Primary hyperoxaluria type 1 (PH1) is a rare autosomal recessive disorder of metabolism caused by a functional deficiency of the liver-specific, pyridoxal-phosphate-dependent enzyme alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44) [1]. In the peroxisomes of normal human hepatocytes, AGT catalyses the transamination of the intermediary metabolite, glyoxylate, to glycine. This can be considered to be a detoxification reaction because its dysfunction in PH1 allows glyoxylate to escape from the peroxisomes into the cytosol where it is oxidised to oxalate, catalysed by lactate dehydrogenase, and reduced to glycolate, catalysed by glyoxylate/hydroxybutyrate reductase (Fig. 1) [2,3]. In humans, at least, oxalate cannot be further metabolised and its increased synthesis and urinary excretion leads to the progressive deposition of insoluble calcium oxalate (CaOx) in the kidney and urinary tract, as various combinations of nephrocalcinosis (diffuse deposition throughout the renal parenchyma) and/or urolithiasis (calculi or stones). This eventually leads to renal failure, following which the combined effects of increased oxalate synthesis and failure to remove it from the body results in the deposition of CaOx almost anywhere. The clinical presentation of PH1 is heterogeneous, for example with respect to age of first symptom, levels of urinary oxalate and glycolate excretion, relative contributions of nephrocalcinosis and urolithiasis, and rate of progression of renal pathology.

2. Relationship between genotype and enzyme phenotype

2.1. The structure of normal AGT

Human AGT is a homodimeric protein, each subunit being composed of 392 amino acids with a molecular mass of about...
43 kDa [4]. Its recently-described crystal structure [5] (PDB 1H0C) (Fig. 2) shows that each subunit can be subdivided into three recognizable structural/functional domains. The first 20 or so residues make up an N-terminal extension that wraps over the surface of the other subunit. The next 260 or so residues form the ‘large domain’ that contains most of the active site and the dimerization interface. The final C-terminal ‘small domain’ consists of about 110 residues, which contains among other things the principal and ancillary peroxisomal targeting information [6,7] (see below). Each subunit binds one pyridoxal phosphate, which forms a Schiff base with Lys209. The large surface area of the dimerization interface explains the high stability of the AGT dimer. Although the inter-subunit interaction mediated by the N-terminal extension is unlikely to make any major contribution to stability of the dimer once formed, it is believed to play a significant role in the dimerization process per se [5]. Dimerization of AGT is very important, not only because monomeric AGT has vastly reduced catalytic activity [8], but also because it is unstable leading to aggregation and rapid degradation [9]. Knowledge of the crystal structure of AGT has provided insights into the processes of AGT folding, dimerization and catalytic activity, as well as allowing rationalisation of the effects mutations and polymorphisms on these processes (see below).

2.2. Enzyme phenotypes in PH1

The clinical heterogeneity of PH1 is matched, or possibly even exceeded, by its enzymatic heterogeneity. Three overt categories can be identified:-deficiency of AGT catalytic activity
but not AGT immunoreactivity, deficiency of both catalytic activity and immunoreactivity, and deficiency of neither catalytic activity nor immunoreactivity. Patients in the latter category can have AGT levels up to half the mean normal level, which is well within the normal range and greater than found in many asymptomatic carriers [2]. Intermediate categories also exist, in which patients have markedly reduced, but still detectable levels of catalytic activity and/or immunoreactivity.

More detailed analysis shows the presence of two enzyme phenotypes unique to PH1. In a small group of patients with almost no AGT catalytic activity and markedly reduced AGT immunoreactivity, AGT appears to have aggregated into intraperoxisomal ‘cores’ [9]. In another, much larger, group of patients who have significant levels of AGT catalytic activity and immunoreactivity, an even more remarkable enzyme phenotype is found in which AGT is mistargeted from its normal location in the peroxisomes to the mitochondria [10]. Although mistargeted AGT remains catalytically active, it appears to be metabolically inefficient. This is because efficient glyoxylate detoxification must occur at its site of synthesis (i.e. in the peroxisomes), thus preventing its oxidation to oxalate, catalysed by cytosolic LDH. Such organelle-to-organelle mistargeting, in which both the normal and abnormal compartments require the protein to contain specific, yet different, targeting information, is without parallel in human genetic disease.

2.3. The normal AGXT gene

In humans, AGT is encoded by the AGXT gene, which consists of 11 exons spanning about 10 kB on chromosome 2q37.3 [11]. There are two main allelic variants, the frequencies of which vary between different populations. The less common “minor” AGXT allele differs from the more common “major” allele by the presence of a 32C→T (Pro11Leu) replacement in exon 1, a 1020A→G (Ile340Met) replacement in exon 10, and a 74 bp duplication in intron 1 [12,13]. In uncharacterized European and North American populations, the minor allele has a frequency of about 15–20%. However, it rises to 28% in the Sama in Northern Sweden and falls to only 2–3% in some far eastern populations in India, China and Japan [14,15]. Although the Ile340Met replacement and the intron 1 duplication have no obvious effects on the expression or properties of AGT, the Pro11Leu polymorphism certainly does [8,12]. Firstly, it leads to a decrease in AGT stability, which is manifested by slower dimerization, especially at elevated temperatures [8,16]. Secondly, it decreases specific catalytic activity of recombinant purified AGT by a factor of two to three [8]. Thirdly, it redirects a small proportion (~5%) of AGT away from peroxisomes towards the mitochondrial matrix [12]. Fourthly, it sensitises AGT to the untoward effects of many of the mutations found in PH1 that are predicted to be innocuous in its absence [8] (see below).

2.4. Mutations in the AGXT gene

Well over fifty mutations have been identified so far in the AGXT gene, about half of which are point missense mutations [17]. Some of the most common co-segregate and functionally interact with the common Pro11Leu polymorphism of the minor allele. Many of the better-studied mutations, or mutation–polymorphism combinations, are associated with specific enzyme phenotypes, such as loss of AGT catalytic activity, AGT aggregation, accelerated AGT degradation, and peroxisome-to-mitochondrion AGT mistargeting. The most extensively studied genotype–enzyme phenotype relationships are described in more detail below.

2.4.1. Gly82Glu

The Gly82Glu mutation is associated with complete loss of AGT catalytic activity, without any effect on enzyme stability, dimerization or targeting [18,19]. Structural analysis shows this to be due to the mutant glutamate side chain filling the space that should be occupied by the cofactor pyridoxal phosphate [5]. This prevents Schiff base formation with the ε-amino group of Lys209, thereby abolishing catalytic activity [8]. Despite lack of enzyme activity, patients homozygous for Gly82Glu have completely normal levels of normally compartmentalised immunoreactive AGT protein. Unlike many of the missense mutations in PH1, the Gly82Glu replacement is always found on the background of the major AGXT allele.

2.4.2. Gly41Arg

The Gly41Arg mutation has been found on the background of both the major and minor AGXT alleles [9,20]. However, there is some evidence to suggest that when present on the minor allele (i.e. in the presence of the Pro11Leu polymorphism) the effects of this mutation are more severe [8]. At least on the minor allele, Gly41Arg destabilizes AGT so that it is rapidly degraded. The protein that manages to escape degradation aggregates into intra-peroxisomal core-like structures [9]. Structural analysis shows that Gly41 sits right in the middle of the dimerization interface, making contact with its opposite number in the other subunit [5]. There is not enough room at this location to accommodate the large arginine side-chain without causing major disruption to the interface, thereby inhibiting dimerization. The monomer has greatly reduced specific catalytic activity [8] and is predicted to be unstable, one consequence of which would be aggregation due to artefactual polymerization. The presence of aggregates only within the peroxisomal matrix suggests that either aggregation is slow and does not occur until after peroxisomal import or, perhaps more probably, aggregates forming in the cytosol, but not the peroxisomes, are rapidly degraded.

2.4.3. Ile244Thr

The second most common missense mutation is an Ile244Thr replacement which segregates with the minor allele (i.e. with the Pro11Leu polymorphism). This mutation has an overall allelic frequency of about 6–9% in PH1 patients [21], but in some parts of Spain, such as La Gomera in the Canary Islands, it is very much higher [22]. The combined presence of the polymorphism and the mutation appears to destabilize AGT leading to its rapid degradation. Homozygous patients have little or no enzyme activity or immunoreactive protein.

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2.4.4. Gly170Arg

By far the most common mutation found so far in PH1 is a Gly170Arg replacement [12], with an estimated allelic frequency between 15 and 35%, depending on the particular population [12,17]. This mutation segregates exclusively with the minor AGXT allele, and is predicted to have no untoward effects in the absence of the Pro11Leu polymorphism [8]. Gly170Arg and Pro11Leu, together, are responsible for the mistargeting of 90–95% of the AGT from its normal location in the peroxisomes to the mitochondrial matrix [10]. The mechanism by which this remarkable trafficking defect is achieved highlights some of the most fundamental differences between the peroxisomal and mitochondrial protein import pathways (see Section 3 below).

3. Molecular and cell biological basis for AGT targeting and mistargeting

3.1. Normal peroxisomal targeting of human AGT

AGT in humans and guinea pigs has been shown to be post-translationally imported into the peroxisomal matrix in a Pex5p-dependent and Pex7p-independent manner [6,23]. Like many other peroxisomal enzymes [24–26], AGT folds and dimersizes in the cytosol, and is imported into peroxisomes already in a fully active state [27]. Despite these similarities, it is clear that AGT is not a typical PTS1 protein. The C-terminal tripeptide in human AGT is KKL. Although this varies from the prototypical PTS1 SKL only at the −3 position, all the evidence suggests that this tripeptide should not be able to act as a PTS1. For example, although KKL is necessary for the peroxisomal targeting of human AGT [6], it is insufficient to direct the peroxisomal import of reporter proteins, such as firefly luciferase, jellyfish green fluorescent protein (GFP), and bacterial chloramphenicol acetyltransferase [6,7,28]. KKL is even unable to direct the peroxisomal import of non-mammalian AGT, such as that from the amphibian Xenopus laevis [29]. This difficulty is compounded by the observations obtained from the crystal structure of the TPR domain of human Pex5p that lysine at −3 could not possibly fit [30].

These findings, together with the observations that the peroxisomal import of human AGT can actually be inhibited by over-expression of Pex5p [31] and that the C-terminal tripeptides of mammalian AGTs are very poorly conserved compared to other PTS1 proteins (see Table 1), suggested that human AGT might contain additional peroxisomal targeting information that allows such an unconventional PTS1 to work. A putative region of human AGT that might contain this ancillary peroxisomal targeting information (termed PTS1A) has been identified in the small domain, 47–68 residues upstream of the C-terminus [7] (Fig. 2). Although further upstream than any other sequence currently known to influence the efficiency of a PTS1, reference to the crystal structure of human AGT [5] (Fig. 2) shows that the PTS1A is actually in close proximity to the C-terminal KKL. How the PTS1A might work is unclear, but it might involve increasing the stability of the KKL-TPR interaction, or possibly directly or indirectly allosterically modifying the TPR domain so that KKL fits.

3.2. Abnormal mitochondrial targeting of human AGT

The peroxisome-to-mitochondrion mistargeting of AGT in PH1 requires the combined presence in cis of the common Pro11Leu polymorphism and the disease-specific Gly170Arg mutation [12]. Surprisingly, however, the polymorphism appears to have a much greater effect on the properties of AGT than does the mutation (see above). The Pro11Leu replacement generates a functionally-weak N-terminal mitochondrial matrix targeting sequence (MTS), the effective strength of which is enhanced by the additional presence of the Gly170Arg replacement [16,32].

MTSs are very different from PTS1s. They are much larger (typically 20–30 residues), N-terminal, and, although in different mitochondrial proteins they show little or no sequence conservation, they all have a tendency to fold into positively-charged amphiphilic α-helices [33]. MTSs bind to a hydrophobic pocket in the outer-membrane mitochondrial import receptor TOM20 [34,35]. Also unlike PTS1s, MTSs are almost invariably cleaved after import [36]. The N-terminal sequence of human AGT (MASHKLLVPKKALLKLPSI) has some similarities to a MTS, for example, absence of acidic residues, and a good distribution of basic, neutral or hydrophobic, and hydroxyl amino acids. However, the presence of two neighboring helix breakers (i.e. prolines) at residues 10 and 11 is likely to prevent this sequence from folding into an α-helix. A

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme</th>
<th>Number of different PTS1s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human and other primates</td>
<td>AGT</td>
<td>6</td>
</tr>
<tr>
<td>Rat and other murine rodents</td>
<td>CAT</td>
<td>2</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>UO</td>
<td>1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>ACO</td>
<td>1</td>
</tr>
<tr>
<td>Cat</td>
<td>GO</td>
<td>2</td>
</tr>
<tr>
<td>Dog</td>
<td>DAO</td>
<td>1</td>
</tr>
<tr>
<td>Cow</td>
<td>Koala</td>
<td>1</td>
</tr>
<tr>
<td>Pig</td>
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Table 1 C-terminal tripeptides of some PTS1 mammalian enzymes

Although it has not yet been found in patients liver in situ, AGT containing Pro11Leu and Ile244Thr has been found to aggregate in transfected COS cells [22], as it does when over-expressed in E. coli [8].
single Pro11Leu replacement is predicted to allow the N-terminus of human AGT to better adopt the conformation required for a MTS, even though circular dichroism showed that the tendency of the purified 20-mer polypeptide to form an \( \alpha \)-helix was hardly any different with or without the replacement. When both prolines were replaced by leucines, the purified 20-mer showed a strong tendency to form an \( \alpha \)-helix [16].

The interesting thing about this polymorphic MTS (i.e. with the single Pro11Leu replacement) is that it is only functionally weak when attached to AGT, whereas it is functionally strong when attached to GFP. However, when both prolines are replaced by leucine, it is strong irrespective of what it is attached to [16]. The reason for this appears to be due to the fact that AGT folds quickly and rapidly forms a stable dimer that does not readily exchange subunits [27]. This fully-folded dimeric conformation, although compatible with peroxisomal protein import (see above), is incompatible with mitochondrial protein import [37,38]. Therefore, despite the presence of an N-terminal MTS, most of the polymorphic AGT is actually targeted to peroxisomes due to the presence of the C-terminal PTS1 (and PTS1A). In other words, polymorphic AGT still folds and dimerizes faster than mitochondrial import. This kinetic partitioning of polymorphic AGT and its perturbation due to the additional presence of the Gly170Arg mutation is crucial to the understanding of the peroxisome-to-mitochondrion mistargeting of AGT in PH1 (Fig. 3).

On its own, the disease-specific Gly170Arg mutation provides no additional mitochondrial targeting information, does not directly interfere with peroxisomal targeting, and has no major effect on AGT stability or dimerization [8,27]. However, the combined effect of the Gly170Arg and Pro11Leu replacements is to severely delay productive folding and dimerization long enough to allow the import of AGT into the mitochondria mediated by the polymorphic MTS.

Surprisingly, unlike almost all other proteins targeted to the mitochondrial matrix, including AGT in many other species (see below), the polymorphic MTS of mistargeted human AGT is not cleaved after import. The polymorphic MTS is located in the N-terminal segment which wraps over the surface of the companion subunit and is thought to be important in the process of dimerization (see above and Fig. 2). The fact that it is not cleaved means that mistargeted AGT is still able to eventually fold and dimerize within the mitochondrial matrix, where it is able to acquire relatively normal catalytic activity, if not metabolic efficiency.

This explanation for the peroxisome-to-mitochondrion mistargeting of AGT in PH1 highlights two of the fundamental differences in the peroxisomal and mitochondrial protein import pathways, namely the differences between the natures and locations of the respective targeting sequences, and the different conformational requirements of importable cargos. Interestingly, mistargeted AGT contains both a N-terminal MTS and a C-terminal PTS1 (+PTS1A). Yet, 90–95% of the enzyme ends up in the mitochondria rather than the peroxisomes. The reason for this is unclear, but might be due to there being more mitochondria (and mitochondrial import receptors) than peroxisomes (and peroxisomal import receptors), or the mitochondrial import process might be intrinsically faster than that of the peroxisomes, or the N-terminal location of the MTS might make it potentially available for operation before the C-terminal PTS1 has been synthesized. This apparent hierarchical dominance of the MTS over the PTS1 is also found with more conventional MTSs, such as those in other mammalian AGTs (see below).

![Fig. 3. Kinetic partitioning of AGT. Most of the newly-synthesized normal AGT encoded by the major and minor AGXT alleles folds, dimerizes and acquires catalytic activity rapidly in the cytosol before import into peroxisomes. About 5% of normal polymorphic (Pro11Leu) AGT and about 90% of mutant (Pro11Leu+Gly170Arg) AGT are imported into mitochondria before having a chance to fold and dimerize. After import into mitochondria, relatively normal folding and dimerization occurs. It is presumed that homologous sets of molecular chaperones mediate folding in the cytosol and mitochondrial matrix, but not the peroxisomes. Retardation of AGT folding and dimerization in the cytosol (*) leads to its redirection along the other pathways, including import into mitochondria (+) if the polymorphic (Pro11Leu) MTS is present.](attachment:image-url)
Luckily, in the absence of the Gly170Arg mutation, the Pro11Leu replacement does not interfere enough with AGT folding and dimerization to generate a functionally strong MTS. If it did, then PH1 would have a frequency of 1 in 25 (the frequency of Pro11Leu homozygosity) rather than somewhere between 1 in 10^5 and 10^6. The same would apply if it were not the case that mitochondria are only able to import unfolded or loosely-folded monomeric polypeptides [37], unlike peroxisomes that seem to be able to import almost anything as long as it contains a PTS [39].

3.3. Evolutionary history of AGT targeting

The finding that AGT is mistargeted from peroxisomes to mitochondria in a large subset of PH1 patients was surprising not least because there are many mammals which have most of their AGT in mitochondria anyway without apparently succumbing to CaOx kidney stone disease [40,41].

For the vast majority of non-cytosolic eukaryotic proteins, compartmentalisation is presumed to have occurred early in eukaryotic evolution, the consequence of this being that the same protein has the same subcellular distribution in all organisms. AGT, on the other hand, can be found in peroxisomes, mitochondria, peroxisomes and mitochondria, peroxisomes and cytosol, and mitochondria and cytosol [29,40,42]. During the evolution of mammals, and possibly vertebrates in general, AGT targeting has been under the influence of strong, yet episodic, dietary selection pressure. Thus AGT tends to be mitochondrial in carnivores, peroxisomal in herbivores, and both peroxisomal and mitochondrial in omnivores. The peroxisomal location of AGT in humans is probably a hangover from our mainly herbivorous Great Ape ancestry [43].

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 [44], whereas in carnivores it is thought to be hydroxyproline [45]. 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 [46], occurs in the mitochondria. Thus, the best place for AGT to be is in the peroxisomes in herbivores and mitochondria in carnivores (Fig. 4).

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 [4,11,23,29,47–50]. The archetypal mammalian AGT gene, as found in the rat or marmoset, for example, has the potential to encode an N-terminal mitochondrial matrix targeting sequence (MTS) of 22 amino acids, and an atypical C-terminal type 1 peroxisomal targeting 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 [51]. The eventual localisation of AGT appears to be dependent on whether or not the MTS is contained within the open reading frame, and therefore present in the encoded 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 [52] (Fig. 5). 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

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Fig. 4. Diet and the intracellular compartmentalization of AGT in mammals. The major precursor of glyoxylate in herbivores is glycolate which is converted to glyoxylate in the peroxisomes, catalysed by glycolate oxidase (GO). In carnivores, the major precursor is hydroxyproline, which is converted to glyoxylate by a number of steps, the last of which catalysed by 4-hydroxy-2-ketoglutarate aldolase (HKA) occurs in the mitochondria. Efficient detoxification of glyoxylate must occur at its site of synthesis. Therefore, alanine:glyoxylate aminotransferase (AGT) is best located in the peroxisomes in herbivores and the mitochondria in carnivores. LDH, lactate dehydrogenase.
targeted only to the peroxisomes [48,53]. 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 [4,23,43,48]. In these animals, AGT is exclusively peroxisomal. In other species, such as the domestic cat, almost all nascent polypeptides contain a MTS due to the loss of the more 3′ transcription start site [47]. AGT in the cat is 90–95% mitochondrial. In certain species, such as the giant panda, although AGT contains a MTS, it does not work very efficiently due to the accumulation of interfering mutations that decrease its net positive charge and weaken its potential to fold into an α-helix [41].

The unique evolutionary history of AGT targeting might be, at least in part, responsible for the unusual properties of this PTS1 enzyme, especially the poor conservation of its C-terminus and, in some species at least, its lack of sufficiency [6,7]. Although the presence of the PTS1A might remove some of the selection pressure to conserve the PTS1 (see above), it is equally possible that during the evolution of vertebrates, or mammals in particular, periods of carnivory and strong mitochondrial targeting have diminished the need for efficient peroxisomal targeting, so that the PTS1 might have had to have evolved on more than one occasion.

4. AGT and idiopathic calcium oxalate kidney stone disease

As described above, the Pro11Leu polymorphism has significant effects on the properties of AGT, most of which might be considered detrimental. Yet, in apparently normal European and North American populations its allelic frequency is 20% or more. In such populations, 4% of individuals would be expected to be homozygous for Pro11Leu. Although the presence or absence of Pro11Leu polymorphism might be a contributory factor in an individual’s susceptibility to idiopathic CaOx kidney stones. The consequences of its dysfunction in PH1 clearly demonstrate that AGT is an important determinant of the rate of oxalate synthesis and excretion, and elevated oxalate excretion is a well-known risk factor for CaOx stones [56]. Thus the polymorphism might be beneficial to high meat-eaters, but neutral, or possibly detrimental, to vegetarians.

5. Postscript

AGT, or at least human AGT, fits well into a scholarly volume on peroxisomes. However, in a wider evolutionary context it could just as easily fit into a volume on mitochondria. The variable peroxisomal and mitochondrial targeting of AGT during mammalian evolution, and the peroxisome-to-mitochondrion mistargeting of AGT in human hereditary kidney stone disease, place AGT in a unique position in the comparative study of peroxisomal and mitochondrial protein import. Whether AGT is truly unique, or whether there are any other, as yet unidentified, enzymes that behave in a similar fashion remains to be seen.
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