dependent on the relative use of two transcription start sites and two in-frame translation start sites (Danpure 1997). In many species, including the rat and marmoset, both transcription and translation start sites have been maintained, so that two transcripts and two polypeptides are produced. The longer, containing the MTS and PTS, is targeted mainly to the mitochondria, whereas the shorter, containing only the PTS, is targeted only to the peroxisomes (Oda *et al.* 1990; Purdue *et al.* 1992). In some species, such as human, rabbit, guinea pig and saki monkey, the MTS is permanently excluded from the open reading frame by the evolutionary loss of the more 5′ translation start site (Takada *et al.* 1990; Purdue *et al.* 1992; Birdsey & Danpure 1998; Holbrook *et al.* 2000). In these animals, AGT is exclusively peroxisomal. In other species, such as the domestic cat, almost all nascent polypeptides contain an MTS owing to the loss of the more 3′ transcription start site (Lumb *et al.* 1994). AGT in the cat is almost entirely mitochondrial. In certain species, such as the giant panda, the efficiency of mitochondrial AGT targeting can be compromised by the accumulation of mutations within the MTS that interfere with its function, possibly by decreasing its positive charge or by weakening the α-helix (Birdsey *et al.* 2004).

Studies on the molecular evolution of AGT targeting in mammals have been dominated by three orders—Primates (Takada *et al.* 1990; Purdue *et al.* 1992; Holbrook *et al.* 2000), Rodentia (Oda *et al.* 1990; Birdsey & Danpure 1998) and Carnivora (Lumb *et al.* 1994; Birdsey *et al.* 2004). Analysis of dN/dS ratios in the region of the AGT gene encoding the putative MTS in Primates (Holbrook *et al.* 2000) and Carnivora (Birdsey *et al.* 2004) has suggested the presence of positive selection pressure to lose or diminish mitochondrial AGT targeting as diets in these orders have become more herbivorous. Whether such pressure has determined the AGT distribution in other orders is unknown.

Although the qualitative relationship between AGT distribution and diet is compelling, it has not been subjected to any statistical analysis, especially to determine whether any correlation is simply a reflection of phylogenetic structure. In order to do this across the breadth of Mammalia, it is important as many species from as many orders as possible are included in the analysis, but also that these species have as many different diets as possible. To this end we have, in the present paper, determined the subcellular distribution of AGT in 22 additional mammalian species, including members of three orders not studied before. In particular, we have extended our dataset for Chiroptera and Marsupialia. In addition, we have carried out comparative statistical analysis on the relationship between distribution and diet independently of phylogeny in all 77 mammals in which the distribution is known, using two different methods (Purvis & Rambaut 1995; Pagel 1997, 1999). The results show that AGT distribution is highly correlated with diet independently of phylogeny, a result that is compatible with our hypothesis that the variability of AGT distribution is an unparalleled adaptive response to episodic changes in dietary selection pressure.

2. METHODS

(a) *Data sources*

The 22 new liver samples were obtained from two sources. The bat species *Plecotus auritus*, *Pipistrellus pipistrellus* and *Eptesicus serotinus* were obtained from the Wildlife Veterinary Investigation Centre, Truro, UK. The remaining bats and other mammals were obtained from the pathology tissue archive, Zoological Society of London, London, UK. Samples of tissue were taken at routine necroscopy of animals that had been found dead, or from animals shortly after they had been euthanized on humane grounds.

Fresh liver tissue was fixed in 1% glutaraldehyde in phosphate buffer (pH 7.4). In all other cases, formalin-fixed wax-embedded specimens were recovered, dewaxed and infiltrated with LR white resin, as described previously (Lewin *et al.* 1995). Samples were embedded, sectioned, immunolabelled for immunoreactive AGT, and examined by immunoelectron microscopy (Lewin *et al.* 1995; Birdsey *et al.* 2004). AGT was immunolabelled using rabbit anti-human AGT anti-serum and 10 nm gold conjugated goat anti-rabbit IgG. For well-fixed specimens, mitochondria and peroxisomes were identified on the basis of their morphology—mitochondria being larger double-membrane limited organelles containing cristae, and peroxisomes being smaller ovoid organelles devoid of internal membranes, but frequently containing matrical para-crystalline cores and marginal plates. In poorly fixed specimens, organelle identification was confirmed by double-labelling using rabbit anti-peroxisomal (catalase) anti-serum and rabbit anti-mitochondrial anti-serum, followed by 20 nm gold conjugated goat anti-rabbit IgG (Birdsey *et al.* 2004). For some specimens, formal quantitative morphometry was carried out. Immunoelectron microscope images were captured using an Advanced Microscopy Techniques (AMT) Advantage CCD camera with Amt Image Capture software, v. 5.4.2 (Deben UK Ltd). Quantitative morphometry was performed using Sis Analysis software, v. 1.2. A total number of 158 fields were analysed, with an average of 7 per species (min = 2, max = 16). For each species, the average profile area of mitochondria analysed was 11.66 μm^2 (min = 3.45 μm^2, max = 25.43 μm^2); for peroxisomes the average profile area analysed per species was 1.83 μm^2 (min = 0.16 μm^2, max = 5.63 μm^2).

AGT subcellular distribution was placed into one of three categories: (i) mitochondrial, (ii) mitochondrial and peroxisomal or (iii) peroxisomal. We used the arbitrary criteria similar to those described previously (Birdsey *et al.* 2004), as follows:

(i) if $n\mathrm{M} \geq 10n\mathrm{P}$, *or* if $d\mathrm{M} \geq 10d\mathrm{P}$, then the distribution is 'mitochondrial';
(ii) if $n\mathrm{P} \geq 10n\mathrm{M}$, *or* if $d\mathrm{P} \geq 10d\mathrm{M}$, then the distribution is 'peroxisomal';
(iii) in all other circumstances, the distribution is 'peroxisomal and mitochondrial'.

(*n*, mean number of gold particles per whole organelle profile, *d*, mean organelle gold labelling density (particles per unit profile area), P, peroxisome, M, mitochondria.)

Levels of AGT immunoreactive protein and AGT enzyme activity for the comparative analysis were taken from primary

data, previously published in an abridged form (Danpure *et al.* 1994). Species diets, body mass and gestation length were taken from Nowak (1991).

(b) *Comparative analysis*

We made use of two methods for comparative analysis, the generalized least-squares model as implemented in the computer program CONTINUOUS (Pagel 1997, 1999) and the method of phylogenetically independent contrasts (Felsenstein 1985) using the computer program CAIC (Comparative analysis by independent contrasts, v. 2.6; Purvis & Rambaut 1995).

Owing to the difficulty of finding a phylogeny that included all the species studied, a composite tree was created based on that by Springer *et al.* (2003) at the supraordinal level, Osborne *et al.* (2002) for Marsupialia, Bininda-Edmonds *et al.* (1991) for Carnivora, Jones *et al.* (2002) for Chiroptera, Huchon *et al.* (2002) for Rodentia and Purvis (1995) for Primates. Because branch lengths were not available for some parts of the phylogeny, we assumed all branch lengths were equal.

For the purpose of these analyses, all trait characters were treated as continuous data. AGT subcellular distribution was classed as 1 = mitochondrial, 2 = mitochondrial + peroxisomal, 3 = peroxisomal. Levels of AGT immunoreactive protein were given values of 0 = very low, through to 3 = high levels. Species diets were categorized into five groups: 1 = carnivorous, 2 = carnivorous/omnivorous, 3 = omnivorous, 4 = herbivorous/omnivorous and 5 = herbivorous. AGT enzyme activity, body mass and gestation length were logarithmically transformed.

Comparative analysis of the data using the CONTINUOUS computer package was carried out using maximum likelihood analysis. Hypotheses for trait correlation were tested using the likelihood ratio statistic, with the chi-squared distribution used as an approximation.

Using the CAIC package, independent standardized linear contrasts of the trait characters were calculated using the Crunch algorithm. AGT subcellular distribution was used as the predictor variable in each analysis. Linear regression through the origin was used to statistically analyse the relationship between contrasts.

3. RESULTS

Using the technique of colloidal gold immunoelectron microscopy, we have determined the subcellular distribution of AGT in liver sections from 22 new mammalian species, including nine members of Chiroptera, six Marsupialia, two Rodentia and one each of Monotremata, Cetartiodactyla, Tubulidentata, Perissodactyla and Primates. The immunoelectron micrographs of some of these are shown in the Electronic Appendix to this paper. Of the nine newly studied bats, AGT was peroxisomal in the two herbivorous species, mitochondrial and peroxisomal in five of the carnivorous (carnivorous includes insectivorous in this paper) species, and mitochondrial in two of the carnivorous species, one of which was haematophagous. Of the six newly studied marsupials, AGT was peroxisomal in three of the herbivorous/omnivorous species, and mitochondrial and peroxisomal in one of the herbivorous/omnivorous species as well as the two carnivorous species. In addition, AGT was peroxisomal in the two herbivorous rodents and the only perissodactyl (Przewalski's horse). It was mitochondrial and peroxisomal in the only tubulidente (aardvark) and new primate (slow loris) examined. In the monotreme (short-nosed echidna) and the cetacean (common porpoise), AGT was mitochondrial.

In 15 of these new species, the organelle distribution of immunoreactive AGT was subjected to quantitative morphometry in order to provide a greater understanding of the variation in AGT distribution between species. These results, together with those from some previously studied species (mainly Carnivora), are shown in table 1. There was a high degree of variability in the immunogold labelling both within and between orders. For example, in the Marsupialia there was a much greater amount of AGT labelling in the peroxisomes than the mitochondria, even in species categorized as 'mitochondrial and peroxisomal'. In contrast, in most of the Carnivora the reverse applied. It is noteworthy that the only member of Carnivora with higher levels of peroxisomal than mitochondrial AGT was the only herbivorous species (i.e. the giant panda *Ailuropoda melanoleuca*; Birdsey *et al.* 2004). The widest range of immunogold labelling, however, was found in Chiroptera. In some bats, mitochondrial labelling vastly exceeded that of the peroxisomes, while in others the reverse applied.

Together with previous studies (Danpure *et al.* 1994; Holbrook *et al.* 2000; Birdsey *et al.* 2004), the subcellular distribution of AGT is now known in 77 mammals from 12 different orders. When this data is superimposed on a phylogenetic tree, it can be seen that the subcellular distribution of AGT has changed on numerous occasions during the evolution of mammals, with extensive variability both within and between orders (figure 1). Despite marked heterogeneity within the orders Primates, Rodentia, Marsupialia and Carnivora, it is noteworthy that so far as is currently known there are no members of Primates, Rodentia or Marsupialia with AGT in the 'mitochondrial' category, and no members of Carnivora with AGT in the 'peroxisomal' category. The only order that contains all three distribution categories is Chiroptera.

The results from AGT subcellular distribution were compared with the species' natural diets (figures 1 and 2). The scatter diagram (figure 2) suggests a relationship between AGT subcellular distribution and species diet. Of the 22 species with AGT in the 'peroxisomal' category, nearly two-thirds (14 species) have a herbivorous diet and none have a carnivorous diet. In contrast, of the 20 species with AGT in the 'mitochondrial' category, the majority (18) have a carnivorous diet and none have a herbivorous diet. The dietary preference in those animals with AGT in the 'mitochondrial and peroxisomal' category is less distinctive. However, of the 35 species with this AGT distribution, 23 have diets that include some degree of omnivory.

There is a significant correlation between AGT subcellular distribution and species diet ($n = 77$, $r = 0.78$, $p < 0.001$). However, this takes no account of the influence of phylogeny on the evolution of character traits among related species. To minimize problems associated with taxonomic relatedness and phylogenetic inertia, we carried out comparative analyses of the relationship between AGT subcellular distribution and a range of other character traits using the CONTINUOUS and CAIC computer packages. The results of these analyses are tabulated in the Electronic Appendix to this paper. Using the generalized least-squares method implemented

Table 1. Quantitative morphometric analysis of immunogold labelling.
(A single member of Monotremata (1), six members of Marsupialia (3, 4, 5, 7, 8, 10), seven members of Carnivora (18, 21, 23, 25, 34, 35, 36), and ten members of Chiroptera (39–48) were subjected to quantitative morphometric analysis following immunogold labelling for immunoreactive AGT (see §2 for details). The number of gold particles per organelle is expressed as a ratio of the number in whole peroxisomes divided by that in whole mitochondria. The gold particle labelling density is expressed as a ratio of the labelling density (particles per unit whole or part organelle profile area) in peroxisomes divided by that in mitochondria. P, peroxisomes; M, mitochondria. The numbering of each species is the same as that used in figures 1 and 2.)

	species	diet	ratio of the number of gold particles per whole organelle (P/M)	ratio of the gold labelling densities (P/M)	AGT distribution
	Monotremata				
1	short-nosed echidna	carniv	0.08	0.30	M
	Marsupialia				
3	kowari	carniv	2.85	3.94	M+P
4	dunnarts	carniv	1.98	7.41	M+P
5	leadbeater's possum	herbiv	1.10	6.07	M+P
7	bennett's wallaby	herbiv	6.86	12.68	P
8	cuscus	herbiv–omniv	17.25	67.85	P
10	common wombat	herbiv	20.00	58.48	P
	Carnivora				
18	oriental small-clawed otter	carniv	0.02	0.17	M
21	raccoon	omniv	0.26	1.68	M+P
23	giant panda	herbiv	1.59	2.59	M+P
25	raccoon dog	omniv	0.20	0.60	M+P
34	crab-eating mongoose	carniv–omniv	0.18	0.71	M+P
35	slender-tailed meerkat	carniv–omniv	0.09	0.38	M
36	blotched genet	carniv	0.01	0.12	M
	Chiroptera				
39	short-nosed fruit bat	herbiv	2.82	25.37	P
40	rodriguez fruit bat	herbiv	9.90	93.09	P
41	horseshoe bat	carniv	0.00	0.00	M
42	sebas short-tailed bat	herbiv	5.47	22.03	P
43	common vampire bat	carniv	0.05	0.49	M
44	noctule bat	carniv	0.16	1.53	M+P
45	pipistrelle bat	carniv	0.14	1.78	M+P
46	long-eared bat	carniv	0.97	2.16	M+P
47	big brown bat	carniv	1.02	7.22	M+P
48	serotine bat	carniv	0.37	1.56	M+P

in the CONTINUOUS program, the most significant relationship was found between AGT subcellular distribution and species diet ($p<0.001$). The only other statistically significant association was found between AGT subcellular distribution and body mass ($p=0.024$). However, it is well known that body mass itself is not unrelated to diet. There was no association between AGT distribution and the other character traits (i.e. AGT enzyme activity, levels of immunoreactive AGT protein or gestation length). Similar results were obtained using the phylogenetically independent contrasts method implemented by the CAIC program. The most significant relationship was found between AGT subcellular distribution and species diet ($p<0.001$), as well as a significant correlation between AGT distribution and body mass ($p=0.024$). No statistically significant associations were found between AGT distribution and the other traits.

4. DISCUSSION

In this paper we have determined the subcellular distribution of AGT in 22 new mammalian species, including members of three orders not studied before (i.e. Monotremata, Tubulidentata and Perissodactyla). In addition, we have added nine more Chiroptera, including the first examples of carnivorous, insectivorous and haematophagous bats, six new Marsupialia, including the first carnivorous species, and one new Cetartiodactyla which is the first cetacean studied. These new data, together with those already published (Danpure *et al.* 1994; Holbrook *et al.* 2000; Birdsey *et al.* 2004), mean that the subcellular distribution of AGT is now known in 77 different mammals, from 12 different orders. Of these mammals, 18 are entirely or almost entirely herbivorous, including species that eat leaves, fruit, flowers, nectar, sap and other plant exudates, 26 are entirely or almost entirely carnivorous, including species that eat vertebrates, insects and other invertebrates, and blood, and 33 are omnivorous. From an examination of this wide range of mammals, it is clear that AGT distribution is related to diet both between and within orders. The total dataset of 77 species has been subjected to two different statistical analyses in order to distinguish between a fortuitous relationship between AGT distribution and diet owing to quirks of phylogeny, and real relationships independent of phylogeny. Analyses by both CONTINUOUS and CAIC clearly show that the intracellular compartmentalization of AGT in mammalian liver cells is correlated with diet independently of phylogeny. This is completely compatible with the idea that changes

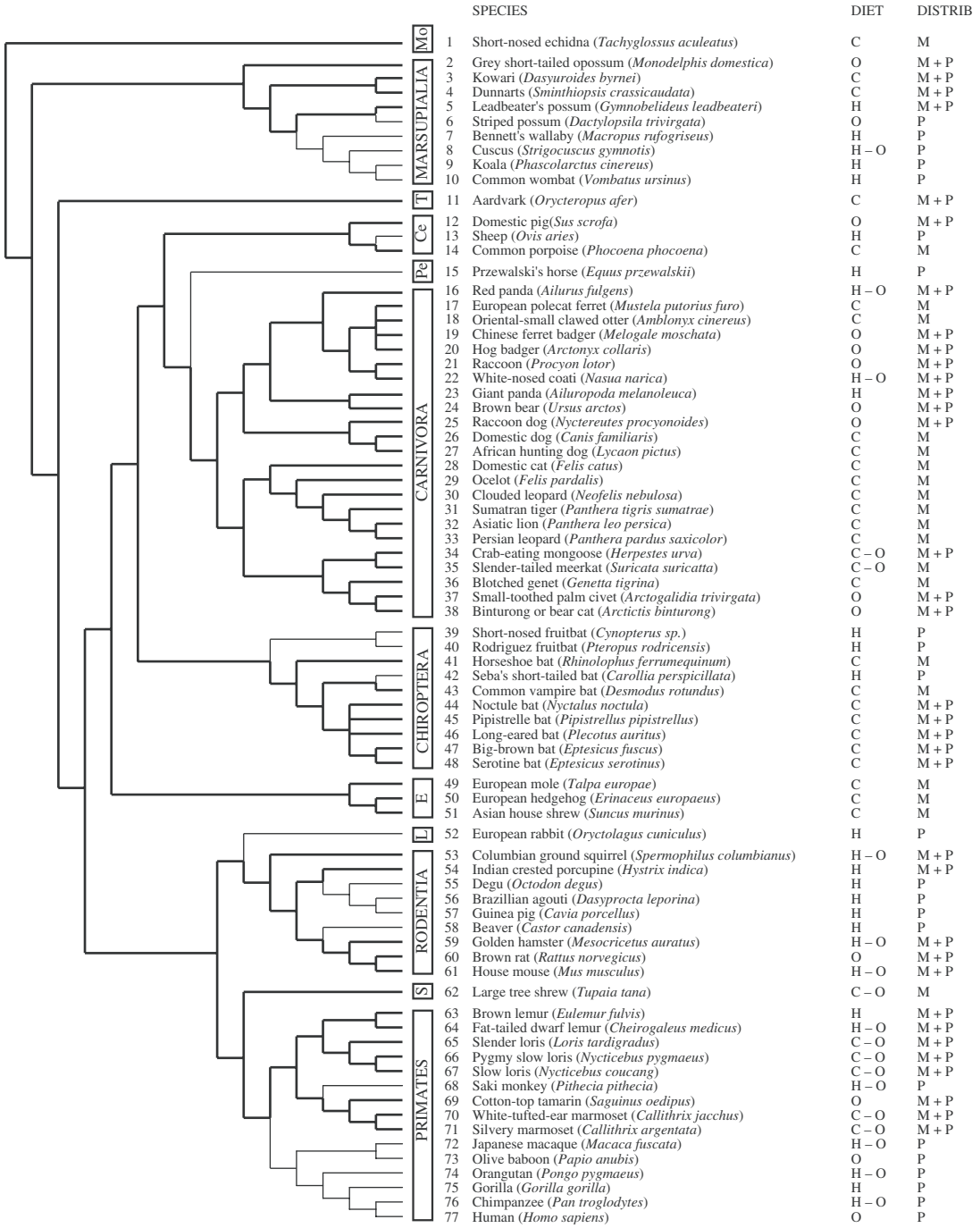

Figure 1. AGT distribution and diet in 77 mammals. The phylogenetic tree is based on those of Bininda-Edmonds *et al.* (1991), Purvis (1995), Huchon *et al.* (2002), Jones *et al.* (2002), Osborne *et al.* (2002) and Springer *et al.* (2003), and shows all mammals in which the distribution of AGT is known. Orders are indicated or abbreviated as follows: S, Scandentia; L, Lagomorpha; E, Eulipotyphla; Pe, Perissodactyla; Ce, Cetartiodactyla; T, Tubulidentata; Mo, Monotremata. Natural diets (DIET) are taken from Nowak (1991) and are categorized into herbivorous (H), herbivorous–omnivorous (H–O), omnivorous (O), carnivorous–omnivorous (C–O), and carnivorous including insectivorous (C). AGT distribution (DISTRIB) is divided into mitochondrial (M), mitochondrial and peroxisomal (M + P), and peroxisomal (P), as defined in the text. On the assumption that the ancestral distribution of AGT was at least partially mitochondrial (i.e. M or M + P), the thick lines in the tree indicate branches where at least some mitochondrial AGT has remained (i.e. M or M + P), whereas the thin lines indicate branches in which it has been lost (i.e. P). From the tree it can be seen that mitochondrial AGT has been lost or greatly diminished on at least 11 occasions during the evolution of mammals.

in AGT distribution are adaptive responses to changes in diet. Although direct evidence for positive selection at a molecular level has only been obtained for two orders so far (i.e. Carnivora (Birdsey *et al.* 2004) and Primates (Holbrook *et al.* 2000)), it is likely that such pressure is spread widely across Mammalia. The consequence of this is that dietary selection pressure has probably exerted repetitive (episodic) influences on the distribution of AGT throughout mammalian evolution.

Large changes in AGT distribution, such as the complete loss or major diminution of mitochondrial targeting, have occurred on at least 11 occasions during the evolution of mammals. Smaller scale changes have probably occurred even more often. At least four

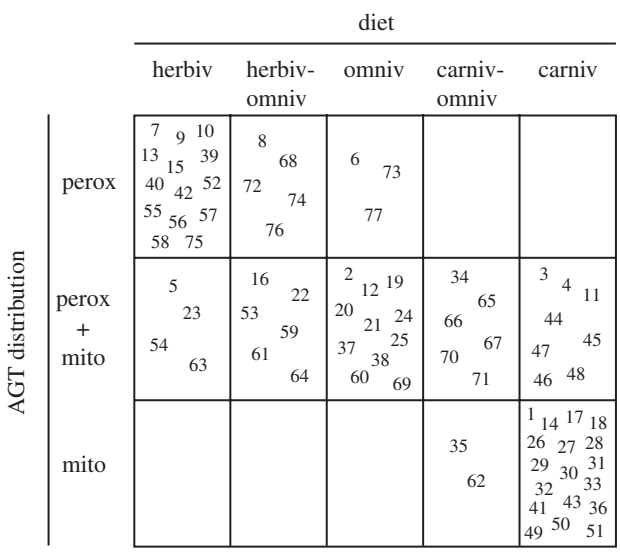

Figure 2. Relationship between AGT distribution and diet. Scatter diagram of the relationship between diet and AGT distribution for all of the 77 species tabulated in figure 1. Each number represents an individual species, using the same system as that in figure 1. All positions within any one box are equivalent.

mechanisms have been identified so far by which AGT can change its distribution—(i) loss of the first ancestral translation start site, (ii) loss of the first ancestral transcription start site, (iii) loss of the second ancestral transcription start site and (iv) accumulation of mutations that diminish the efficiency of the MTS (see §1). However, a full description of the molecular bases of changes to AGT targeting requires knowledge of transcription site usage. As this requires mRNA extraction and transcript mapping, it is only possible on the rare occasions when fresh, good quality liver material can be obtained. Without this knowledge, a prediction of AGT distribution simply from the sequence of the ancestral MTS could be misleading. Although absence of the more 5′ translation start site with subsequent permanent exclusion of the ancestral MTS from the open reading frame indicates, with a fair degree of certainty, that the distribution of AGT will not be wholly or partly mitochondrial (Oatey *et al.* 1996), its presence does not necessarily mean the opposite. For example, in the case of Przewalski's horse, the more 5′ translation start site is present and the nascent polypeptide is predicted to contain an efficient MTS (Birdsey *et al.* 2004; Birdsey *et al.*, unpublished observations). However, data presented above clearly shows that the AGT is peroxisomal. The most likely explanation for this is that the horse has lost the first ancestral transcription start site fairly recently in its evolutionary history, so that the ancestral MTS, now part of the 5′-UTR, has not had enough time to accumulate mutations that would stop it 'looking like' an MTS. This is unlike the situation in the baboon, which is also thought to have lost the more 5′ transcription start site but has had enough time to accumulate many mutations which would prevent it acting as a MTS, even if it was within the open reading frame (Holbrook *et al.* 2000; Birdsey *et al.* 2004). In these cases, whatever the molecular mechanisms leading to changes in AGT distribution, the effect is the same, i.e. to diminish or abolish mitochondrial targeting with a consequent increase in peroxisomal targeting.

Several pieces of evidence suggest that the AGT distribution in the ancestral mammal was at least partly mitochondrial, but whether it was also partly peroxisomal is less easily deduced. The most closely studied outgroup to Mammalia is Amphibia, particularly *Xenopus laevis* (Holbrook & Danpure 2002). AGT in amphibian liver appears to be mitochondrial and cytosolic. Although the C-terminus of AGT in *X. laevis* is similar to that in mammals, it does not contain a PTS. Additionally, species that branch off early from the main mammalian stem, such as the echidna (*Tachyglossus aculeatus*) fit firmly into the 'mitochondrial' category, as do species whose lifestyles appear to have changed little from those of ancestral mammals, such as the Eulipotyphla and Scandentia. Also, all orders in which more than one species have been studied contain at least some members with 'mitochondrial' or 'mitochondrial and peroxisomal' AGT. However, several orders in which multiple species have been studied, such as Carnivora and Eulipotyphla, have no members with 'peroxisomal' AGT. More formal ancestral reconstruction of AGT distribution using maximum parsimony (Mesquite, v. 1.05; W. & D. Maddison, http://mesquiteproject.org) indicates that the distribution of AGT in the ancestral mammal was most likely to be either 'mitochondrial' or 'mitochondrial and peroxisomal'. From this, it follows that if the ancestral distribution was 'mitochondrial' or 'mitochondrial and peroxisomal', then the ability to target AGT to the mitochondria must have been lost or greatly diminished on at least 11 different occasions. In addition, if ancestral AGT was 'mitochondrial and peroxisomal', then its distribution must have changed at least 21 times (10 times into the 'peroxisomal' category and 11 times into the 'mitochondrial' category). Whatever the ancestral distribution of AGT, it is clear that the distribution of AGT must have changed on numerous occasions during the evolution of mammals.

Knowledge of the diets of extant and extinct mammals, together with knowledge of AGT distribution in extant species and predicted MTS efficiency in ancestral species (Birdsey *et al.* 2004), suggests a number of general trends during mammalian evolution (see figure 3). In either of the possibilities indicated in this figure, there is an evolutionary shift from omnivory to herbivory, and either carnivory to omnivory (figure 3*a*) or omnivory to carnivory (figure 3*b*). The dietary shifts are paralleled by shifts in AGT distribution, from 'mitochondrial and peroxisomal' to 'peroxisomal', and either 'mitochondrial' to 'mitochondrial and peroxisomal' (figure 3*a*) or 'mitochondrial and peroxisomal' to 'mitochondrial' (figure 3*b*). The extent to which these dietary and AGT distribution transitions are reversible on evolutionary time-scales is unclear. However, it is noteworthy that secondary carnivory (a shift from herbivory to carnivory) appears to be a rare event in mammals, which might be a reflection of the difficulty (unlikelihood) of reacquiring an MTS once it has been lost.

Species that maintain the MTS in the open reading frame and target AGT to both the mitochondria and peroxisomes would be expected to maintain some form of adaptive flexibility on evolutionary and, in certain cases, lifetime time-scales. An example of the latter type of

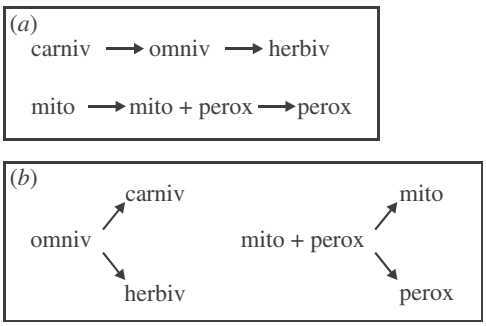

Figure 3. Dietary changes paralleled by changes in AGT distribution. Putative parallel nature of the presumed changes in diet and AGT distribution during the evolution of mammals. Evolutionary time flows from left to right. In panel (*a*), the mammalian ancestral states are carnivorous diets and mitochondrial AGT; in panel (*b*), they are omnivorous diets and mitochondrial + peroxisomal AGT. In either case, it is the omniv → herbiv and mito + perox → perox transitions that might be nearly irreversible.

adaptive phenotypic flexibility (Lister 2004) is found in certain murine rodents, such as the rat, hamster and mouse, which have evolved the ability to alter the subcellular distribution of AGT according to changes in diet. Thus, rats fed a high protein diet will specifically increase the expression of mitochondrial, rather than peroxisomal, AGT (Oda *et al.* 1982). This results in a shift of AGT distribution from being mainly peroxisomal to mainly mitochondrial. Over longer periods of evolutionary time, the possession of both an MTS and a PTS would provide a species with greater adaptive potential to alter the subcellular distribution of AGT in response to environmental changes. Species that lose their MTS by excluding it from the open reading frame will lose much of their flexibility, because from a molecular/cellular biology point of view reacquisition of the MTS is much less likely than its loss in the first place. This is especially likely to be the case in species such as the rabbit (Purdue *et al.* 1992), where the time since MTS loss is such that this region of the gene (now part of the 5′-UTR) has accumulated numerous point mutations that would render it ineffective as an MTS even if it were to be reintroduced back into the open reading frame. On the other hand, humans and horses (see above) have probably only recently lost the ability to target AGT to mitochondria because the ancestral MTS in the AGTs of these species is predicted to be still able to target AGT to mitochondria if reintroduced back into the open reading frame (Oatey *et al.* 1996; Lumb *et al.* 1999; Birdsey *et al.*, unpublished observations).

The human, in fact, is remarkable because, after having lost the ability to target AGT to mitochondria following a single mutation to the more 5′ ancestral translation start site (Takada *et al.* 1990), some individuals have reacquired the ability to target a small amount of their AGT back to mitochondria. However, this is not owing to reintroduction of the ancestral MTS back into the open reading frame. Instead, it is owing to the presence of a very common polymorphism which creates a new MTS in a region downstream of the ancestral MTS (Purdue *et al.* 1990). Whether this reacquisition of mitochondrial AGT targeting in some humans is related to increased meat-eating is unknown.

The variable intracellular compartmentalization of AGT and its evolutionary relationship to diet is without parallel. It is self-evident that diet provides one of the most potent selection forces in evolution. Nonetheless, although there are numerous examples of *macroevolutionary* events related to diet, such as changes in size, dentition, alimentary tract and so on, far fewer *micro-evolutionary* events are known to have resulted from dietary selection pressure. The variable intracellular compartmentalization of AGT could be considered to be a *mini-evolutionary* event as it is dependent on a small number of micro-evolutionary events (i.e. mutations). Bearing in mind the large metabolic differences dictated by carnivorous and herbivorous diets, it is perhaps surprising that no other micro-/mini-evolutionary change is so consistently related to diet across Mammalia. One of the better-studied micro-evolutionary responses to dietary selection pressure is the evolution of acid resistance in mammalian lysozymes. However, this occurs only in a very restricted number of species (i.e. some artiodactyls and one group of primates; Stewart *et al.* 1987; Messier & Stewart 1997).

There are probably several properties of AGT, the combined effects of which give rise to its unique evolutionary behaviour. Firstly, unlike many other enzymes with dual localization, AGT is encoded by a single gene. The structure of the gene (i.e. two in-frame translation start sites straddling the MTS, and two transcription start sites) allows a single mutational event to influence the expression of both mitochondrial and peroxisomal forms, such that as one form is decreased the other is increased. Secondly, AGT has different diet-dependent metabolic roles in the two compartments—in mitochondria it transaminates glyoxylate derived from hydroxyproline, while in peroxisomes it transaminates glyoxylate derived from glycolate. Thirdly, the transamination of glyoxylate to glycine is a detoxification reaction. In other words, if its efficiency is diminished it is potentially lethal.

The variable intracellular targeting of AGT in mammals is a unique example of molecular adaptation. It shows for the first time that Darwinian natural selection operates at the molecular level in at least one gene across a whole class (i.e. Mammalia). It also shows that molecular contingencies and constraints can shape the direction of evolutionary events. Thus, the rarity of secondary carnivory might be related to the perceived greater difficulty of regaining an MTS compared with losing it in the first place.

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