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Molecular aetiology of primary hyperoxaluria and its implications for clinical management

Christopher J. Danpure and Gill Rumsby

The primary hyperoxalurias type 1 (PH1) and type 2 (PH2) are autosomal recessive calcium oxalate kidney stone diseases caused by deficiencies of the metabolic enzymes alanine:glyoxylate aminotransferase (AGT) and glyoxylate/hydroxypyruvate reductase (GR/HPR), respectively. Over 50 mutations have been identified in the *AGXT* gene (encoding AGT) in PH1, associated with a wide variety of effects on AGT, including loss of catalytic activity, aggregation, accelerated degradation, and peroxisome-to-mitochondrion mistargeting. Some of these mutations segregate and interact synergistically with a common polymorphism. Over a dozen mutations have been found in the *GRHPR* gene (encoding GR/HPR) in PH2, all associated with complete loss of glyoxylate reductase enzyme activity and immunoreactive protein. The crystal structure of human AGT, but not human GR/HPR, has been solved, allowing the effects of many of the mutations in PH1 to be rationalised in structural terms. Detailed analysis of the molecular aetiology of PH1 and PH2 has led to significant improvements in all aspects of their clinical management. Enzyme replacement therapy by liver transplantation can provide a metabolic cure for PH1, but it has yet to be tried for PH2. New treatments that aim to counter the effects of specific mutations on the properties of the enzymes could be feasible in the not-too-distant future.

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Oxalate is an end product of metabolism that is of no known use to the body. In fact, it is potentially detrimental because of the low solubility of its calcium salt. Under certain conditions, calcium oxalate (CaOx) can crystallise out of solution to form deposits that can obstruct ducts, damage cells and cause inflammatory reactions. Almost all oxalate produced by the body is removed by urinary excretion and it is the kidney where CaOx deposition is most likely to occur, either as urolithiasis (calculi in the kidney or urinary tract) or nephrocalcinosis (diffuse interstitial deposition of crystals in the renal parenchyma). In extreme cases, CaOx deposition in the kidney results in renal failure, after which CaOx might deposit at almost any site within the body (systemic oxalosis). The causes of CaOx kidney stones are largely unknown but are generally considered to be multifactorial in nature, with both genetic and environmental determinants. Two CaOx kidney stone diseases with well-established monogenic origins are primary hyperoxaluria type 1 (PH1) and type 2 (PH2).

Primary hyperoxaluria type 1 (PH1)

Primary hyperoxaluria type 1 (PH1, MIM 259900) is an autosomal recessive disorder characterised by increased synthesis and excretion of oxalate and, in the majority of patients, glycolate. Increased levels of oxalate result in the renal deposition of CaOx as urolithiasis and/or nephrocalcinosis. Following kidney failure, the increased synthesis of oxalate together with a failure to remove it from the body leads to systemic oxalosis (Refs 1, 2, 3).

AGT deficiency in PH1

PH1 is caused by a deficiency of the intermediary metabolic enzyme alanine:glyoxylate aminotransferase (AGT, EC 2.6.1.44) (Ref. 4). AGT is a peroxisomal liver-specific enzyme, dependent on pyridoxal phosphate (PLP), that catalyses the transamination of the intermediary metabolite glyoxylate to glycine (Fig. 1). Failure to do so in PH1 allows increased amounts of glyoxylate to be oxidised to oxalate, catalysed mainly by lactate dehydrogenase (LDH), and reduced to glycolate, catalysed by glyoxylate/hydroxypyruvate reductase (GR/HPR).

PH1 is very heterogeneous at the level of AGT deficiency. Three basic enzyme phenotypes can be recognised: (1) absence of AGT catalytic activity as well as immunoreactivity; (2) absence of catalytic activity but presence of immunoreactivity; and (3) presence of both catalytic activity and immunoreactivity (Ref. 5). Frequently, intermediate phenotypes are found. For example, AGT immunoreactivity can vary from undetectable to supranormal levels, and AGT catalytic activity can vary from 0% to ~50–60% of the mean normal level. Several more-specific and highly unusual enzyme phenotypes fit into this overall classification. For example, in a few patients with low levels of AGT immunoreactive protein and no catalytic activity, AGT appears to have aggregated inside the peroxisomal matrix to form core-like structures (Ref. 6). Even more unusual is the finding that in most patients who have both AGT catalytic activity and immunoreactivity, ~90% of the AGT is mistargeted from its normal location in the peroxisomes to mitochondria (Ref. 7). Although AGT remains catalytically active in the mitochondria, it is metabolically ineffective because its substrate, glyoxylate, is thought to be synthesised principally in peroxisomes.

The AGT gene (*AGXT*)

AGT is encoded by the *AGXT* gene, which consists of 11 exons spanning about 10 kb on chromosome 2q37.3 (Ref. 8). The open reading frame encodes a polypeptide of 392 amino acids, which homodimerises to yield a protein of ~86 kDa (Ref. 9). Each subunit binds one molecule of the cofactor PLP. AGT is imported into the peroxisomal matrix, probably in its fully active dimerised cofactor-bound form, using the Pex5p-dependent pathway as a result of an atypical C-terminal type 1 peroxisomal targeting sequence (PTS1, Lys-Lys-Leu) (Refs 10, 11). Although this tripeptide is similar to the prototypical PTS1 Ser-Lys-Leu, it is unusual insofar as it seems to require additional, as yet unidentified, targeting information within the AGT molecule in order to work.

Polymorphic variants of the *AGXT* gene

Two main polymorphic variants of the *AGXT* gene exist at high frequency in normal European and North American populations. The functionally significant (see below) difference between the proteins encoded by the 'major' *AGXT* allele (frequency ~80%) and the 'minor' *AGXT* allele (frequency ~20%) is the presence of a Pro11Leu polymorphism in the latter (Ref. 12). A number of

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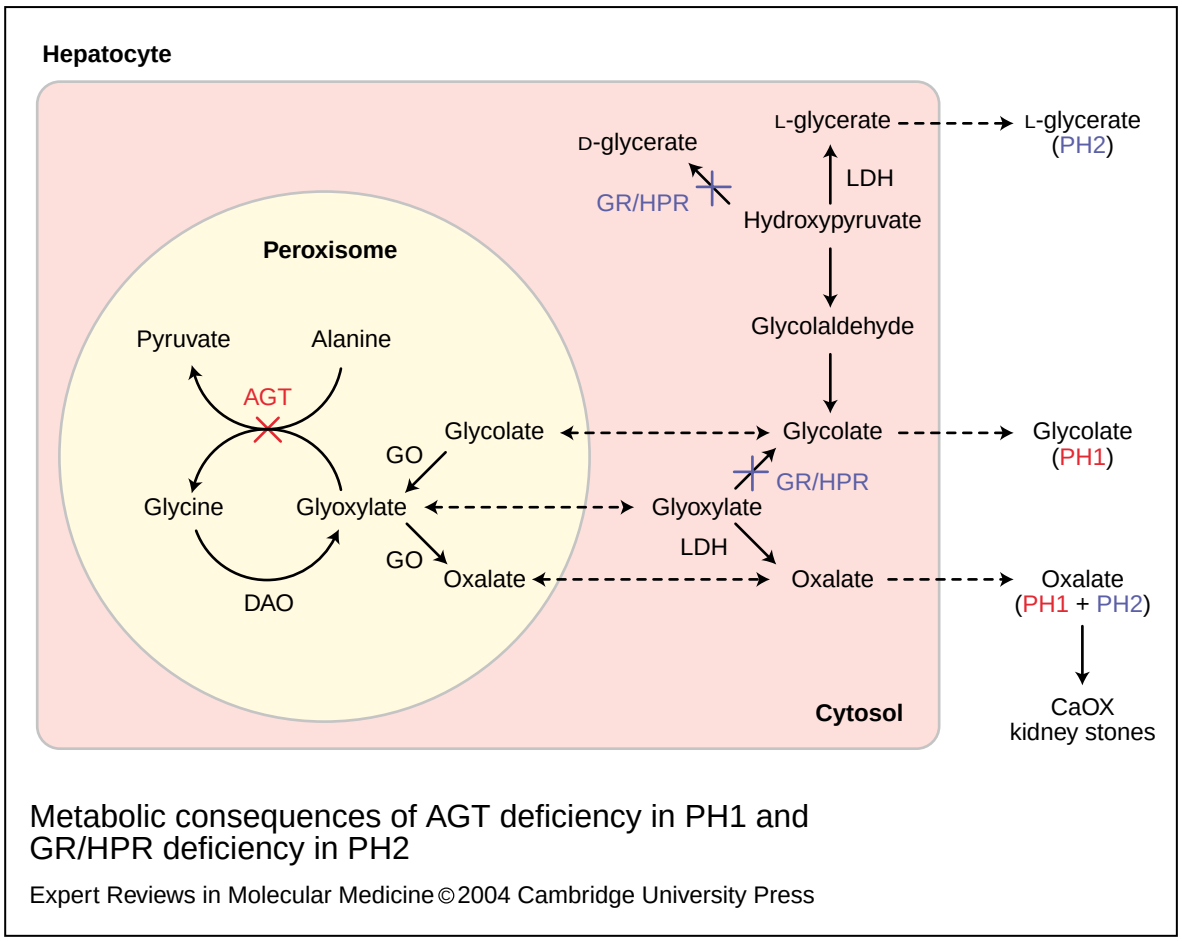

Figure 1. Metabolic consequences of AGT deficiency in PH1 and GR/HPR deficiency in PH2. AGT deficiency in PH1 blocks the transamination (detoxification) of glyoxylate to glycine in hepatocyte peroxisomes. Instead, glyoxylate diffuses into the cytosol where it is oxidised to oxalate, catalysed by LDH, and reduced to glycolate, catalysed by GR/HPR. The elevated synthesis and excretion of oxalate and glycolate are characteristic of PH1. GR/HPR deficiency in PH2 prevents the reduction of glyoxylate to glycolate and hydroxypyruvate to D-glycerate in the cytosol. This leads to increased synthesis of oxalate and L-glycerate, both catalysed by LDH. Concomitant hyperoxaluria and hyper-L-glycericaciduria are characteristic of PH2. Solid arrows indicate metabolic conversions; dashed arrows indicate membrane transport (diffusion or active). Abbreviations: AGT, alanine:glyoxylate aminotransferase; CaOx, calcium oxalate ; DAO, D-amino acid oxidase; GO, glycolate oxidase; GR/HPR, glyoxylate/hydroxypyruvate reductase; LDH, lactate dehydrogenase; PH1, primary hyperoxaluria type 1; PH2, primary hyperoxaluria type 2.

other intragenic polymorphisms are in linkage disequilibrium with Pro11Leu, including an Ile340Met substitution, a 74 bp duplication in the first intron (Ref. 13) and an Epstein–Barr-virus-like VNTR in the fourth intron (Ref. 14).

Although the Pro11Leu polymorphism is not known to cause disease, it does have at least four measurable effects on the properties of AGT. First, it decreases the specific catalytic activity of purified recombinant AGT by 70% (Ref. 15). Second, at elevated temperatures it decreases the efficiency of dimerisation of AGT translated in vitro (Ref. 15). Third, when present homozygously it causes a small proportion (~5%) of hepatic AGT to be rerouted from the peroxisomes to the mitochondria (Ref. 12). Fourth, it appears to sensitise AGT to the untoward effects of some of the most common mutations found in PH1 (see below).

Mutations in the *AGXT* gene

About 50 mutations have been identified so far in the *AGXT* gene; most of these are missense point mutations, but nonsense point mutations,

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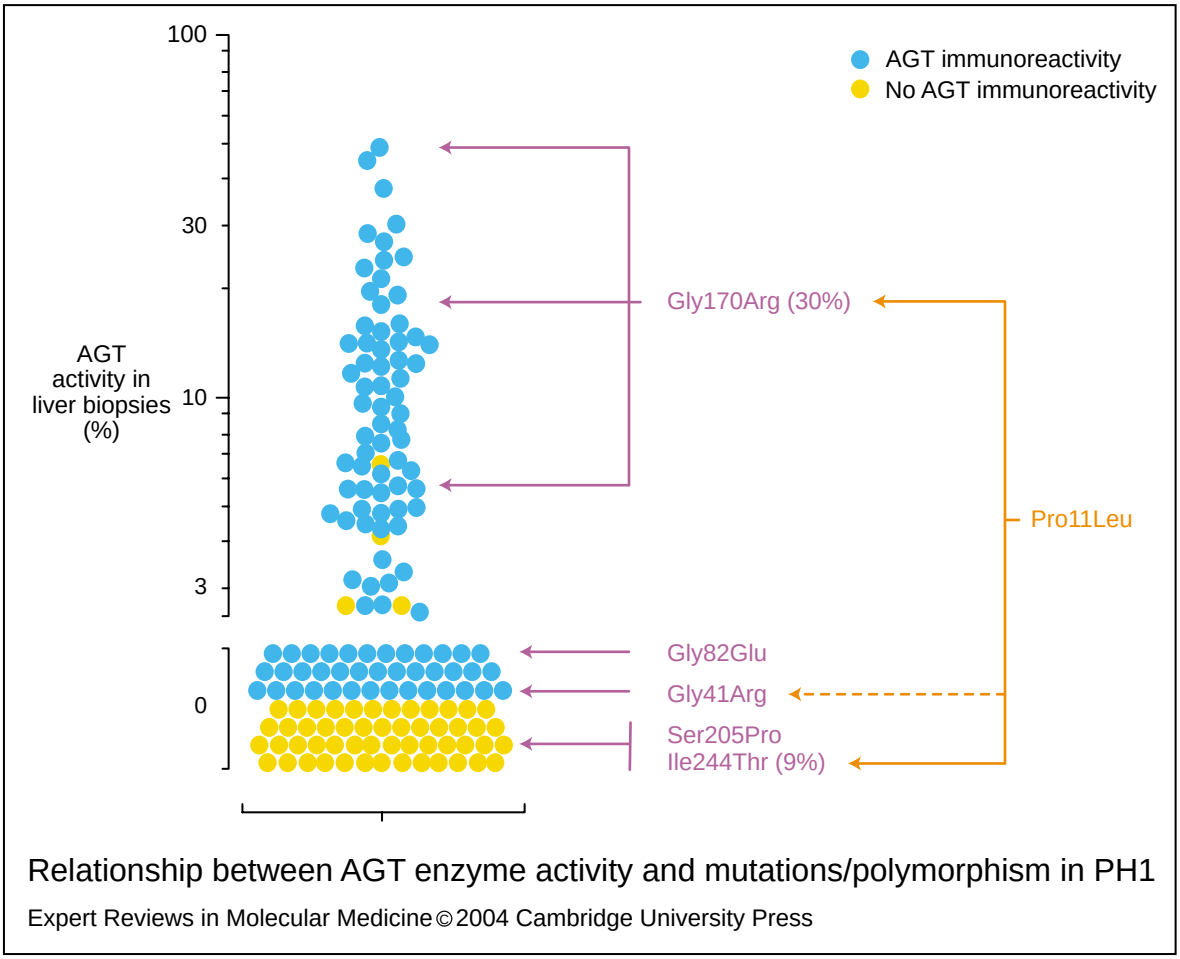
insertions and deletions have also been found (Ref. 1). The most common mutation, and the first to be discovered, is a Gly170Arg replacement found in 30% of mutant alleles in European and North American PH1 patients (Ref. 12). The second most common mutation is an Ile244Thr replacement, which has a frequency of 9% (Ref. 16). Most other mutations have much lower frequencies (<1%). The frequency of at least some mutations is very population-specific: for example, Gly170Arg is rare in Japanese populations, and Ile244Thr is much more common in Spanish populations, especially of the Canary Islands (Ref. 17). Some mutations, including Gly170Arg and Ile244Thr, segregate with the Pro11Leu polymorphism and are predicted to be completely innocuous in its absence (see below) (Ref. 15).

Relationships between genotype and enzyme phenotype in PH1

The relationships between genotype and enzyme phenotype in PH1 have been well studied in five cases (Fig. 2). Gly170Arg is usually associated with



Relationship between AGT enzyme activity and mutations/polymorphism in PH1

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Figure 2. Relationship between AGT enzyme activity and mutations/polymorphism in PH1. AGT catalytic activity can vary widely in PH1 liver biopsies (from 0% to 50–60% of the mean normal level). In many cases, specific mutations, some of which segregate with the Pro11Leu polymorphism, are associated with particular levels of catalytic activity and immunoreactivity. In the figure, the specific mutations discussed in the text are shown in purple, and segregation with Pro11Leu is represented by the orange arrows (the dashed orange arrow indicates that Gly41Arg does not completely segregate with Pro11Leu). AGT catalytic activity (μmol pyruvate/h/mg protein) is expressed on a logarithmic scale as a percentage of mean normal value. Blue circles indicate significant levels of immunoreactive AGT protein; yellow circles indicate undetectable, or barely detectable, levels of immunoreactive AGT protein. Abbreviations: AGT, alanine:glyoxylate aminotransferase; PH1, primary hyperoxaluria type 1.

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the presence of significant amounts of both AGT catalytic activity and immunoreactivity, whereas Gly82Glu is associated with zero catalytic activity but normal or supranormal levels of immunoreactivity. Ile244Thr, Gly41Arg and Ser205Pro are associated with absent or greatly reduced catalytic activity and immunoreactivity. Gly170Arg and Ile244Thr segregate absolutely, and Gly41Arg partially, with the Pro11Leu polymorphism; Gly82Glu and Ser205Thr do not.

Gly170Arg

All PH1 patients so far studied in whom AGT is mistargeted from peroxisomes to mitochondria express at least one allele containing both the Gly170Arg mutation and the Pro11Leu polymorphism (Ref. 12). Pro11Leu generates an inefficient mitochondrial targeting sequence (MTS) at the N-terminus of polymorphic AGT, the efficiency of which is increased by the additional presence of the Gly170Arg mutation (Fig. 3). The inefficiency of the Pro11Leu-generated MTS is shown by the fact that only ~5% of AGT is targeted to mitochondria in hepatocytes of normal individuals homozygous for the minor *AGXT* allele (Ref. 12) and none at all is targeted to mitochondria in transfected tissue culture cells (Refs 10, 18) (Fig. 4a, d). The enhanced efficiency imparted by Gly170Arg (Fig. 4b, e) results from the fact that the mutation and the polymorphism together block AGT dimerisation. This leads to mistargeting because, although peroxisomes can import fully folded cofactor-bound oligomeric proteins (Refs 19, 20), mitochondria can import only unfolded, or loosely folded, monomers (Refs 21, 22). The N-terminus of Pro11Leu AGT acts only as a weak MTS because, although it provides all the necessary topogenic information, the protein dimerises so rapidly into a mitochondrial-import-incompetent form that mitochondrial import is blocked. Interestingly, when attached to a reporter protein that does not dimerise with such avidity, such as green fluorescent protein (GFP), the N-terminal 20 amino acids of Pro11Leu AGT act as a very strong MTS (Ref. 18). It is a sobering thought that if Pro11Leu AGT dimerised a little more slowly, then even this AGT would be significantly mistargeted to mitochondria – the result being that PH1 would have a frequency in the population of about 4% (the frequency of Pro11Leu homozygotes) instead of 0.001–0.0001%.

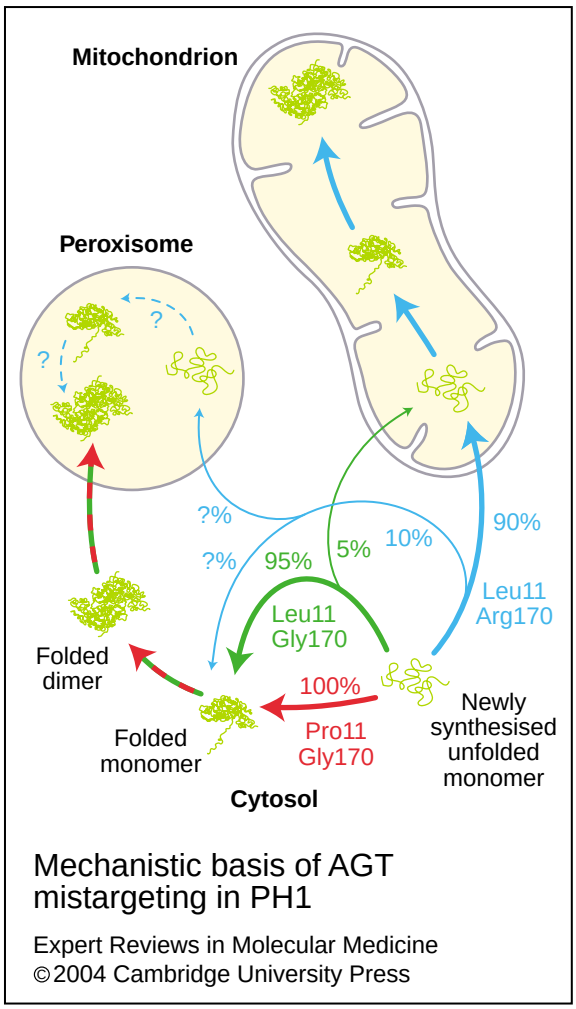

Figure 3. Mechanistic basis of AGT mistargeting in PH1. Normal AGT encoded by the major *AGXT* allele (Pro11, Gly170) follows the red pathway. It folds and dimerises rapidly and is imported into the peroxisomes. Polymorphic AGT encoded by the minor *AGXT* allele (Leu11, Gly170) follows the green pathway. Most (~95%) folds and dimerises rapidly and is imported into peroxisomes, but a small amount (~5%) is imported into mitochondria before it has chance to fold and dimerise. Once in the mitochondria, polymorphic AGT eventually folds and dimerises. PH1 mutant AGT (Leu11, Arg170) follows the blue pathway. Only a small proportion (~10%) is imported into peroxisomes, either as an unfolded monomer, folded monomer, or a dimer. The majority (~90%) is imported into mitochondria before it has chance to fold and dimerise. Once imported, it does eventually fold and dimerise. Although catalytically active, mitochondrial AGT is metabolically inefficient because its substrate (glyoxylate) is synthesised mainly in the peroxisomes. Abbreviations: AGT, alanine:glyoxylate aminotransferase; PH1, primary hyperoxaluria type 1.

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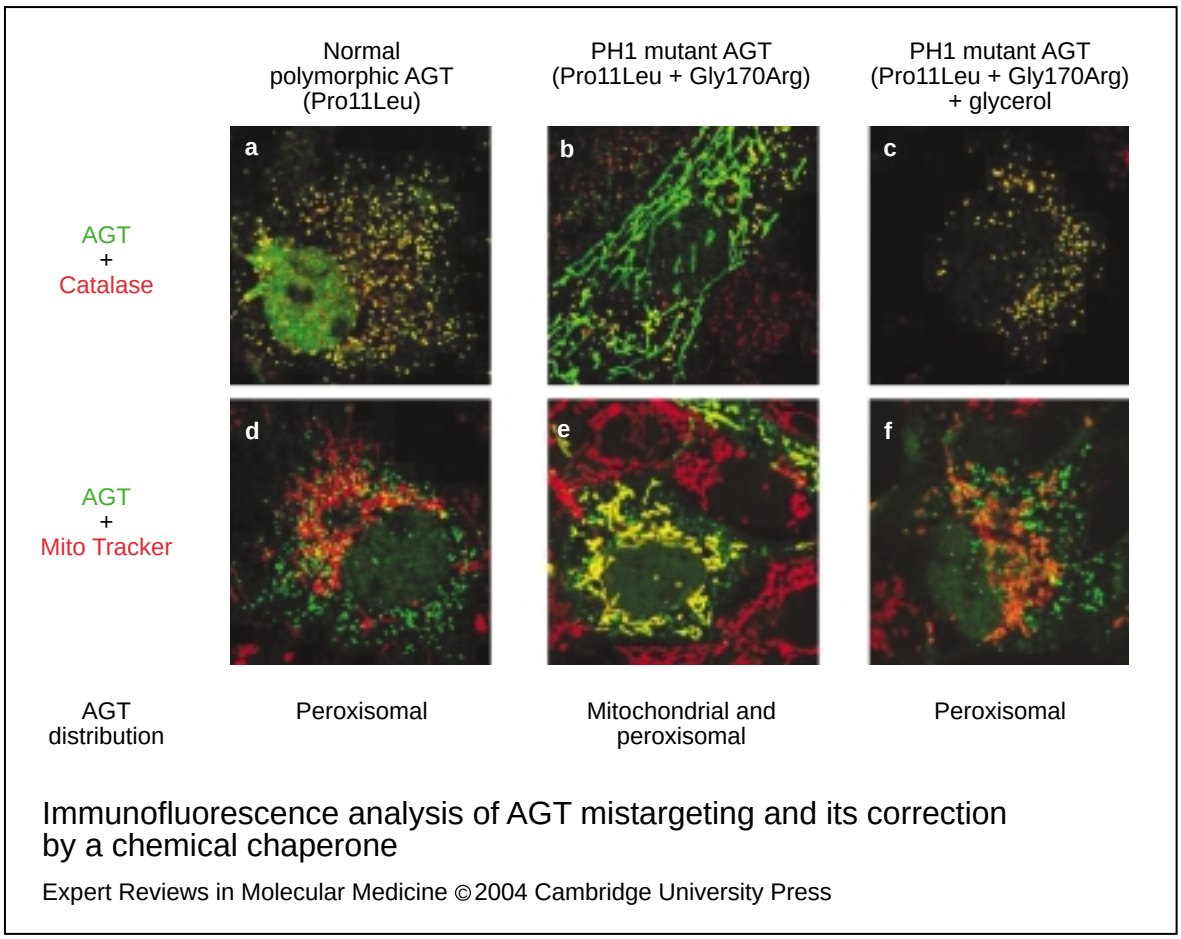

Figure 4. Immunofluorescence analysis of AGT mistargeting and its correction by a chemical chaperone. COS cells were transfected with normal and mutant AGT and analysed after 24 h by confocal immunofluorescence microscopy: (a, d) normal polymorphic AGT encoded by the minor *AGXT* allele (i.e. containing the Pro11Leu polymorphism); (b, e) PH1 mutant AGT containing the Pro11Leu polymorphism and the Gly170Arg mutation; (c, f), as (b, e) but in the presence of 5% glycerol. AGT was indirectly immunolabelled using a green fluorochrome (fluorescein); the peroxisomal marker, catalase, was indirectly immunolabelled using a red fluorochrome (Texas Red) (a–c); and mitochondria were labelled with the red dye MitoTracker (d–f). Colocalisation results in a yellow colouration (red + green = yellow). Normal AGT (containing the Pro11Leu polymorphism) targets to peroxisomes, but the additional presence of the Gly170Arg mutation results in mistargeting to mitochondria. This aberrant targeting can be corrected by the addition of the nonspecific chemical chaperone glycerol. Abbreviations: AGT, alanine:glyoxylate aminotransferase; PH1, primary hyperoxaluria type 1.

Ile244Thr

The second most common PH1 mutation, Ile244Thr, also segregates with Pro11Leu and is predicted to have no pathological effect in its absence (Ref. 15). When expressed in prokaryotic or eukaryotic heterologous expression systems, AGT containing both the mutation and the Pro11Leu polymorphism is unstable and forms aggregates (Refs 15, 17). The reason why such aggregates have not been found in PH1 liver biopsies is unclear. One possibility is that AGT containing Ile244Thr is rapidly degraded by the hepatocyte proteolytic machinery before the aggregates have time to form.

Bearing in mind that this mutant contains the Pro11Leu-generated MTS, it is not clear why none is mistargeted to mitochondria, either in patients or when it is expressed in tissue culture cells (Ref. 17). It might be simply a question of degree – this mutant might be so unstable that it is degraded before it has a chance to be imported.

Accession information: DOI: 10.1017/S1462399404007203; Vol. 6; Issue 1; 9 January 2004

Gly41Arg

Gly41Arg is a rare mutation that has been found in three compound heterozygote patients on the background of the minor *AGXT* allele (Ref. 6). However, there has been at least one case reported of this mutation being present in the homozygous state on the background of the major *AGXT* allele (Ref. 23). At least in the former situation, Gly41Arg results in AGT aggregating into core-like structures in the peroxisomal matrix. When expressed in *Escherichia coli*, AGT containing both the mutation and the Pro11Leu polymorphism aggregates into inclusion bodies. However, when expressed with the mutation alone the protein is partially soluble, although its specific catalytic activity is markedly reduced (Ref. 15). This partial functional linkage between Gly41Arg and Pro11Leu is compatible with the findings, admittedly in a very small number of patients, that patients with Gly41Arg alone are more mildly affected than those in which both Gly41Arg and Pro11Leu are present.

Gly82Glu

The Gly82Glu mutation does not segregate with the Pro11Leu polymorphism (Ref. 24). Its presence leads to a complete loss of AGT catalytic activity, but it has no effect on protein stability or targeting (Refs 25, 26). Purified recombinant AGT containing Gly82Glu is stable but it fails to bind the cofactor PLP (Ref. 15), which explains perfectly its loss of activity.

Ser205Pro

Ser205Pro is a rare mutation identified in Japanese PH1 patients (Ref. 27). It does not segregate with the Pro11Leu polymorphism. AGT containing Ser205Pro is highly unstable and prematurely degraded (Ref. 28), a finding that is completely compatible with the absence of both AGT immunoreactivity and catalytic activity in PH1 patients homozygous for this mutation.

Crystal structure of AGT

Although the mechanistic relationship between the various mutations found in PH1, with or without the Pro11Leu polymorphism, can be largely understood in biochemical and cell biological terms, a full understanding is impossible without knowledge of the three-dimensional structure of AGT. This can now be achieved following the solution of its crystal structure to 2.5Å (Refs 29, 30) (Fig. 5).

Normal human AGT crystallises as an intimate dimer, each subunit of which can be divided into two main domains. The larger, N-terminal domain is composed of the first 283 residues and contains the catalytic site and the bulk of the dimerisation interface. Apart from the fact that it contains the PTS1, the function of the smaller, C-terminal domain (residues 284–392) is unknown. The first 20 or so residues of the N-terminal domain of one subunit wrap round the surface of the other subunit, probably not only contributing to the stability of the dimer but also to the dimerisation process per se. Biochemical evidence supports the idea that the N-terminal 'clamp' is involved in dimerisation, because the protein remains monomeric when this region is deleted from AGT translated in vitro (Ref. 18). Pro11 is positioned right in the middle of this N-terminal clamp and its replacement by Leu might make it more difficult for it to bind to the surface of the other subunit (Ref. 30). In addition, the Pro11Leu polymorphism generates a sequence that more closely fits the requirements for binding to the mitochondrial import receptor TOM20 (Refs 31, 32).

The crystal structure of AGT shows that each subunit contains one catalytic site in which the cofactor PLP forms a Schiff base with the ε-amino group of Lys209. Gly82 is situated in the middle of the catalytic site where it makes hydrogen-bond contact with the cofactor. Its replacement by the much larger amino acid Glu would not give enough room for PLP to fit into the catalytic cavity. This is compatible with the enzymatic phenotype associated with the Gly82Glu mutation – namely, inhibition of PLP binding and complete loss of catalytic activity.

Gly41 sits right in the middle of the dimerisation interface and interacts with its counterpart in the other subunit (Fig. 5). Replacement of the smallest amino acid (i.e. Gly) with one of the largest (i.e. Arg) would prevent the formation of the interface and prevent dimerisation. This would inevitably destabilise AGT, leading to its aggregation and accelerated degradation. This again fits exactly with the enzymatic phenotype associated with the Gly41Arg mutation (i.e. loss of catalytic activity, very low immunoreactivity, and intraperoxisomal aggregation).

Gly170 is located on the surface of AGT about as far away from the dimerisation interface as it is possible to get (Fig. 5). For example, whereas

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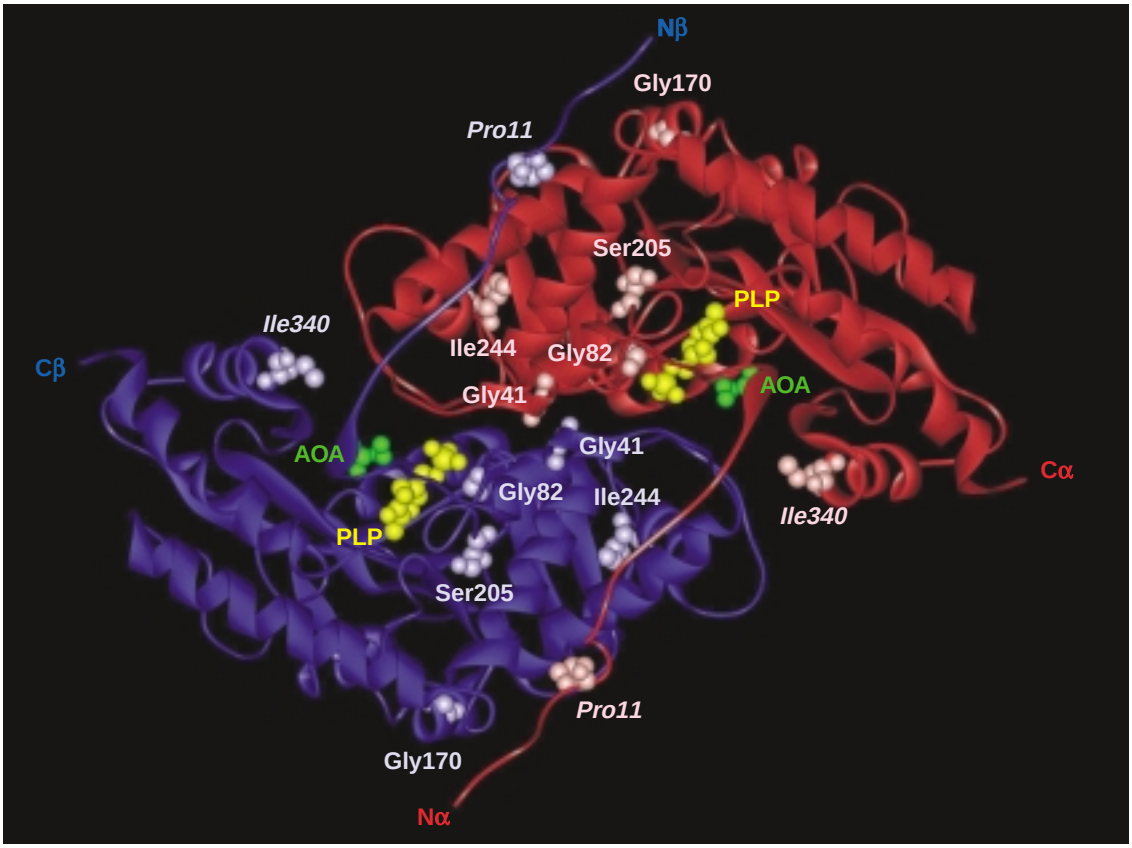

Crystal structure of human AGT

Figure 5. Crystal structure of human AGT. Normal human AGT crystallises as an intimate dimer. The two identical subunits, arbitrarily designated α (red) and β (blue), are held together by the large dimerisation interface and the N-terminal extensions that wrap over the surfaces of the opposing subunits. The cofactor pyridoxal phosphate (PLP) is coloured yellow, and the competitive inhibitor amino-oxyacetic acid (AOA) is coloured green. The sites of the Pro11Leu and Ile340Met polymorphisms are indicated (in italics) as are the sites of the various mutations mentioned in the text. Such analysis helps to explain how many of the mutations achieve their untoward effects on the properties of AGT as well as being the starting point for rational design of drugs that might counter these effects. Abbreviation: AGT, alanine:glyoxylate aminotransferase.

Gly41α and Gly41β (i.e. the Gly41 residues in the α and β subunits) are less than 5Å apart, Gly170α and Gly170β are 50Å apart. Surprisingly, although Gly170Arg has untoward effects on the properties of AGT only when Pro11Leu is also present in cis (i.e. on the same allele), their functional interaction appears to be in trans (i.e. Gly170Arg in one subunit functionally interacts with Pro11Leu on the other) (Ref. 30). The strength of the interaction between the N-terminal clamp of one subunit and the surface of the other is likely to affect the dimerisation process and to a lesser extent the stability of the dimer once formed. The presence of the Pro11Leu polymorphism already weakens slightly this interaction, and the Gly170Arg mutation weakens it further. The resulting decrease in the rate of dimerisation, together with the presence of the Pro11Leu-generated MTS, results in the unparalleled peroxisome-to-mitochondrion mistargeting of AGT.

Although the replacement of Ile244 by Thr would generate only a small defect in helix packing, it might indirectly affect the intersubunit binding of the N-terminal clamp as suggested for Gly170Arg. This could destabilise AGT enough for it to aggregate and be rapidly degraded.

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The replacement of Ser205 by Pro would necessitate a large conformational change in the backbone of one of the β-strands of AGT that would completely disrupt the main-chain hydrogen bonding of the central β-sheet. This would disrupt AGT folding and lead to accelerated degradation.

Primary hyperoxaluria type 2 (PH2)

Primary hyperoxaluria type 2 (PH2, MIM 260000) is an autosomal recessive disorder characterised by increased synthesis and excretion of oxalate and L-glycerate. The increased levels of oxalate lead to CaOx deposition in the kidney and pathological sequelae similar to those of PH1 (see above).

GR/HPR deficiency in PH2

PH2 is caused by a deficiency of the intermediary metabolic enzyme GR/HPR (EC 1.1.1.26). This cytosolic enzyme is primarily hepatic, but some is also found in the kidney, heart, pancreas and leukocytes (Refs 33, 34). GR/HPR catalyses the reduction of glyoxylate to glycolate (glyoxylate reductase activity, GR) and of hydroxypyruvate to D-glycerate (hydroxypyruvate reductase activity, HPR), using NADH and NADPH as cofactors. Failure of these reactions in PH2 allows glyoxylate to be oxidised to oxalate and hydroxypyruvate to be reduced to L-glycerate, both catalysed by LDH (Fig. 1). Although concomitant hyperoxaluria and hyper-L-glycericaciduria have been regarded as pathognomonic for PH2, two siblings who have GR/HPR deficiency without hyper-L-glycericaciduria have recently been described (Ref. 35).

Unlike PH1, there is currently little known about the enzymic phenotypes in PH2. All the liver biopsies analysed to date have had undetectable GR catalytic activity and negative immunoreactivity although the numbers analysed are still relatively few (Refs 34, 36). Hepatic HPR activity, by contrast, tended to be at the lower end of normal, illustrating a contribution to this activity by other enzymes, possibly LDH.

The GR/HPR gene (*GRHPR*)

GR/HPR is encoded by the *GRHPR* gene, which maps to the pericentromeric region of chromosome 9; it is composed of 9 exons ranging in size from 72 to 264 bp, spanning 9 kb of genomic DNA (Refs 37, 38). The open reading frame encodes a polypeptide of 328 amino acids, which homodimerises to yield a protein of ~73 kDa (Ref. 37) possessing GR, HPR and D-glycerate dehydrogenase (D-GDH) enzyme activities (Refs 37, 38). Several transcripts potentially encoding GR/HPR have been identified by northern blot analysis and might represent splice variants. In addition, a transcript lacking the last 28 bp of exon 1 has been demonstrated (Ref. 34). At the present time, the functional significance of these variants is unclear.

Polymorphic variants of the *GRHPR* gene

A 579A>G polymorphism has been described in exon 6 (Ref. 38) but, as expected for a silent change, it appears to have no functional consequences (Ref. 34).

Mutations in the *GRHPR* gene

Fourteen mutations have been described in the gene to date and are spread throughout the nine exons. These mutations consist of minor deletions/insertions and point mutations, causing both missense changes, premature termination codons and aberrant splicing (Refs 33, 34, 38, 39). Two point mutations within the splice acceptor sites of introns 1 and 7 (IVS1-2A>G and IVS7-1G>A, respectively), and a 4 bp deletion at the boundary of exon 4 and intron 4, are predicted to affect splicing of the mRNA as a result of either exon extension or exon skipping. Abnormal transcripts have been demonstrated for IVS1-2A>G and the 4 bp deletion (Fig. 6) (Ref. 34). The latter occurs in a repetitive region and might be the result of loss of the last two nucleotides of exon 4 and the first two of the intron (AAGT), or the first four nucleotides of the intron (GTAA), or nucleotides 3–7 of intron 4 (AAGT) (Ref. 39). Four point mutations have been described. One of these, 295C>T, encodes the nonsense mutation Arg99X, while three others – 494G>A, 904C>T and 965T>G – encode the amino acid replacements Gly165Asp, Arg302Lys and Met322Arg, respectively.

The crystal structure of the human enzyme is not available, but when modelled on the three-dimensional structure of D-GDH from *Hyphomicrobium methylovorum*, Gly165 occurs in the putative cofactor-binding site in the central portion of the polypeptide chain. This residue is highly conserved throughout the species, and replacement by an aspartate residue would be predicted to impair cofactor binding. The other

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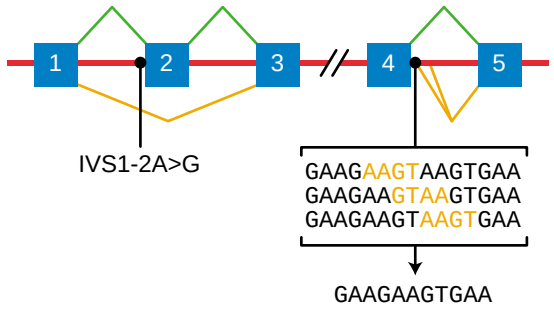

Splicing defects in the *GRHPR* gene in PH2

Expert Reviews in Molecular Medicine

Figure 6. Splicing defects in the *GRHPR* gene in PH2. Exons 1–5 are denoted by blue boxes, introns by red lines, normal splicing by green lines, and aberrant splicing by orange lines. The IVS1-2A>G mutation destroys the splice acceptor site at the end of intron 1, resulting in skipping of exon 2. The 4 bp deletion at the boundary of exon 4 and intron 4 can occur at three possible sites yielding the same contiguous sequence. Cryptic splice sites within intron 4 are activated, one at 20 and the other at 81 nucleotides from the original exon–intron boundary, leading to exon extension. The extended products each contain a premature termination codon and would therefore fail to encode a recognisable protein. Abbreviations: GR/HPR, glyoxylate/hydroxypyruvate reductase; PH2, primary hyperoxaluria type 2.

two mutations occur in the catalytic domain located at the N- and C-termini and appear to affect function and stability of the protein. A further five minor deletions have been identified – namely 103delG, 375delG, 540delT, 608-609delCT and 862-863delTG – that all lead to frame shift mutations and premature termination codons. The high incidence of minor deletion or insertion mutations (7/14) is atypical and very different from that found in PH1 where point missense mutations are in the majority.

The 103delG mutation occurred in 39% of alleles overall in the two reported series of patients (Refs 34, 39). It was notable that the mutation was restricted to those of Caucasian descent. The next most commonly occurring change was the 4 bp deletion at the boundary of exon 4 and intron 4. In one patient cohort it was restricted to those of Asian origin (Ref. 34), in whom it occurred in 50% of alleles from seven unrelated families. However, this mutation was also described in two Caucasians of Italian background (Ref. 39) and therefore is not specific to one ethnic group.

Relationships between genotype and enzyme phenotype in PH2

Because of the much smaller number of PH2 patients analysed, compared with PH1 patients, there is little or no information on the relationships between genotype and enzyme phenotype in most cases. However, most patients appear to have undetectable levels of GR catalytic activity and immunoreactivity. Those mutant forms of GR/HPR with amino acid replacements expressed in vitro also have significantly reduced GR and HPR activities (Refs 34, 39). Of interest is the finding of a 2 bp deletion in two siblings without the characteristic hyper-L-glycericaciduria of the disease. This genotype leads to absence of functional protein, excluding the possibility that the unusual biochemical phenotype is caused by a mutation in GRHPR affecting the substrate specificity of the enzyme. Instead, this finding reinforces the idea that phenotypic differences in these diseases (PH1 as well as PH2) owe as much to the contribution of other genetic loci and/or the environment as to the primary disease.

Clinical implications/applications

Up until the mid1980s, the clinical management of PH1 and PH2 was based on knowledge only of the more distal pathological events, such as elevated oxalate excretion and the formation of CaOx kidney stones. However, over the past 15–18 years, it has increasingly taken into account our increased understanding of the more proximal molecular processes, such as mutations and enzyme deficiencies. Until the mid1980s, diagnosis was largely dependent on the measurement of urinary metabolites – oxalate and glycolate for PH1, and oxalate and L-glycerate for PH2. Although very successful, it was not without its drawbacks. For example, some laboratories found urinary glycolate and L-glycerate difficult to measure accurately, and in any case some PH1 patients do not have elevated glycolate excretion (Ref. 40) and some PH2 patients do not have elevated L-glycerate excretion (Ref. 35). In addition, urinary metabolite levels become erratic and eventually not measurable as kidney function deteriorates. Prenatal diagnosis was not possible at all, probably because the metabolic

Accession information: DOI: 10.1017/S1462399404007203; Vol. 6; Issue 1; 9 January 2004

consequences of AGT or GR/HPR deficiency are not manifested in utero. Treatment was concerned mainly with: (1) the prevention of stone formation, by increasing hydration or the use of crystallisation inhibitors; (2) the removal of stones once formed, by surgery or lithotripsy; and (3) dealing with the consequences of renal failure – that is, dialysis and kidney transplantation. Unfortunately, kidney transplantation provides only temporary relief. Because the enzyme defect is not in the kidney but in the liver (entirely so for PH1, and mainly so for PH2), the newly transplanted kidney will eventually go the same way as the original one (i.e. fill up with insoluble CaOx). Although the rationale for its original use 40 years ago is unclear, the use of pyridoxine in the treatment of PH1 turned out to be remarkably prescient given that PLP is the cofactor of AGT. So, although its use in the treatment of PH1 has not increased as a result of our greater understanding of the molecular aetiology of PH1, at least we now know how it might work. For example, it might lead to increased conversion of the inactive apoenzyme to the active holoenzyme.

Diagnosis

The discovery of the basic enzyme defect in PH1 has led to the introduction of an AGT assay in percutaneous needle liver biopsies as the definitive diagnostic method (Ref. 41). This can distinguish PH1 from all other hyperoxaluric syndromes (including PH2) and other CaOx stone diseases. In addition, it is independent of the clinical state of the patient. Probably over 500 enzymatic diagnoses for PH1 have been made worldwide over the past 18 years.

The history of our understanding of PH2 has been rather different. The enzyme defect was discovered 35 years ago, but the enzyme was identified by one of its minor reactions as D-GDH (Ref. 42), which has unfavourable kinetics. The discovery that D-GDH was the same enzyme as GR/HPR, and that PH2 patients have GR deficiency as well as D-GDH deficiency (Ref. 43), opened up the possibility that PH2 could be diagnosed using the kinetically much more favourable, and metabolically much more relevant, GR reaction.

Improvements in the techniques over the past few years mean that AGT and GR can be assayed, and therefore PH1 and PH2 diagnosed, on the same liver biopsy.

Because some mutations in AGT, such as Gly170Arg and Ile244Thr, are so common, it is feasible, under certain circumstances, to use mutational analysis, rather than enzyme analysis, as a definitive diagnostic method for PH1. This approach is especially useful in the diagnosis of siblings of previously diagnosed patients.

Prenatal diagnosis

Prenatal diagnosis for PH1 became available first by the measurement of AGT levels in foetal liver biopsies in the second trimester (Refs 44, 45, 46) but nowadays by the use of mutational or linkage analysis from chorionic villus samples in the first trimester (Refs 46, 47, 48). We estimate that more than 60 prenatal diagnoses for PH1 have been carried out worldwide over the past 15 years.

Although prenatal diagnosis of PH2 has not yet been carried out, it is certainly possible. Again, this could use either mutation or linkage analysis. Several microsatellite markers close to the *GRHPR* gene have been described and found to be of value for linkage analysis (Refs 39, 49); in addition, a microsatellite has been identified within the *GRHPR* gene at the 3' end of intron 8 and might prove useful for further studies (Ref. 34).

Treatment

Probably the greatest changes in the clinical management of PH1 have been in its treatment. AGT expression is mostly confined to liver parenchymal cells (hepatocytes). Therefore, even though PH1 is classified as a kidney disease at the clinical or pathophysiological level, it is a liver disease at the level of the basic enzyme defect. It is exactly for this reason that kidney transplantation is unlikely to provide a long-term solution for PH1. However, liver transplantation is a very effective form of enzyme replacement therapy because it not only provides the total body's requirement of AGT, but also provides it already in the correct organ, cell and intracellular compartment (Refs 50, 51, 52, 53, 54). Liver transplantation can provide a metabolic cure for PH1, although some metabolic parameters, such as urinary oxalate excretion, can take several years to normalise. The longest-surviving transplanted PH1 patient was transplanted in 1986 (Ref. 50) and has led a relatively normal and productive life for over 17 years. Because most patients in line for liver transplantation are also in renal failure, most procedures have been coupled with kidney transplantation. We estimate that over 200 liver

Accession information: DOI: 10.1017/S1462399404007203; Vol. 6; Issue 1; 9 January 2004

(with or without kidney) transplantations have been carried out for PH1 worldwide since the mid1980s (Refs 55, 56).

Although it has yet to be put into practice, the finding that most, but not all, GR/HPR is located in the liver suggests that enzyme replacement therapy by liver transplantation might also work for PH2.

Research in progress and outstanding research questions

Outstanding unanswered questions

Although the past two decades have seen many advances, not only in our understanding of molecular aetiology and pathophysiology of PH1 and PH2, but also in our development of more rational approaches to their clinical management, there remain several puzzles yet to be solved and challenges yet to be addressed.

As far as the molecular aetiology and pathophysiology of PH1 is concerned, although we know a great deal about the relationship between genotype and enzyme phenotype, we know much less about the relationship between enzyme phenotype and clinical phenotype. For example, for a particular combination of mutations and polymorphisms in PH1, we might be able to predict the approximate residual level of AGT catalytic activity, immunoreactivity, and even subcellular distribution, but we would be much less successful at predicting disease progression and future severity. This is very important because knowledge of the prognosis for a patient with a particular genotype or enzyme phenotype would make it much easier to decide on the relative merits of kidney or liver transplantation, for example. The difficulty in relating mutations to the severity of the disease is probably due to the presence of other genetic and environmental factors in the disease process. Identification of these factors could be important because they might be the same ones that determine an individual's susceptibility to much more common conditions, such as idiopathic CaOx stone disease (Ref. 57).

The clinical heterogeneity of PH is poorly understood. For example, it is not known what determines whether CaOx deposits in the kidney as urolithiasis or nephrocalcinosis, whether patients have hyperglycolic aciduria as well as hyperoxaluria in PH1, or more recently whether they have hyper-L-glycericaciduria and hyperoxaluria in PH2. There are a few reports of individuals who have hyperoxaluria and hyperglycolic aciduria, or isolated hyperglycolic aciduria, but do not have AGT deficiency (Refs 58, 59). Some patients with CaOx stones and all the hallmarks of metabolic disease do not have either PH1 or PH2, as determined by enzyme deficiency (Ref. 60).

Treatments for the future

Liver transplantation is as much a form of gene therapy as it is a form of enzyme replacement therapy. After all, the long-term success of the procedure is more dependent on the replacement of the mutant gene with a normal gene that continues to produce normal AGT over many years, than it is with the normal AGT enzyme that happens to be in the donor liver at the time of transplantation. A disadvantage of this sort of gene therapy is that it is very inefficient, as 10 000s of perfectly normal genes have to be replaced at the same time as one defective gene. Liver transplantation is not without its problems, both in the short- and long-terms – not least the scarcity of suitable donor livers. A good case could be made for more 'conventional' gene therapy (Ref. 61), although vectors that could achieve the necessary transduction efficiency and long-term stability are probably still a long way off.

Another approach to treatment, which is being actively pursued in many other genetic diseases, is to try to counteract the adverse effects of specific mutations on the properties of the enzyme using pharmacological agents. This requires intimate structural and biochemical knowledge of how particular amino acid substitutions alter the protein's stability, folding, oligomerisation, and subsequent downstream intracellular events. In the case of AGT and PH1, this information already exists to a large extent. The potential of this kind of approach has been demonstrated in principle in the case of the two most common mutations – namely Gly170Arg and Ile244Thr (Refs 17, 62).

It is generally recognised that most missense point mutations in hereditary diseases achieve their adverse effects by disrupting protein-folding pathways and protein stability. Although in most cases this leads to protein misfolding, aggregation, and accelerated degradation, more spectacular consequences can occur, such as peroxisome-to-mitochondrion mistargeting in the case of AGT. Aberrant folding and stability is clearly at the heart of AGT mistargeting (Ref. 30), so it is perhaps unsurprising that nonspecific treatments

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known to improve protein stability and improve productive folding and oligomerisation, such as lowering the temperature or the addition of chemical chaperones like glycerol, also correct the targeting defect, at least in vitro (Ref. 62) (Fig. 4c, f). Similarly, another chemical chaperone, betaine, can decrease the aggregation of AGT containing the Ile244Thr mutation and the Pro11Leu polymorphism in transfected tissue culture cells (Ref. 17). Although many of the mutant forms of AGT, especially those containing the Pro11Leu polymorphism, might be amenable to this approach, some, such as that containing the Gly82Glu mutation that abolishes PLP binding, are clearly not.

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Further reading, resources and contacts

Reviews

Recent reviews on the molecular aetiology and clinical management of PH1, PH2 or CaOx stones in general:

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Websites

Website of the Oxalosis and Hyperoxaluria Foundation:

http://www.ohf.org/.

On-line Mendelian Inheritance in Man web pages for PH1 and PH2:

http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?259900 (PH1)
http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?260000 (PH2)

Features associated with this article

Figures

Figure 1. Metabolic consequences of AGT deficiency in PH1 and GR/HPR deficiency in PH2.
Figure 2. Relationship between AGT enzyme activity and mutations/polymorphism in PH1.
Figure 3. Mechanistic basis of AGT mistargeting in PH1.
Figure 4. Immunofluorescence analysis of AGT mistargeting and its correction by a chemical chaperone.
Figure 5. Crystal structure of human AGT.
Figure 6. Splicing defects in the *GRHPR* gene in PH2.

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